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SUMMARY

"*Air pollution and health*" is one the most important environmental and public health issues. Although air pollution levels have been decreasing in recent decades, the levels are still relatively high and harmful. Over the last decade, the impact of air pollution on cognitive functioning has become an issue of worry in epidemiological research, not the least because studies showed discrepancies on cognitive outcomes. In addition to the lack of consistency, potential underlying mechanisms of air pollution neurotoxicity are still scarce. In this context, neonates and children are particularly vulnerable to the detrimental effects of air pollution because of a higher respiratory rate compared to adults, and the dynamic modulation of the blood-brain barrier mechanisms to provide the necessary microenvironment for the developing brain. As a consequence, toxicants can enter the brain through the highly active transport systems that are evolved to transport nutrients and other molecules essential for the maintenance and development of the CNS. During the prenatal period, the placental barrier may play aside from its traditional role for transport of nutrients, growth factors, and hormones, also an important role in supporting CNS development through adaptive responses to the maternal environment. Therefore, the neurodevelopmental effects of air pollution early in life might present a serious health risk, not only to the fetus but also in childhood and later stages of life.

This PhD project falls into two parts, overall consisting of 5 chapters. In **PART 1** (3 chapters), we investigated molecular signatures of prenatal air pollution exposure and (neuro)development. We focused on the placenta as surrogate tissue for studying biomolecular processes of fetal (neuro)development. The specific objectives were to investigate the association between prenatal air pollution exposure and:

- A nitrosative stress marker in placental tissue, *i.e.* 3-nitrotyrosine,
- Transcriptional differences of the *Brain-derived neurotrophic factor* pathway in the placenta, an important signaling route in neurodevelopment,
- Changes in DNA methylation of *Leptin*, an energy-regulating hormone involved in fetal growth and development

In **PART 2** (2 chapters), we assessed cognitive performance in children in association with recent or chronic exposure to air pollution. Furthermore, we evaluated urinary carbon load as a novel biomarker of internal exposure to residential ambient air pollution.

A short description of each chapter is summarized in **Table 1**. This doctoral dissertation contributes to the evidence that air pollution exposure, as currently experienced in Belgium, may adversely affect cognitive functioning. Our findings of placental molecular signatures are likely to improve the understanding of the mode of action of how air pollution exposure may affect (neuro)development in the early phase of life. Furthermore, novel biomarkers that reflect ambient air pollution together with the current air pollution models will improve assessment of individual exposure and susceptibility. It should be noted that these findings were observed at levels below the current European Union guidelines, which increases the pressure on government and individuals to continue to reduce air pollution levels.

Table 1. Summary of this doctoral dissertation

PART 1: Placenta, (neuro)development, and air pollution

Chapter	What is known	What this study adds	Conclusions and perspectives
Chapter 1	▪ The placenta plays a crucial role in fetal growth and development ▪ Oxidative and nitrosative stress are two putative mechanisms by which air pollutants may exert their toxic effects	▪ Placental 3-nitrotyrosine (3-NTp), a biomarker of nitrosative stress, is associated with fine particle air pollution exposure during gestation	▪ Our findings highlight the relevance of placental 3-NTp as a biomarker of cumulative fine particle-induced prenatal oxidative stress. ▪ The potential health consequences will be investigated in a follow-up study of the ENVIR*ON*AGE birth cohort
Chapter 2	▪ Experimental evidence showed that developmental processes in the placenta and fetal brain are shaped by the same biological signals ▪ Adaptive responses of the placenta to the maternal environment may influence central nervous system development in the fetus	▪ Placental expression of genes within the brain-derived neurotrophic factor pathway, important in normal neurodevelopmental trajectories, is inversely associated with first trimester fine particle ($PM_{2.5}$) exposure	▪ Altered expression of genetic targets could be part of the molecular mechanism through which fine particle air pollution might affect placental and (neuro)developmental processes ▪ Future studies should corroborate these findings in other study populations ▪ Potential long-term health consequences remain to be elucidated
Chapter 3	▪ Placental Leptin (LEP) is involved in fetal growth and development ▪ Fine particle ($PM_{2.5}$) exposure affects fetal development, potentially through oxidative stress mechanisms	▪ Placental *LEP* methylation status is negatively associated with $PM_{2.5}$ exposure during the second trimester, and with placental nitrosative stress	▪ Epigenetic modification of LEP may interfere with normal placental processes and subsequent fetal development, but needs further exploration ▪ Additional research is needed to corroborate whether nitrosative stress might contribute to $PM_{2.5}$-induced placental epigenetic events

Table 1. (continued)

PART 2: Children, cognition and air pollution

Chapter	What is known	What this study adds	Conclusions and perspectives
Chapter 4	▪ Different studies on chronic air pollution exposure show association with endpoint measures of neurobehavioral performance ▪ Still insufficient evidence on consistency of these findings ▪ Studies on recent air pollution exposure and neurobehavioral performance are scarce	▪ Differential neurobehavioral changes are associated with recent and chronic air pollution exposure: ➢ Recent exposure: decreased visual information processing speed ➢ Chronic exposure: diminished sustained and selective attention	▪ Our findings aid to underscore the association between neurobehavioral performance and air pollution exposure, and highlight the importance of investigating recent air pollution exposures ▪ Future efforts should focus on different time periods of air pollution exposure and a more precise characterisation of the specific behavioral domains of cognitive function
Chapter 5	▪ Experimental studies demonstrated the translocating nature of ultrafine particles from the lungs to the systemic circulation ▪ Issue of particle translocation in humans is still controversial	▪ A novel technique to measure carbonaceous particles in urine ▪ Urinary carbon load can be used as an internal exposure marker for chronic combustion-related air pollution exposure	▪ Urinary carbon load reflects internal systemic black carbon particles, and provides a new marker to unravel the complexity of particulate-related health effects ▪ Development of a dedicated instrument and extrapolation to other fluids in the human body would be of interest for future studies ▪ Experimental studies are still needed to better understand the toxicokinetic fate of inhaled carbonaceous particles

SAMENVATTING

"*Luchtvervuiling en gezondheid*" is sinds enkele jaren één van de belangrijkste milieu- en volksgezondheidsproblemen. Hoewel de luchtverontreiniging in de afgelopen decennia afgenomen is, zijn de concentraties nog relatief hoog en schadelijk. In de afgelopen tien jaar is de invloed van luchtvervuiling op het cognitief functioneren een gebied van interesse geworden voor epidemiologisch onderzoek, niet in het minst omdat studies tegenstrijdige cognitieve uitkomsten hebben aangetoond. Naast het gebrek aan samenhang is de kennis van mogelijke onderliggende mechanismen van door luchtvervuiling geïnduceerde neurotoxiciteit nog steeds beperkt. Pasgeborenen en kinderen zijn bijzonder kwetsbaar voor de nadelige effecten van luchtvervuiling enerzijds als gevolg van een hoger ademhalingsritme in vergelijking met volwassenen, en anderzijds door de dynamische variatie in de bloed-hersenbarrière-mechanismen. Deze mechanismen zijn noodzakelijk om de micro-omgeving van de ontwikkelende hersenen optimaal te ondersteunen. Toxische stoffen kunnen hierdoor voornamelijk in de hersenen komen via actieve transportsystemen, welke voedingsstoffen en andere moleculen transporteren die essentieel zijn voor het onderhoud en ontwikkeling van het centraal zenuwstelsel. Tijdens de prenatale periode kan de placentabarrière, afgezien van zijn traditionele rol voor transport van voedingsstoffen, groeifactoren en hormonen, ook een belangrijke rol spelen in de ondersteuning van de ontwikkeling van het centrale zenuwstelsel door middel van adaptieve reacties op de maternale omgeving. Daarom kunnen (neuro)ontwikkelingseffecten van luchtvervuiling vroeg in het leven een ernstig gezondheidsrisico vormen, niet alleen bij de foetus maar ook in de kindertijd en in latere levensfasen.

Dit doctoraatsproject bestaat uit twee delen met daarin 5 hoofdstukken. In **DEEL 1** (3 hoofdstukken) onderzochten we mogelijke onderliggende actiemechanismen van prenatale blootstelling aan luchtvervuiling op foetale (neuro)ontwikkeling. Hiervoor richtten we ons op de placenta als surrogaatweefsel voor het bestuderen van biomoleculaire processen in foetale (neuro)ontwikkeling. De specifieke doelstellingen waren het onderzoeken van de associatie tussen prenatale blootstelling aan luchtvervuiling en:

- Een nitrosatieve stress-merker in placentaweefsel, nl. 3-nitrotyrosine
- Genexpressie-veranderingen van de *Brain-derived neurotrophic factor* signaleringsroute in de placenta, een belangrijke route in neuro-ontwikkeling

- Veranderingen in DNA-methylering van *Leptine*, een energie-regulerend hormoon betrokken bij foetale groei en ontwikkeling.

In **DEEL 2** (2 hoofdstukken) hebben we cognitieve prestaties bij kinderen beoordeeld in combinatie met recente of chronische blootstelling aan luchtvervuiling. Verder hebben we urinair koolstof als een nieuwe biomerker van interne blootstelling aan luchtvervuiling in het leefmilieu geëvalueerd.

Een korte beschrijving van elk hoofdstuk is samengevat in **Tabel 1**. Dit proefschrift versterkt de recente bewijsgaring dat blootstelling aan luchtvervuiling, zoals momenteel ervaren in België, schadelijk kan zijn voor de cognitieve functie. Onze bevindingen van placentaire moleculaire signalen dragen bij tot het voortschrijdende inzicht over hoe blootstelling aan luchtvervuiling in de vroege fase van het leven de (neuro)ontwikkeling kan verstoren. Bovendien zullen de nieuwe biomerkers, die luchtvervuiling van de omgeving reflecteren, samen met de huidige modellen van externe blootstelling de beoordeling van individuele blootstelling en susceptibiliteit verbeteren. Het is belangrijk erop te wijzen dat onze bevindingen slaan op Belgische populatiegroepen met blootstelling aan luchtvervuilings-concentraties die lager zijn dan de huidige EU-richtlijnen. Hierdoor neemt de druk op overheid en particulieren toe om te zorgen voor adequate maatregelen die de luchtverontreinigingsniveaus verder verlagen.

Table 1. Samenvatting van dit proefschrift

DEEL 1: Placenta, (neurologische) ontwikkeling, en luchtvervuiling

Hoofdstuk	Wat is er geweten	Wat deze studie bijbrengt	Conclusies en Perspectieven
Hoofdstuk 1	▪ De placenta is cruciaal voor de foetale groei en ontwikkeling ▪ Oxidatieve en nitrosatieve stress zijn twee mogelijke mechanismen voor hoe luchtverontreinigende stoffen hun toxische effecten kunnen uitoefenen	▪ Placentair 3-nitrotyrosine (3-NTp), een biomerker van nitrosatieve stress, houdt verband met fijnstof blootstelling tijdens de zwangerschap	▪ Onze bevindingen beklemtonen de relevantie van placentair 3-NTp als pseudo-biomerker van cumulatieve fijnstof-geïnduceerde prenatale oxidatieve stress ▪ De mogelijke gevolgen voor de gezondheid zullen worden onderzocht in een vervolgstudie van het Limburgs geboortecohort
Hoofdstuk 2	▪ Experimenteel bewijs toont aan dat ontwikkelingsprocessen in de placenta en foetale hersenen gevormd worden door dezelfde biologische signalen ▪ De placenta past zich aan veranderingen in de maternale omgeving aan, en kan zo de ontwikkeling van het centrale zenuwstelsel van de foetus mogelijk beïnvloeden	▪ Placentaire expressie van genen in de *Brain-derived neurotrophic factor* signaleringsroute, die belangrijk zijn voor normale neurologische ontwikkeling, waren negatief geassocieerd met de fijnstof-blootstelling van het eerste trimester van de zwangerschap.	▪ Veranderde expressie van genen kan deel uitmaken van het moleculaire werkingsmechanisme hoe fijnstof de placentaire en neurologische ontwikkelingsprocessen kan beïnvloeden ▪ Toekomstige studies zouden deze bevindingen moeten bevestigen in andere studiepopulaties ▪ Mogelijke gevolgen op lange termijn voor de gezondheid dienen nog opgehelderd te worden
Hoofdstuk 3	▪ Placentair *Leptin* (*LEP*) is betrokken bij foetale groei en ontwikkeling ▪ Fijnstof ($PM_{2.5}$) beïnvloedt de ontwikkeling van de foetus, mogelijk door oxidatieve stress mechanismen	▪ De methylatiestatus van placentair *LEP* is negatief geassocieerd met $PM_{2.5}$ blootstelling van het tweede trimester van de zwangerschap, alsook met placentaire nitrosatieve stress	▪ Epigenetische wijzigingen in *LEP* kunnen invloed hebben op normale placentaire processen en ontwikkeling van de foetus, maar deze bevindingen dienen verder onderzocht te worden ▪ Aanvullend onderzoek is nodig of nitrosatieve stress bijdraagt aan de $PM_{2.5}$-geïnduceerde placentaire epigenetische signaturen

Tabel 1. (vervolg)

DEEL 2: Kinderen, cognitie en luchtvervuiling

Hoofdstuk	Wat is er geweten	Wat deze studie bijbrengt	Conclusies en Perpectieven
Hoofdstuk 4	▪ Verschillende studies tonen aan dat blootstelling aan langdurige luchtverontreiniging geassocieerd is met het cognitieve prestatievermogen ▪ Onvoldoende bewijs van éénduidigheid van deze bevindingen ▪ Studies naar recente blootstelling aan luchtverontreiniging en het cognitieve prestatievermogen zijn schaars	▪ Verschillende veranderingen in cognitieve prestatie staan in verband met recente en langdurige blootstelling aan luchtverontreiniging: ➢ Recente blootstelling: verminderde snelheid van visuele informatieverwerking ➢ Langdurige blootstelling: vermindering van aanhoudende en selectieve aandacht	▪ Onze bevindingen ondersteunen de bewijsvoering van de associatie tussen het cognitieve prestatievermogen en blootstelling aan luchtverontreiniging, en benadrukken het belang van het onderzoek naar recente blootstelling ▪ Toekomstige studies moeten zich richten op verschillende leeftijdsperiodes van blootstelling aan luchtvervuiling en een nauwkeurigere karakterisering van de specifieke gedragsdomeinen van cognitieve functie
Hoofdstuk 5	▪ Experimentele studies hebben de translocatie van ultrafijne deeltjes van longen naar de circulatie aangetoond ▪ Studies omtrent translocatie van deeltjes bij mensen is nog steeds controversieel	▪ Een innovatieve techniek om koolstofhoudende deeltjes in urine te meten ▪ De hoeveelheid koolstofdeeltjes in urine kan gebruikt worden als een interne blootstellingsmerker voor langdurige verkeers-gerelateerde luchtverontreiniging	▪ De hoeveelheid koolstofdeeltjes in urine weerspiegelt interne blootstelling aan zwarte koolstofdeeltjes, en kan gebruikt worden om de complexiteit van deeltjes-gerelateerde gezondheidseffecten te ontrafelen ▪ Ontwikkeling van een semi-automatisch meetinstrument, alsook de extrapolatie van de techniek naar andere biologische specimens zou van belang zijn voor toekomstige studies ▪ Experimentele studies zijn nodig om de toxicokinetiek van ingeademde koolstofachtige partikels beter te begrijpen

LIST OF ABBREVIATIONS

$-CH_3$	Methyl group
3-NTp	3-Nitrotyrosine
ADHD	Attention-deficit/hyperactivity-disorder
AKT1	V-akt murine thymoma viral oncogene homolog 1
AKT2	V-akt murine thymoma viral oncogene homolog 2
AKT3	V-akt murine thymoma viral oncogene homolog 3
BC	Black carbon
BDNF	Brain-derived neurotrophic factor
BMI	Body mass index
CI	Confidence interval
CNS	Central nervous system
CO	Carbon monoxide
COGNAC	COGNition and Air pollution in Children
COX2	Cyclo-oxygenase 2
DEP	Diesel exhaust particles
DMRs	Differentially methylated regions
DNA	Deoxyribonucleic acid
DOHaD	Developmental Origins of Health and Disease
ELISA	Enzyme-linked immunosorbent assay
eNOS	Endothelial nitric oxide synthase
ENVIR*ON*AGE	ENVIRonmental influence *ON* early AGEing
EU	European Union
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GIS	Geographical information system
IARC	International Agency for Research on Cancer
IGF2	Insulin-like growth factor 2
IL1β	Interleukin 1β
iNOS	Inducible nitric oxide synthase
IPO8	Importine 8
IQR	Interquartile range
LEP	Leptin
MAPK	Mitogen-activated protein kinase
MIQE	Minimum information for publication of quantitative real-time PCR experiments
mRNA	Messenger ribonucleic acid
NES3	Neurobehavioral Evaluation System 3
NH_3	Ammonia
NH_4^+	Ammonium
NO	Nitric oxide
NO_2	Nitrogen dioxide
NOS2	Nitric oxide synthase 2
NO_x	Nitrogen oxides
O_3	Ozone
PAH	Polycyclic aromatic hydrocarbon

PCR	Polymerase chain reaction
PGCs	Primordial germ cells
PLCG1	Phospholipase C gamma 1
PLCG2	Phospholipase C gamma 2
PM	Particulate matter
PM_{10}	Particulate matter with an aerodynamic diameter ≤ 10 μm
$PM_{2.5}$	Particulate matter with an aerodynamic diameter ≤ 2.5 μm
POLR2A	Polymerase (RNA) II, polypeptide A
qPCR	quantitative real-time polymerase chain reaction
ROS	Reactive oxygen species
RPMR	Residential proximity to major roads
SD	Standard deviation
SE	Standard error
SOS1	Son of sevenless homolog 1
SOS2	Son of sevenless homolog 2
SO_x	Sulfur oxides
SYN1	Synapsin 1
t,t-MA	trans,trans-muconic acid
TiO_2	Titanium dioxide
TRKB	Neurotrophic tyrosine kinase receptor type 2
UBC	Ubiquitine C
UFPs	Ultrafine particles
US	United States
WHO	World Health Organization

TABLE OF CONTENTS

GENERAL INTRODUCTION

From the late 1970s, air pollution has been one of Europe's main socio-political concerns as it harms both the environment and human health.[1] Although emissions of many air pollutants decreased substantially over the past decades, concentrations of many air pollutants are still too high. Hitherto, a significant proportion of Europe's population lives in areas with exceedance of current air quality standards representing a great concern for public health, especially for vulnerable population groups such as the fetus, children, and elderly.[2] It has been estimated that ambient air pollution was responsible for 4.2 million premature deaths worldwide, representing 13.1% of the total deaths between 1990 and 2015.[3] The cardiovascular and respiratory health effects of air pollution have already been extensively investigated.[4-6] Recently, air pollution has been proposed as a suspected developmental neurotoxicant[7] and is classified by the International Agency for Research on Cancer (IARC) as carcinogenic for humans.[8]

AMBIENT AIR POLLUTION

Origin and composition of air pollutants

Air pollution is a complex mixture of gases/fumes, liquid droplets and solid particles derived from natural (volcanic activity, geological dust, methane and wildfires), and anthropogenic (industrial activities and fuel combustion from traffic and domestic heating) sources that modify the natural characteristics of a wholesome atmosphere (**Figure 1**). Air pollutants are grouped into primary pollutants that are emitted directly in the air [*e.g.* volatile organic compounds such as benzene, nitrogen oxides (NO_x), sulfur oxides (SO_x), carbon monoxide (CO), ammonia (NH_3), volcanic ash, toxic metals such as lead, and particulate matter (PM)], and secondary pollutants that are formed by chemical reactions (*e.g.* ozone (O_3), gaseous pollutants including SO_3, and ammonium (NH_4^+), and volatile organic compounds such as polycyclic aromatic hydrocarbons).

PM is a heterogeneous mixture of small particles that may vary in size, mass, and chemical composition, and which is mainly categorized into three main fractions on the basis of particle size (**Figure 2**). Particles with an aerodynamic diameter between 2.5 and 10 μm ($PM_{2.5-10}$) are called coarse particles and mainly consist of sea salt, wind-blown dust, pollen, mold, and spores. Fine particles

(aerodynamic diameter between 0.1 and 2.5 µm; $PM_{0.1-2.5}$) include sulfate, nitrate, organic compounds, endotoxins and toxic metals such as lead. Ultrafine particles (UFPs) is the generic term of all particles with a diameter ≤ 0.1 µm.

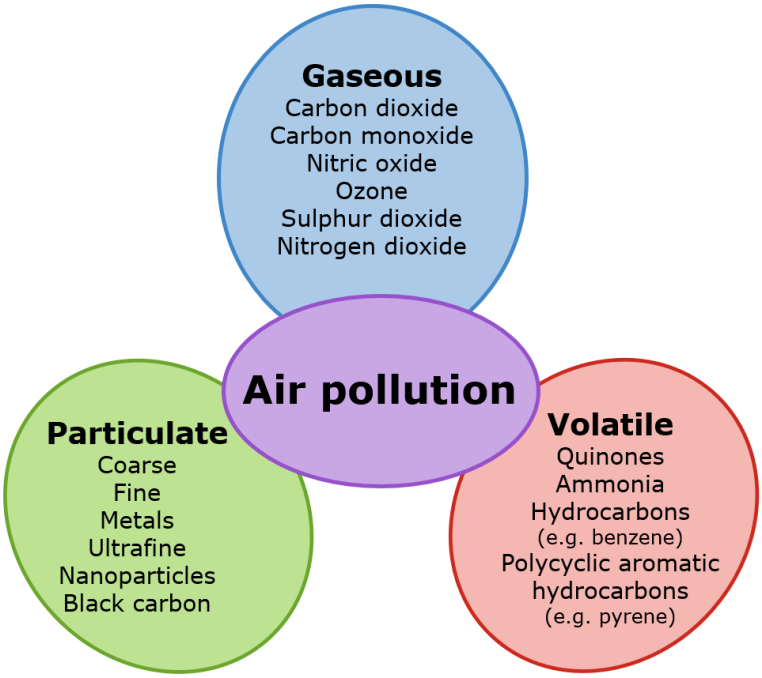

Figure 1: **:** Air pollution consists of different atmospheric fractions, i.e., gases, volatile components and particulate matter

Black carbon versus *Carbon black*

Black carbon (BC), another constituent of the fine particle fraction, is a product of incomplete combustion of fossil fuels, biofuel, and biomass, that mainly reflects traffic-related air pollution. BC should not be confused with carbon black (CB), which is the manufactured product found in vehicle tires and rubber automotive products. CB is simply a quasi graphitic form of nearly pure elemental carbon that is distinguished by its verly low quantities of extractable organic compounds and total inorganics (< 1%). BC on the other hand has a higher load of solvent extractable organic matter (>20%), and more PAHs (1%), oxygen and hydrogen on the surface, making it fundamentally different from CB with respect to reactivity and oxidation behaviour.[9]

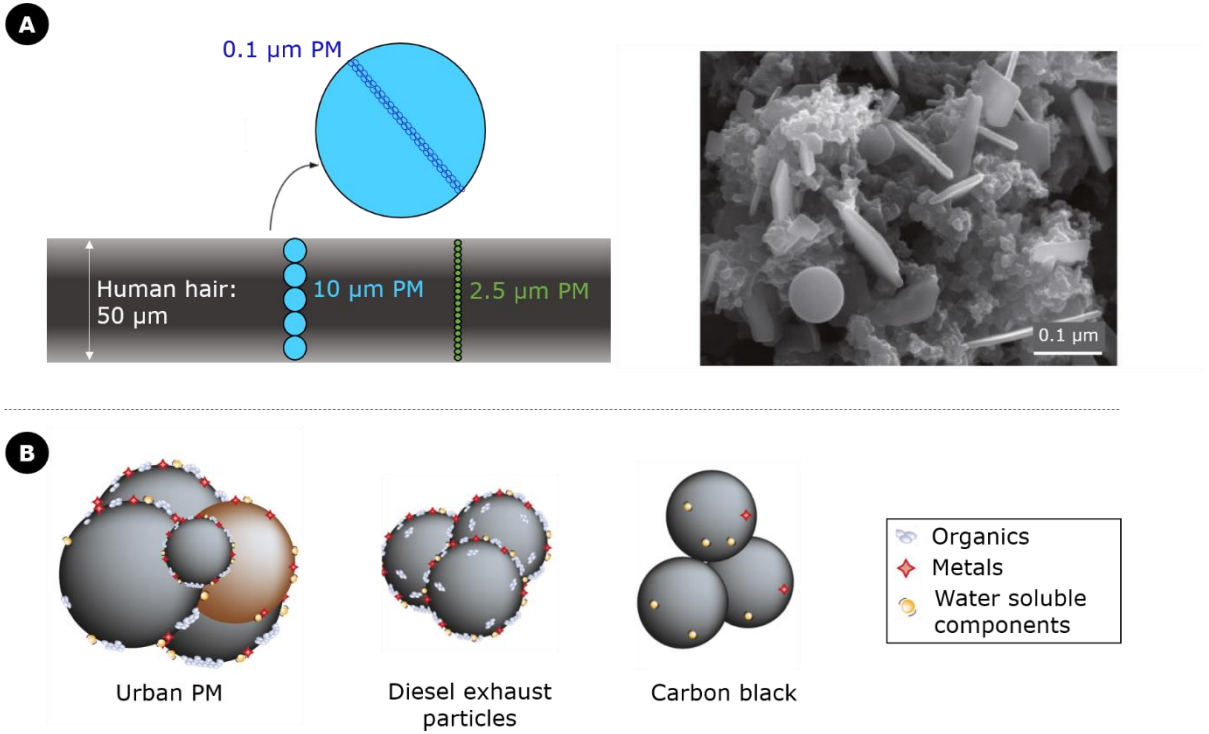

Figure 2: Categorization of air pollution. (**A**) Different size ranges of PM compared to human hair. The electron micrograph shows urban PM, highlighting its wide range of particle sizes, shapes and surface characteristics. (**B**) Differences in composition of several types of environmental and model compound PM. Urban PM represents a mixed and variable set of particles. A large proportion of the mass will be large particles (with micrometer diameter); however, a sizable proportion of the number of particles is often in the nanometer range (<100 nm diameter). Diesel exhaust particulate (DEP) are aggregates of particles with a primary particle size of between 20–100 nm. The composition will vary considerably depending on the source of DEP, however, often they are rich in metal and organic constituents. Carbon black is a 'pure' model carbon particle, often used as a 'clean' source of carbon or negative control particle. It can be produced in specific size ranges; however, most frequently used is the ultrafine fraction of these carbon particles (adapted from Miller *et al.* 2012[10]).

Air quality standards and legislation

According to the WHO assessment of the burden of disease due to air pollution, more than 3 million premature deaths (based on the report of 2012) can be attributed to the effects of urban outdoor air pollution and indoor air pollution. The WHO updated the Air Quality Guidelines for PM, NO_2, SO_2, and O_3 in 2005. These guidelines are based on evidence derived from air quality and adverse health effects in humans. For PM, the annual mean concentrations are set at 10 μg/m³ for $PM_{2.5}$ (particles with an aerodynamic diameter ≤ 2.5 μm) and 20 μg/m³ for PM_{10} (particles with an aerodynamic diameter ≤ 10 μm). The European Union has set less strict air quality standards because of a substantial body of Community legislation that has been adopted aside from the human health effects (**Table 1**).

Table 1: Air quality guidelines for particulate matter

Annual average (μg/m³)	European guidelines	WHO guidelines
PM_{10}	40	20
$PM_{2.5}$	25	10

Particle deposition, clearance and extrapulmonary translocation

Depending on their size, inhaled particles may deposit more or less deeply in the lungs, may translocate to the systemic circulation, and can even cross the blood-brain barrier (**Figure 3**). Coarse particles can enter all lung compartments but mainly deposit in the upper airways, i.e., pharynx, larynx, and upper trachea. Fine and ultrafine particles penetrate more deeply into the lower respiratory tract, particularly in the smaller airways (bronchioles and alveoli). The larger particles predominantly settle by gravity or impact directly onto airway walls, while UFP deposition largely depends on diffusion.[11] Hereby, UFPs are more homogeneously distributed onto the epithelia over the various pulmonary regions.

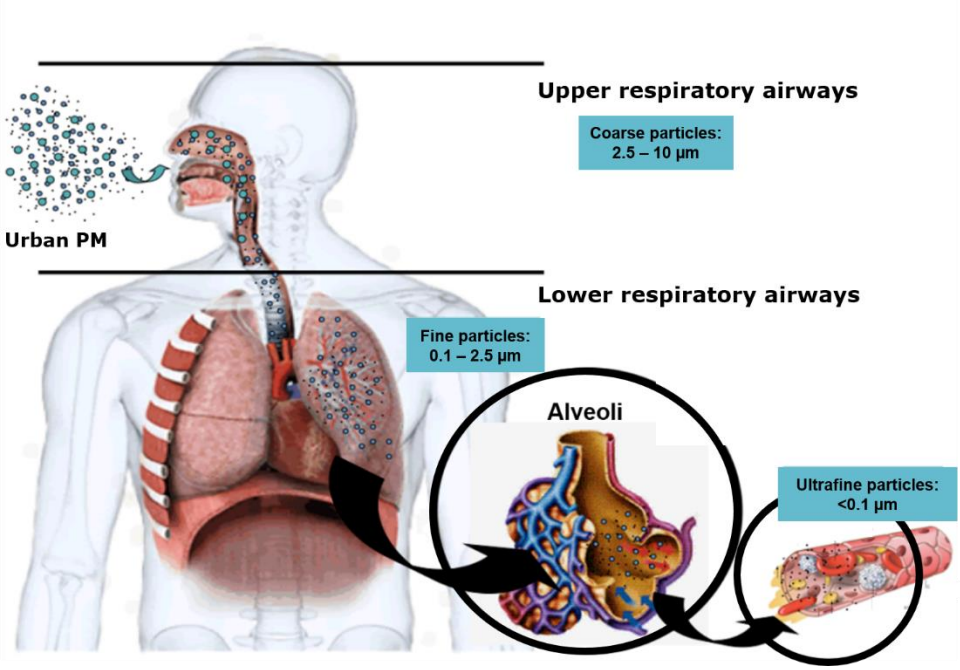

Figure 3: Particle-size-dependent pulmonary deposition of particulate matter (adapted from Lefol Nani Guarieiro L. and Lefol Nani Guarieiro A. 2013 [12]).

The clearance of deposited particles is an important aspect of lung defense. The specific mechanisms available for the removal of particles from the respiratory tract include both physical and biological processes (e.g. mucociliary movement, epithelial endocytosis, leaching or protein binding) and vary with the site of the deposition.[13] Depending on the specific clearance mechanism used, particles are cleared to i) the stomach and gastrointestinal tract from the upper and lower airways with elimination through feces, ii) the lymphatics and lymph nodes where they may be dissolved and entered in the venous circulation, and iii) the systemic blood circulation from where other organs such as bone marrow, spleen, liver, heart, kidneys, and brain can be targeted (**Figure 4**).

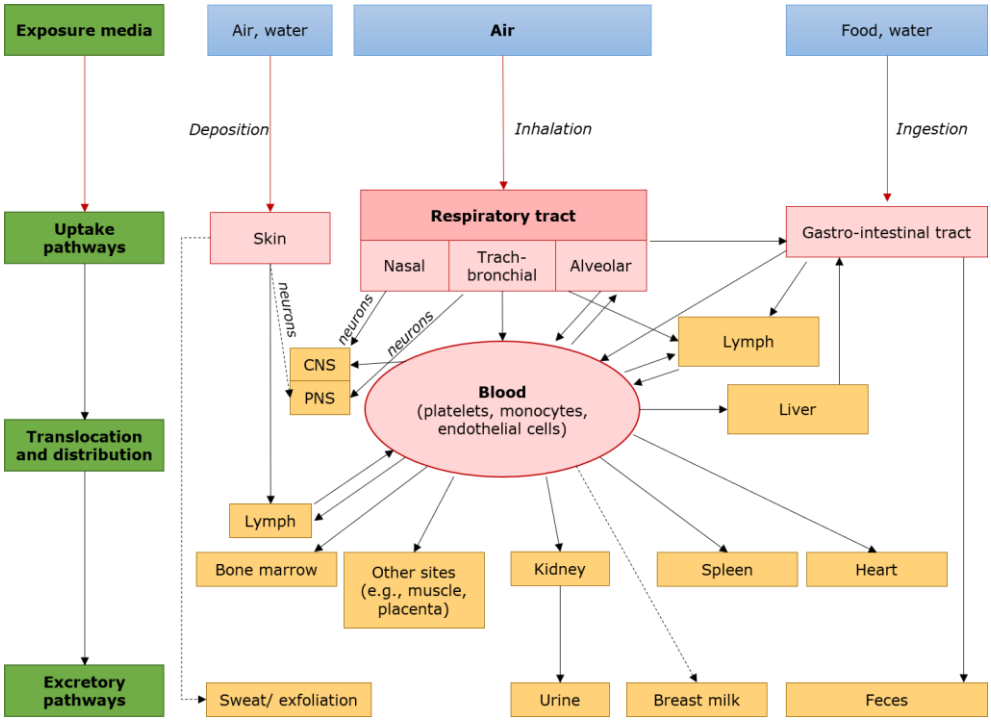

Figure 4: Exposure and biokinetics of nanoparticles (uptake, distribution, elimination). Translocation rates are in general very low. (Adapted from Casarett & Doull's. Toxicology: The Basic Science of Poisons. 8th edition[14])

In this respect, there are several primary ways by which particulate material is removed from the lower respiratory tract once it has been deposited. First, particles trapped on the fluid layer of the conducting airways by impaction are cleared within 24 to 48h upward in the tracheobronchial tree via the mucociliary escalator. Second, particles phagocytized by alveolar macrophages may also be cleared via the mucociliary escalator or via lymphatic drainage. Third, chemical material may dissolve from the surface of particles and be removed via the bloodstream or lymphatics. Fourth, small particles may directly penetrate epithelial membranes (**Figure 5**).[15]

A few minutes after inhalation, fine particles may be found in alveolar macrophages. Many of these particle-laden alveolar macrophages are ultimately transported to the mucociliary escalator of the bronchioles via the alveolar fluid that contributes to the fluid layer in the airways. In humans, the retention half-time of solid particles in the lower respiratory airways may be up to two years. When particles are not cleared from the lung they can be retained for prolonged periods, often in macrophages located in the interstitium, resulting in accumulation in the lung tissue. [13, 15]

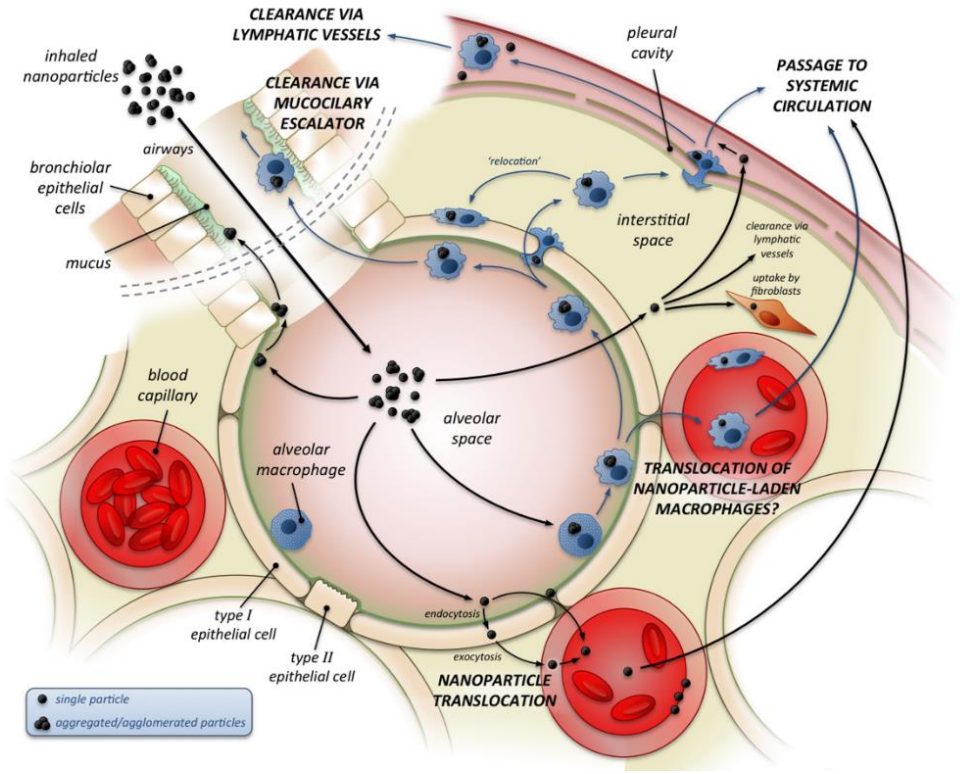

Figure 5: Pathways of relocation of inhaled nano-sized particles in lungs based on animal models (from Stone V. *et al.* 2017[16])

UFPs are cleared less efficiently from the lungs as alveolar macrophages are incapable to phagocytose these small particles. In rats, the phenomenon of 'overload pathology' occurs following exposure to high concentrations of airborne particles,[17] and when particles accumulate in the lungs to a point where there is failure of clearance, increased build up of dose, inflammation, proliferation, fibrosis, and tumour production.[18] Even low toxicity particles, such as carbon black and titanium dioxide (TiO_2), can cause this overload pathology in rats. It has been shown that the particle surface area is the measure that controls overload.[19] This is especially relevant as ultrafine particles at relatively low mass have a high surface area (**Table 2**). When rats were exposed to an overload airborne mass concentration of fine and ultrafine TiO_2 (23 mg/m³), there was far more bronchoalveolar inflammation in the group with ultrafine exposure. Furthermore, these rats showed a notable increase in interstitial and lymph node transfer compared to those exposed with fine particles.[20] At low dose airborne mass concentration (1 mg/m³), rats exposed to ultrafine carbon black had detectable proinflammatory local and systemic effects whereas non-ultrafine carbon black did not.[21] Why particle surface area is the most appropriate measure of dose toxicity is not well understood, but may be related to free radical activity and oxidative stress resulting from biological interaction with the particle surface.[17]

Table 2: Physical characteristics of a cloud of particles with airborne mass concentration of 10 µg/m³ (from Donaldson *et al*. 2001[17])

Airborne mass concentration (µg/m³)	Particle diameter (µm)	Particles/ml of air	Particle surface area (µ³/ml air)
10	2	1.2	24
10	0.5	153	120
10	0.02	2,400,000	3016

Through diffusion, UFP particles can readily gain access to the lung epithelium and penetrate interstitial sites. Once in the pulmonary interstitial sites, uptake in the blood circulation can occur. Animal studies have shown that a substantial fraction of intratracheally introduced ultrafine particles could translocate into the systemic circulation,[22] and may even translocate via the olfactory nerve to the brain when deposited in the nose.[23] Moreover, examination of brains of individuals who died suddenly and resided in cities with high air

pollution exposure revealed the presence of ultrafine particles in cerebral tissue.[24, 25] Although experimental evidence shows the translocating potential of inhaled particles, further studies are still needed to fully elucidate the toxicokinetics of extrapulmonary translocation of UFP to other organs in humans.

Health effects of PM-related air pollution

Clean air is considered to be a basic requirement of human health and well-being. However, air pollution continues to pose a significant threat to health worldwide. Current concentrations of ambient air pollution have been associated with a range of adverse health effects, particularly mortality and morbidity due to cardiovascular [4, 5] and respiratory diseases.[26, 27] Recently, air pollution has been classified as an IARC Group 1 carcinogen to humans.[8, 28]

Adverse health effects from air pollution are not equally distributed among populations and individuals. Differences in vulnerability and genetic susceptibility may affect the risk of developing an adverse health effect and its severity. Many of these differences are linked to population characteristics including 1) life stage, specifically children and elderly; 2) co-morbidity, such as preexisting cardiovascular (e.g. hypertension, coronary heart disease) or respiratory diseases (e.g. chronic obstructive pulmonary disease, asthma); 3) socioeconomic status; and 4) specific genetic predisposition.[29]

With regard to life stage, specific populations may be more prone to PM-induced health effects than the active adult population (20-60 years) as a result of behavioral and physiological differences. Children for example spend more time outdoors, and have a higher respiratory and activity level compared to adults, which can lead to an increased PM dose per lung surface area and adversely influence the developing lungs.[29] Epidemiological studies pointed to increasing respiratory symptoms and effects, such as wheezing,[30] cough,[31] or acute bronchitis,[32] in association with PM exposure in children. Likewise, fetuses and newborns are physiologically immature, and are especially vulnerable to environmental toxicants in sensitive periods of development because of higher rates of cell proliferation, or varying metabolic capabilities.[33] Accordingly, exposure to ambient PM air pollution has been found a determinant of an increased risk of low birth weight and prematurity.[34-36]

Air pollution: a suspected developmental neurotoxicant

Increasing evidence suggests that environmental agents, including air pollution, influence *in utero* programming of the developing central nervous system (CNS), and may even induce permanent long-lasting changes in disease physiopathology later in life.[37] Experimental studies demonstrated a wide range of biological CNS effects of air pollution exposure. Rodents exposed to diesel exhaust by inhalation demonstrated changes in expression of inflammatory and immune response-related genes, neurotrophins,[38] pro-inflammatory cytokines,[38-40] microglial activation,[41, 42] and oxidative stress[43]. Mice instilled with nanoparticles showed changes in neurotransmitter levels and expression of proinflammatory cytokines.[44, 45] Furthermore, rodents which were prenatally exposed to diesel exhaust showed gene expression differences of stress-related proteins and steroid hormones,[46] and the level and turnover of neurotransmitters, including dopamine,[47, 48] noradrenaline[48], and serotonin[48]. One study in dogs living in Mexico City showed chronic brain inflammation and neurodegeneration compared to dogs living in clean(er) areas.[49]

With regard to humans, two studies investigating the brains of humans who suddenly died revealed that residential air pollution was associated with increased inflammation in the brain.[25, 50] Moreover, epidemiological studies have reported that prenatal and early-life air pollution exposure is associated with altered cognitive function in children. Kim *et al.*[51] observed altered psychomotor and mental development in association with PM_{10} exposure during pregnancy. Children from different European cohorts showed altered psychomotor development in early childhood in association with residential NO_2 concentrations during pregnancy.[52] The Project Viva Cohort, a longitudinal pre-birth cohort in eastern Massachusetts that examines which events during early development affects health, showed that children living less than 50 m from a major roadway at the time of birth had lower nonverbal IQ compared to children living at least 200m away.[53] Furthermore, prenatal exposure to polycyclic aromatic hydrocarbon (PAH) air pollutants may contribute to slower mental processing speed, and attention-deficit/hyperactivity-disorder (ADHD) symptoms in urban youth by disruptions in the development of hemispheric white matter.[54]

Although these studies are suggestive of a neurobehavioral performance deficit associated with fine particle air pollution, there is still insufficient evidence

on the consistency of these associations [55] and the link with underlying mode(s) of action.

UNDERLYING MODE(S) OF ACTION OF AIR POLLUTION

Oxidative stress and inflammation: toxicity mediators of air pollution

Potential biological mechanisms through which air pollutants may exert their toxic effects are oxidative stress and inflammation (**Figure 6**). Air pollutants, such as PM, nitrogen oxides, and transition metals are able to generate reactive oxygen species, thereby inducing oxidative stress. More specifically with regard to PM, different pathways may be responsible for the production of free radicals and reactive oxygen species: 1) particles may interact with receptors on the alveolar surface, eliciting redox reactions,[17] 2) particles, small enough to translocate from the alveolar membrane into the bloodstream, can interact with vascular endothelium cells via stimulation of enzymes,[10] or 3) particle-induced activation of inflammatory cells releasing oxidative and nitrosative mediators with concomitant increase of oxidative stress-induced DNA damage.[56] These different oxidative stress-related pathways depend on the chemicals present on the surface of PM and can affect the human body either indirectly via a proinflammatory response that leads to increased systemic inflammation, or directly via translocation of inhaled particles from the lung into the bloodstream, leading to oxidative stress in blood cells and surrounding organs.

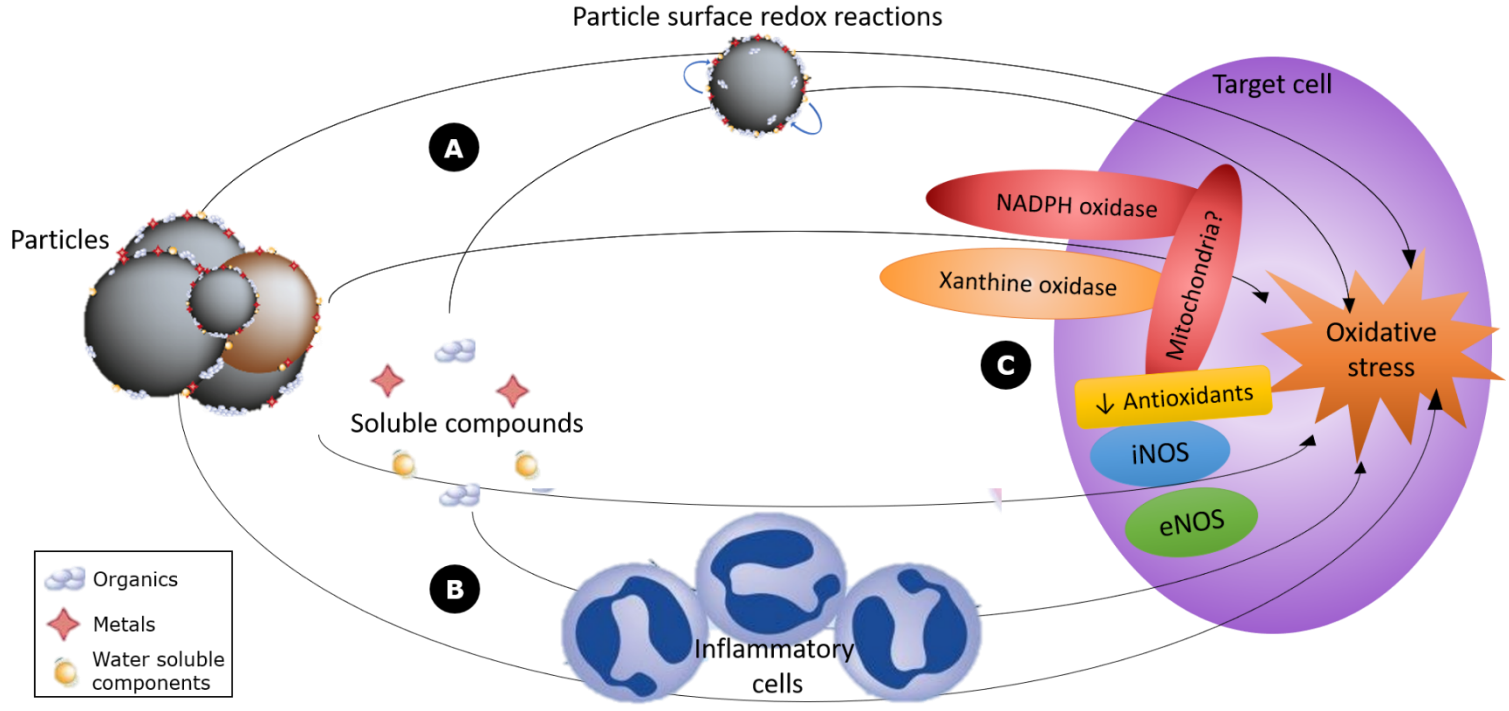

Figure 6. Different pathways through which contact between particulate matter and cells can induce cellular oxidative stress. **A**) Innate generation of free radicals by particulate matter (PM) in the absence of biological tissue. Free radicals may be produced by redox reactions between different chemicals on the surface of the particulate. **B**) Release of cytokines and oxidative mediators from PM-induced activation of inflammatory cells. **C**) Free radical generation from interaction between PM and cells (e.g., from stimulation of enzymes, such as NADPH oxidase, xanthine oxidase, uncoupling of eNOS, iNOS, exacerbation of free radicals from mitochondrial inefficiency or depletion of antioxidant defences). These pathways may be stimulated by particulate itself or through release of soluble constituents of PM in biological fluids. eNOS: Endothelial nitric oxide synthase; iNOS: Inducible nitric oxide synthase (adapted from Miller et al. 2012[10]).

Epigenetic mechanisms

Epigenetics deal with heritable variation in gene expression that is not genetically determined.[57, 58] There are four interacting systems ensuring epigenetic control: DNA methylation, histone modification, non-coding RNA's, and chromatin remodelling. These communicating mechanisms ensure tissue-specific gene expression, genomic imprinting, and stem-cell maintenance.

The most extensively studied epigenetic mechanism is DNA methylation. It occurs as a covalent addition of methyl groups ($-CH_3$) to cytosines that precedes a guanine in the DNA sequence (the CpG dinucleotide) and thereby determines the process by which genes are converted to mRNA.[59] The pattern of methylation strongly influences gene expression. While methylated gene promoters usually lead to transcriptional silencing, it has been shown that gene body methylation is a feature of transcribed genes.[60] Methylation patterns can be clustered in differentially methylated regions (DMRs), which are contiguous genomic regions that respond to regulatory signals. DMRs are involved in imprinting, ageing and diseases such as cancer.[58]

During *in utero* development, a process called '*epigenetic reprogramming*' occurs involving erasure and re-establishment of chromatin modifications, mainly by differential DNA methylation of promoter regions of imprinted genes. It serves to remove random changes in epigenetic marks (*i.e.*, epimutations) that have occurred in the germ cells (*i.e.*, gametes) and restores the ability of the zygote to develop into different cell types and tissues.[61] Epigenetic modifications are modulated in a temporal and spatial manner and act as reversible switches of gene expression that can lock genes into active or repressed states. In addition, these modifications allow the zygote to give rise to the cellular lineages that will form the embryo. Reprogramming occurs in two distinct phases during *in utero* development, one shortly after fertilization and the other in the developing gametes of the fetus (**Figure 7**).[62] After fertilization, the first phase takes place in the blastocyst where embryonic epigenetic patterns are erased and re-established in normal body cells (*i.e.*, somatic cells). The second phase occurs in the gametes where both imprinted and non-imprinted *loci* become demethylated. This total erasure of epigenetic information is required for the totipotency of future germ cells, imprinting switching, and prevention of inheritance of epigenetic defects. Then *de novo* DNA methylation begins, in which sex-specific methylation

of imprinted genes is established and reprogramming of non-imprinted genes occurs.[63]

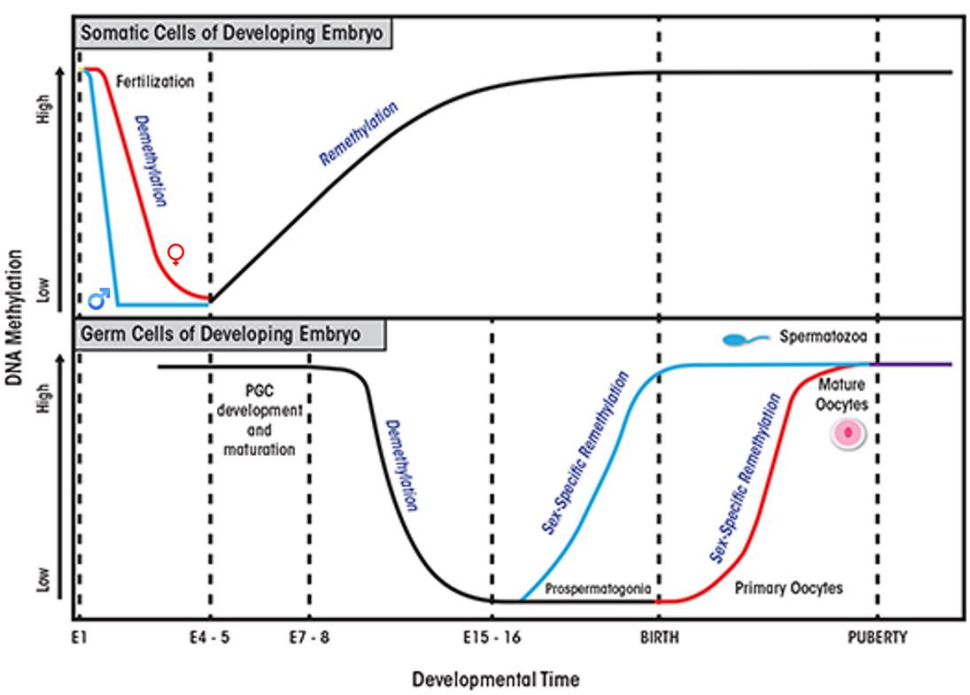

Figure 7: Reprogramming in mammalian development. Two waves of epigenetic reprogramming occur during embryo development. The first phase of reprogramming occurs in the normal body cells (i.e., somatic cells) of the developing embryo. In mice, following fertilization (E1), the embryo undergoes genome-wide demethylation that is completed by embryonic day 5 (E5). The paternal genome (blue line) undergoes rapid, active demethylation, whereas in the maternal genome (red line), demethylation occurs via a passive process. Remethylation of the mouse embryonic genome begins at day E5 and is completed prior to birth. The second phase of epigenetic reprogramming occurs in the germ cells of the developing embryo, which will ultimately give rise to gametes that contain sex-specific epigenetic signatures. The primordial germ cells (PGCs) of the developing embryo contain the methylation signatures of the parental genomes. At approximately E7–8, the PGCs undergo rapid demethylation that is complete by E15–16. Following this, a sex-specific methylation is re-established. In the male germline, reprogramming is complete at birth (blue line), whereas in females, reprogramming continues until puberty (red line). (from Ungerer, M *et al.* 2013[62]).

Epigenetic reprogramming in early embryonic development is a critical period in which external factors can affect the epigenetic regulation. This is illustrated by the Dutch Hunger Winter study showing that individuals who were prenatally exposed to famine in 1944-1945 had less DNA methylation of the imprinted Insulin-like growth factor 2 (*IGF2*) gene compared to their unexposed, same-sex sibling.[64] These results were the first empirical support to highlight that an anomaly in early-life environmental conditions can cause epigenetic changes in humans that persist throughout life. With respect to PM air pollution, prenatal exposure has been shown to affect global DNA methylation[65] as well as gene-specific mitochondrial methylation at birth.[66]

THE PLACENTA: A SURROGATE TISSUE FOR STUDYING FETAL (NEURO)DEVELOPMENT?

Perturbation of developmental adaptive processes in the fetus that are known to be involved in permanent changes in prenatal physiology, structure, and metabolism or early life programming, can adversely affect brain development and impact both brain structure and function.[67] Evidence has shown that transcriptional changes during prenatal development are associated with morphological and functional development of the CNS.[68] Hence, environmentally driven epigenetic alterations in early life, including DNA methylation, may play a determining role in the *in utero* gene expression processes, [69] because epigenetic changes in the developing brain are considered to be robust and lasting when occurring during the prenatal period.[70] Epigenetic alterations are suggested to be involved in the pathogenesis of some neurological disorders in adulthood.[71, 72]

The fetal origins of health and disease

Unfavourable conditions during life in the womb and in childhood do not only affect children's health but also predispose them to increased risk of diseases in adulthood. Adverse influences during fetal life modify structural, hormonal and metabolic processes, a phenomenom called fetal 'programming', that leads to persisting changes with lasting effects.[73] This concept has been introduced by Professor David Barker, who was the first to recognize the potential link between malnutrition during pregnancy and the development of coronary heart disease in

adult life.[74] Since then, many implications of the Developmental Origins of Health and Disease (DOHaD) hypothesis have been reported. Early embryonic development is of special interest in this respect, because this is a crucial period for establishing and maintaining epigenetic marks.[61]

The placenta: a versatile, ephemeral organ

The placenta is the most essential organ during the prenatal stages of life. It is a multifunctional organ that acts both as a gateway for nutrients and oxygen from mother to the fetus, and as exit for waste products such as urea, creatinine and carbon dioxide. During its nine months existence, it performs actions that are later taken over by diverse separate organs, including the lungs, kidneys, heart, gut, liver and endocrine glands. Furthermore, the placenta adopts other strategies that are important for facilitating transfer, such as remodelling of the maternal uterine arteries to ensure optimal perfusion. By virtue of these roles, the placenta plays a pivotal role in fetal programming.

Development of the placenta begins during implantation (**Figure 8**). The placenta is formed from the zygote, early in pregnancy, and thus has the same genetic composition as the fetus. When the blastocyst adheres to, and penetrates the endometrial epithelium, trophoblast cells differentiate into an inner cytotrophoblast layer and an outer syncytiotrophoblast layer (multinucleated syncytium). Trophoblast cells further differentiate into villous and extravillous trophoblast cells. The villous trophoblast cells give rise to chorionic villi, finger-like projections which are responsible for oxygen and nutrient transport between mother and fetus. The extravillous trophoblast cells migrate deeper into the uterine wall to anchor the placenta and penetrate the maternal vasculature. Placentation is completed 8-10 weeks after fertilizaton.[75]

Adequate exchange across the placenta between maternal and fetal circulation is essential for normal fetal metabolism and growth. Respiratory gases, carbohydrates and lipid-soluble substances are exchanged by diffusion; amino acids and some vitamins are actively transported; macromolecules such as immunoglobulins are probably transported by pinocytosis, and water is most likely exchanged in bulk flow in response to small hydrostatic or osmotic pressure gradients. Throughout gestation, the area of placental exchange increases as the villous surface area extends, accompanied with an increase of placental and fetal

weight. As the placenta is permeable to lipid-soluble substances, such as alcohol and nicotine, increased diffusion of these fetotoxic components can be harmful for the developing embryo or fetus.[76]

The placenta: a surrogate tissue for studying early-life (neuro)development

During the prenatal period, the placenta is the paramount organ for proper development and growth of the fetus. Except from its traditional role, there is growing appreciation that the placenta is also an important player in fetal CNS development through adaptive responses to the maternal environment.[77] In response to acute maternal food deprivation, Broad and Keverne (2011) observed in mice a strong co-expression of imprinted genes in both the hypothalamus and placenta during mid-gestation (embryonic day E11-E13), a crucial period of neuronal proliferation and differentiation.[78] Another study using an *ex vivo* model showed that the placenta can convert maternal tryptophan into the neurotransmitter serotonin, providing serotonin as a primary source to the developing mouse forebrain at mid-gestation.[79] In addition, Goeden *et al.* (2016) [80] revealed altered placental serotonin production in mice as a new mechanism by which maternal inflammation disrupts serotonin-dependent neurodevelopmental processes. Taken together, these studies suggest a key role of the placenta in early-life neurodevelopment.

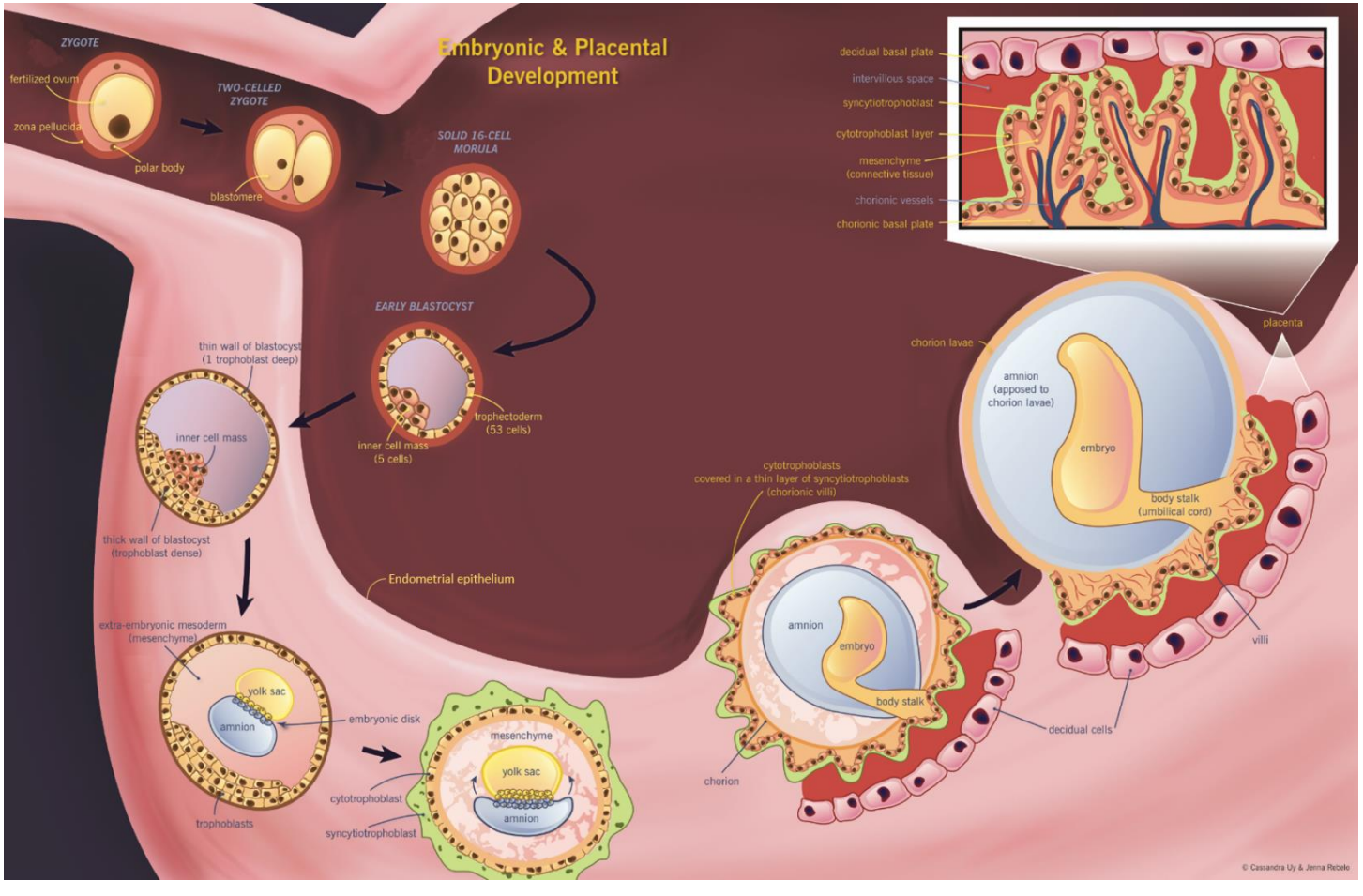

Figure 8. Development of the placenta and embryo (adapted from Gerster K. 2012[81])

AIMS OF THE STUDY

Studies on neurodevelopmental effects of air pollution exposure early in life and their potential underlying indicators are still scarce. In this PhD project, we investigated the association between air pollution and cognitive performance in children, and examined potential molecular signatures during prenatal development using the placenta as a surrogate tissue for studying fetal (neuro)development.

The specific goals of this doctoral dissertation were:

1. To evaluate potential placental molecular signatures associated with air pollution exposure and/or (neuro)developmental processes during prenatal life.

   In **chapter 1**, we investigated an oxidative stress marker, namely 3-nitrotyrosine, in placental tissue in association with air pollution exposure during pregnancy. In **chapter 2**, we evaluated in the placenta whether alterations at transcriptional level of the Brain-derived neurotrophic factor pathway, an important signaling pathway in growth and development of the central and peripheral nervous system, were observed in association with prenatal air pollution exposure.

   In **chapter 3**, we investigated whether prenatal exposure to air pollution was associated with changes in DNA methylation of Leptin, an energy-regulating hormone involved in fetal growth and development.
2. To investigate the association between air pollution exposure and cognitive performance in primary schoolchildren.

   Since evidence of air pollution effects on cognitive performance are still scarce and controversial, we investigated in **chapter 4** the association between recent *versus* chronic air pollution exposure and cognitive performance in primary schoolchildren.
3. To investigate a novel internal marker of residential ambient air pollution exposure

   Different experimental studies have demonstrated that ultrafine particles can translocate from the lung into the circulation. Though, the issue of particle translocation in humans is still controversial. To date, an internal exposure

marker to combustion-related air pollution originating from the circulation does not exist. In **chapter 5**, we summarized the methodology of the detection/determination of carbonaceous particles in urine as a novel internal marker of residential black carbon exposure.

PART 1: PLACENTA, (NEURO)DEVELOPMENT AND AIR POLLUTION

CHAPTER 1

PLACENTAL NITROSATIVE STRESS AND EXPOSURE TO AMBIENT AIR POLLUTION DURING GESTATION: A POPULATION STUDY*

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*Based on: Saenen ND *et al*. *Am J Epidemiol* 2016,184 (6): 442-449

ABSTRACT

Background: The placenta plays a crucial role in fetal growth and development through adaptive responses to perturbations of the maternal environment. We investigated the association between placental 3-nitrotyrosine (3-NTp), a biomarker of oxidative stress, and exposure to air pollutants during various time windows of pregnancy.

Methods: We measured the placental 3-NTp levels of 330 mother-newborn pairs, enrolled in the Environmental Influence *ON* Ageing in Early Life (ENVIR*ON*AGE) Study, a Belgian birth-cohort study (2010 to 2013). Daily concentrations of particulate matter with an aerodynamic diameter ≤2.5 μm ($PM_{2.5}$), black carbon (BC), and nitrogen dioxide were interpolated for each mother's residence using a spatiotemporal interpolation method.

Results: Placental 3-NTp levels, adjusted for covariates, increased by 35.0% (95% confidence interval (CI): 13.9, 60.0) for each interquartile range increment in entire pregnancy $PM_{2.5}$ exposure. The corresponding estimate for BC exposure was 13.9% (95% CI: −0.21, 29.9). These results were driven by the first [$PM_{2.5}$: 29.0% (95% CI: 4.9, 58.6); BC: 23.6% (95% CI: 4.4, 46.4)] and second [$PM_{2.5}$: 39.3% (95% CI: 12.3, 72.7)] gestational exposure windows.

Conclusions: This link between placental oxidative/nitrosative stress and exposure to fine particle air pollution during gestation is in line with experimental evidence on cigarette smoke and diesel exhaust exposure. Further research is needed to elucidate potential health consequences experienced later in life through particle-mediated nitrosative stress incurred during fetal life.

INTRODUCTION

Ambient air pollution has been linked to a variety of adverse health outcomes early in life in both the fetus and the neonate, such as infant mortality,[82] low birth weight,[34, 83] and low birth length and head circumference,[84] as well as diseases arising later in life such as pulmonary and cardiovascular disorders and even mortality.[85, 86] Oxidative and nitrosative stress are two putative main mechanisms by which air pollutants may exert their toxic effects.[87] Fine particles [particulate matter with an aerodynamic diameter ≤2.5 μm ($PM_{2.5}$)], black carbon (BC), ozone, nitrogen oxides, and transition metals are able to generate reactive oxygen species.[88] These pollutants can induce cellular oxidative and nitrosative stress via different pathways. With regard to fine particles, free radicals and reactive oxygen species can be formed through redox reactions between the particles and sensitive receptors on the alveolar surface, or particle-induced activation of inflammatory cells may release oxidative mediators,[56] or small particles could translocate from the alveolar membrane into the bloodstream and interact with vascular endothelium cells via stimulation of enzymes.[10] The excess of reactive oxygen species can lead to the generation of peroxynitrite, a reactive intermediate of the interaction between the superoxide radical and nitrogen oxide. Peroxynitrite is a potent and relatively long-living oxidant that can cause DNA strand-breaks, damage membrane lipids, and modify proteins particularly at the tyrosine residues.[89] The peroxynitrite attack on tyrosine groups of proteins gives rise to the formation of 3-nitrotyrosine (3-NTp). This well-known marker for oxidative stress[90] and inflammation[91, 92] is a molecular fingerprint of peroxynitrite because of its positive association with the rate of protein degradation.[93, 94] Increased 3-NTp levels have been observed in different illnesses and are regarded as a marker for Alzheimer's syndrome[95] and Parkinson's disease[96]. During gestation, 3-NTp formation in the placenta has been demonstrated in high-risk pregnancies including cases of pre-eclampsia and pre-gestational diabetes.[97, 98]

Evidence exists that 3-NTp, as measured in the blood plasma of bus drivers, is associated with traffic-related air pollution exposure[87]; however, to our knowledge, the relationship between 3-NTp in the placenta and exposure to ambient air pollutants during gestation has not yet been investigated. Because maternal exposure to ambient air in addition to pregnancy, a state of increased

oxidative stress, may exacerbate oxidative damage, we hypothesized that overproduction of free radicals may result in accumulation of 3-NTp insults in placental tissue. Therefore, we studied the association between placental 3-NTp and measures of exposure to ambient air pollutants, including $PM_{2.5}$, BC, and nitrogen dioxide during various time windows of pregnancy.

METHODS

Study population

Our investigations were embedded in the Environmental Influence *ON* Ageing in Early Life (ENVIR*ON*AGE) Study, an ongoing birth cohort study in Limburg, Belgium.[66, 99] We recruited expectant mothers on weekends when they arrived at East-Limburg Hospital (Genk, Belgium) for delivery, as during this period no competition with other studies existed. Enrollment in the birth cohort was spread equally over all seasons of the year and 61% of the eligible mothers participated. The inclusion criteria were that mothers 1) were able to fill out a Dutch language questionnaire and 2) delivered a singleton. The ENVIR*ON*AGE study protocol was approved by the ethical committees of Hasselt University (Hasselt, Belgium) and East-Limburg Hospital, and the study has been carried out according to the Declaration of Helsinki. Written informed consent was obtained from all participants at delivery, and a questionnaire inquiring about demographic and lifestyle characteristics was completed in the postnatal ward. We gathered information on maternal age, place of residence, pregestational body mass index [weight (kg)/height $(m)^2$], education, occupation, smoking status, alcohol consumption, use of medication, parity, and newborn's ethnicity. Data on perinatal parameters such as birth date, gestational age, newborn's sex, birth weight and length, Apgar score, and ultrasonographic factors were gathered immediately after delivery from the medical record. All neonates who, 5 minutes after birth, had an Apgar score ranging from 7 to 10 were considered healthy. Maternal educational level was coded as "low" (no diploma or primary school only), "medium" (high school diploma), or "high" (4-year college or university degree). We coded socio-economic status according to the United Kingdom Office for National Statistics using the Standard Occupational Classification hierarchy and

condensed it into "low" (including occupations belonging to groups 7–9), "middle" (groups 4–6), and "high" (groups 1–3).[100]

In the present study, 502 mother-newborn pairs were enrolled in the ENVIR*ON*AGE birth cohort between February 2010 and May 2013. For practical and financial reasons, we had to randomly select 400 placentas which were used for multiple biomolecular assays. From these 400 mother-newborn pairs, we excluded 46 biopsies with insufficient tissue, 7 pairs with missing covariate data, 12 preterm newborns, and 5 mothers with gestational diabetes, which resulted in a final study population of 330 mother-newborn pairs. The study population did not differ from the entire birth cohort or the source population (northern part of Belgium) (Supplemental Material Table S1).[101]

Placental sampling

Placentas were collected and deep-frozen within 10 minutes after delivery. After thawing, the placental tissue samples were taken in the middle region of the fetal side of the placenta, approximately 4 cm away from the implantation of the cord and to the right of the main artery. To avoid membrane contamination, we sampled 1–1.5 cm below this membrane. Since the samples were used for multiple measurements, they were stabilized in RNAlater (Qiagen, KJ Venlo, Netherlands), incubated at 4°C for 24 hours, and stored at −20°C.

3-NTp protein measurement

After thawing, 10 mg of placental tissue (wet weight) was homogenized in a mixture containing lysis buffer [10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, and Complete Mini Protease Inhibitor Cocktail (Hoffman La Roche, Basel, Switzerland)] by sonicating 3 times in bursts of 10 seconds. The samples were allowed to settle for 20 minutes on ice and then centrifuged at 16 000×g for 20 minutes at 4°C. The supernatant was aliquoted and frozen at -20°C.

The total protein concentration of the supernatant was determined with the Bio-Rad protein assay (Bio-Rad Laboratories, Temse, Belgium) according to the manufacturer's instructions. The amount of 3-NTp in each sample was quantified with a competitive ELISA (Oxiselect Nitrotyrosine ELISA kit, CellBioLabs, Inc., San

Diego, CA, USA). Briefly, 50 µl of the samples and nitrated bovine serum albumin standards were first added to a nitrated bovine serum albumin precoated enzyme immunoassay plate. After 10 minutes incubation, 50 µl of anti-3-NTp antibody was added, followed by 1 hour incubation on an orbital shaker and 3 wash steps. Then, 100 µl of horseradish peroxidase conjugated secondary antibody was added to the samples, and they were incubated for 1 hour and washed 3 times. Finally, 100 µL of substrate solution was added and the 3-NTp content of unknown samples was determined by comparing them with a curve of known nitrated bovine serum albumin standards. The 3-NTp content (nM) was normalised to the placental total protein content (mg) and expressed as nM/mg protein.

Exposure estimates

Regional background levels of $PM_{2.5}$, BC, and nitrogen dioxide (µg/m³) exposure were interpolated for each mother's residential address, using a spatiotemporal interpolation method [102] that takes into account land-cover data obtained from satellite images (CORINE land-cover data set[103]) and pollution data from fixed monitoring stations in combination with a dispersion model.[104, 105] This model provided daily interpolated exposure values in a high-resolution receptor grid using data from the Belgian telemetric air quality networks, point sources, and line sources. Overall model performance was evaluated by means of leave-1-out cross-validation including 34 monitoring points for $PM_{2.5}$, 14 for BC, and 44 for nitrogen dioxide. Validation statistics of the interpolation tool gave a spatiotemporal explained variance of more than 0.80 for $PM_{2.5}$[105], 0.74 for BC[106], and 0.78 for nitrogen dioxide[105].

To explore potentially critical exposure windows during pregnancy, we averaged the daily interpolated exposure concentrations (µg/m³) for each pregnancy trimester[107], that is, 1–13 weeks, 14–26 weeks, and 27 weeks to delivery. Date of conception was estimated on the basis of ultrasound data. Exposure throughout the entire pregnancy time window was calculated as the mean value for all days between the estimated date of conception and the date of delivery. When mothers moved during pregnancy, we recalculated exposure windows accounting for the residential changes during this period. Data on BC exposure were available for only 271 mother-newborn pairs.

Statistical analyses

Statistical analyses were carried out using SAS software (version 9.3; SAS Institute, Inc., Cary, North Carolina). Continuous data are presented as mean (standard deviation) and categorical data as frequencies and percentages. Data on 3-NTp level were log_{10}-transformed to improve the normality of the distribution. Pearson correlation coefficients were calculated to evaluate the correlations between placental 3-NTp and gestational exposure to $PM_{2.5}$, BC, or nitrogen dioxide. We performed multiple linear regression analyses between the same variables to assess the independent associations while accounting for significant covariates and covariates based on literature evidence. These included gestational age, maternal age, maternal educational level (low, middle, or high), pregestational body mass index, smoking status (never, past, or current smoker), newborn's sex, newborn's ethnicity (European origin or non-European origin), and seasonality of birth (warm or cold). The Shapiro-Wilk statistic and Q-Q plots of the residuals were used to test the assumptions of model linearity. The effect estimates were calculated for an interquartile-range increment of the independent variable. Results are presented as the percentage difference in 3-NTp content for an interquartile-range increment in $PM_{2.5}$, BC, or nitrogen dioxide exposure. In 4 sensitivity analyses, we performed linear regression to examine the associations between 3-NTp and $PM_{2.5}$ exposure after excluding smokers, induced deliveries, cesarean births, and persons of non-European origin, respectively. To study the relative importance of entire pregnancy $PM_{2.5}$ exposure, we mutually adjusted the main model results for entire pregnancy BC or nitrogen dioxide exposure. In addition to maternal education, we also explored the significance of socioeconomic status.

RESULTS

Study population characteristics

Demographic and lifestyle characteristics of the study population (n = 330) are presented in Table 1. Briefly, mothers had a mean age of 29.4 [standard deviation (SD), 4.6] years and a pregestational body mass index of 24.3 (SD, 4.6). Most of the mothers (55.2%; n = 182) had obtained a higher education degree, and 46 mothers (13.9%) reported having smoked during pregnancy, whereas 68.2% (n = 225) had never smoked cigarettes. The total newborn population [comprising 165 boys (50.0%)] had a mean gestational age of 39.5 weeks (range, 37–42). All newborns were term-born, and the sample included a high number of primiparous (53.0%; n = 175) and secundiparous (37.6%; n = 124) births. Most of the newborns were of European origin (86.7%; n = 286), and overall the mean birth weight and length were 3,443 (SD, 432) g and 50.5 (SD, 2.0) cm, respectively.

Exposure estimates

Table 2 displays the distributions of estimated daily levels of outdoor exposure to $PM_{2.5}$, BC, and nitrogen dioxide for the different time windows of pregnancy. The mean trimester-specific $PM_{2.5}$ exposure was 15.8 [interquartile range (IQR), 8.8] $\mu g/m^3$ for the first trimester of pregnancy, 15.3 (IQR, 7.4) $\mu g/m^3$ for the second trimester, 17.1 (IQR, 9.4) $\mu g/m^3$ for the third trimester, and 16.1 (IQR, 3.5) $\mu g/m^3$ for the entire pregnancy period. The corresponding exposure estimates for the other pollutants during these respective time windows were 0.90 (IQR, 0.54) $\mu g/m^3$, 0.95 (IQR, 0.56) $\mu g/m^3$, 1.05 (IQR, 0.46) $\mu g/m^3$, and 0.97 (IQR, 0.36) $\mu g/m^3$ for BC exposure and 19.8 (IQR, 9.1) $\mu g/m^3$, 20.2 (IQR, 8.8) $\mu g/m^3$, 21.7 (IQR, 8.4) $\mu g/m^3$, and 20.5 (IQR, 5.8) $\mu g/m^3$ for nitrogen dioxide exposure. For the entire pregnancy, nitrogen dioxide exposure and BC exposure correlated well with $PM_{2.5}$ exposure ($r = 0.75$ and $r = 0.64$, respectively).

Table 1. Characteristics of 330 mother-newborn pairs from the ENVIR*ON*AGE study, Limburg, Belgium, 2010-2013

Characteristics	Mean ± SD or number (%)
Maternal	
Age, years	29.4 ± 4.6
Pregestational body mass index[a]	24.3 ± 4.6
Educational level	
Low (less than high school)	38 (11.5)
Middle (high school diploma)	110 (33.3)
High (college or more)	182 (55.2)
Socioeconomic status[b]	
Low	87 (26.3)
Middle	187 (56.7)
High	56 (17.0)
Smoking status	
Never smoker	225 (68.2)
Former smoker	59 (17.9)
Current smoker	46 (13.9)
Parity	
1	175 (53.0)
2	124 (37.6)
≥ 3	31 (9.4)
Newborn	
Male sex	165 (50.0)
European-origin ethnicity	286 (86.7)
Gestational age, weeks	39.5 ± 1.1
Born at term (≥ 37 w)	330 (100)
Cesarean birth	16 (4.9)
Induced delivery	26 (7.9)
Seasonality	
Cold (autumn or winter)	190 (57.6)
Warm (spring or summer)	140 (42.4)
Apgar score after 5 minutes	
7	7 (2.1
8	18 (5.4)
9	87 (26.4)
10	218 (66.1)
Birth weight, g	3,443 ± 432
Birth length, cm	50.5 ± 2.0

[a] Body mass index was calculated as weight (kg)/height $(m)^2$.

[b] Socioeconomic status was coded according to the United Kingdom Office for National Statistics using the Standard Occupational Classification hierarchy and condensed into "low"(including occupations belonging to groups 7–9), "middle" (groups4–6), and "high" (groups 1–3) [100].

Table 2 Distribution of Interpolated Air Pollution Exposures (μg/m^3) During Pregnancy From a Spatiotemporal and Dispersion Model Among 330 Mother-Newborn Pairs From the ENVIR*ON*AGE Study, Limburg, Belgium, 2010–2013

Exposure and Time window	Mean ± SD	5th percentile	25th percentile	Median (IQR)	75th percentile	95th percentile
$PM_{2.5}$						
Entire pregnancy	16.1 ± 2.4	12.4	14.2	16.1 (3.5)	17.6	20.3
Trimester 1	15.8 ± 5.6	9.1	11.2	13.7 (8.8)	20.1	26.0
Trimester 2	15.3 ± 4.8	9.6	11.3	14.6 (7.4)	18.8	24.4
Trimester 3	17.1 ± 5.7	9.3	12.0	16.8 (9.4)	21.5	27.0
BC[a]						
Entire pregnancy	0.97 ± 0.28	0.56	0.77	0.92 (0.36)	1.12	1.53
Trimester 1	0.90 ± 0.39	0.40	0.59	0.85 (0.54)	1.13	1.61
Trimester 2	0.95 ± 0.41	0.38	0.63	0.91 (0.56)	1.18	1.74
Trimester 3	1.05 ± 0.37	0.52	0.78	1.03 (0.46)	1.24	1.72
NO_2						
Entire pregnancy	20.5 ± 4.5	14.0	17.3	20.2 (5.8)	23.2	29.1
Trimester 1	19.8 ± 6.3	10.5	14.9	19.4 (9.1)	24.0	31.7
Trimester 2	20.2 ± 6.3	11.3	15.6	19.8 (8.8)	24.4	30.8
Trimester 3	21.7 ± 6.1	12.0	17.3	21.6 (8.4)	25.7	32.0

[a] Data were available for 271 mother-newborn pairs.

Association between placental 3-NTp and ambient air pollution during various time windows of pregnancy

The geometric mean value for placental 3-NTp was 3,735 (range, 212 – 23,682) nM/mg protein. There was no association between placental 3-NTp and the covariates shown in Table 1, except for pregestational body mass index (-3.2%, 95% confidence interval (CI): -5.2, -1.3; P = 0.006) and season of birth (cold season vs. warm season) (-21.8% (95% CI: -35.1, -5.8; P = 0.01)). We observed a positive and significant correlation between placental 3-NTp and entire pregnancy exposure to $PM_{2.5}$ (r = 0.27, P < 0.0001), BC (r = 0.13, P = 0.03) or nitrogen dioxide (r = 0.15, P = 0.007) (Figure 1). After adjustment for gestational age, maternal age, maternal education, pregestational body mass index, smoking status, newborn's sex, newborn's ethnicity, and seasonality (Table 3), we observed similar results as in the unadjusted model for the association between placental 3-NTp and entire pregnancy $PM_{2.5}$ or BC exposure. For an interquartile range increment in $PM_{2.5}$ exposure over the entire pregnancy period, placental 3-NTp increased by 35.0% (95% CI: 13.9, 60.0; P < 0.0006). The corresponding increase in placental 3-nitrotyrosine for black carbon exposure was 13.9% (95% CI: -0.21, 29.9; P = 0.05), however, no association was found between 3-NTp and entire pregnancy nitrogen dioxide exposure (P = 0.17).

To assess which time window of pregnancy is driving the results of the entire pregnancy exposure, we examined the association between placental 3-nitrotyrosine and each gestational window of exposure. For $PM_{2.5}$ exposure, the associations were only significant across the first and second trimesters of pregnancy, i.e., first trimester [29.0% (95% CI: 4.9, 58.6; P = 0.02)] and second trimester [39.3% (95% CI: 12.3, 72.7; P = 0.003)]. For black carbon exposure, the results were driven by the association between placental 3-nitrotyrosine and the first trimester exposure window [23.6% (95% CI: 4.4, 46.4, P = 0.01)], whereas for nitrogen dioxide exposure only a tendency was observed for the first trimester exposure window [14.7% (95% CI: -1.3, 33.4, P = 0.07)].

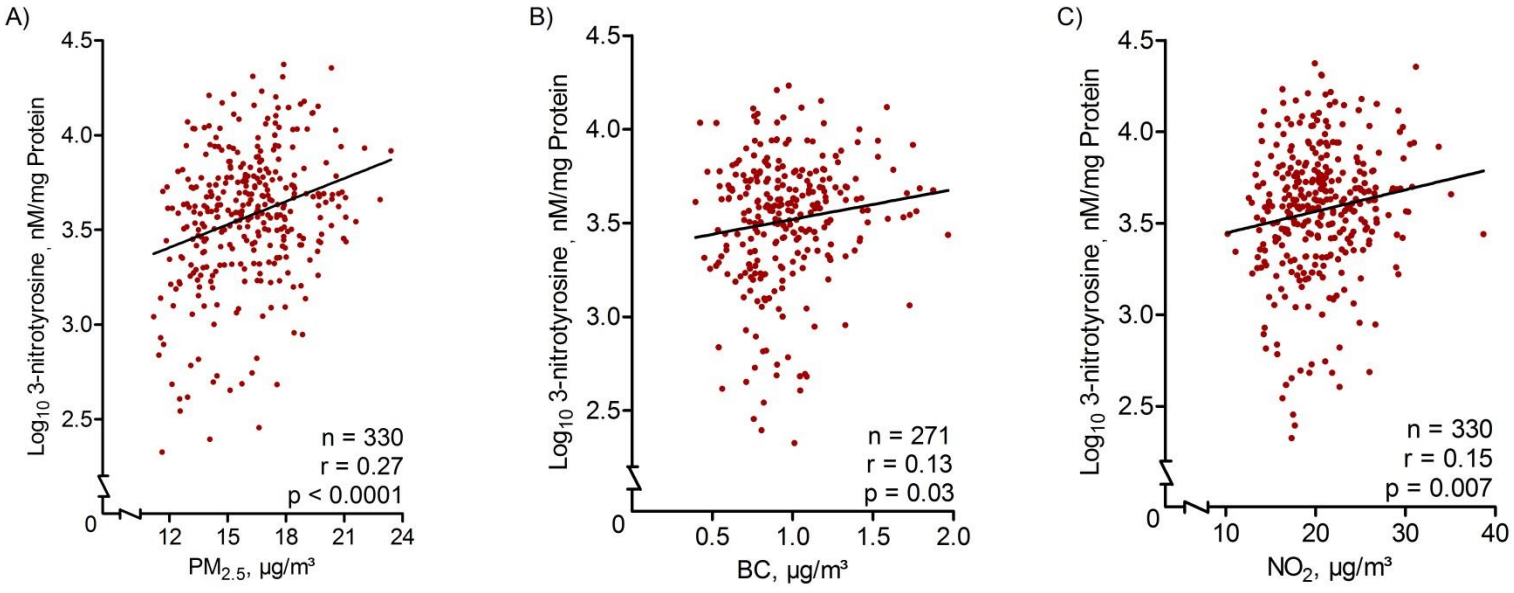

Figure 1 Pearson correlations between placental 3-nitrotyrosine levels and entire pregnancy exposure to particulate matter with an aerodynamic diameter ≤ 2.5 µm ($PM_{2.5}$) (n = 330, r = 0.27, *P* < 0.0001) (A), black carbon (n = 271, r = 0.13, *P* = 0.03) (B), and nitrogen dioxide (n = 330, r = 0.15, *P* = 0.007) (C) among mother-newborn pairs from the ENVIR*ON*AGE Study (Limburg, Belgium, 2010-2013).

Table 3 Estimated increase (%) in placental 3-nitrotyrosine content associated with an interquartile range increment in air pollution exposure during pregnancy among 330 mother-newborn pairs from the ENVIR*ON*AGE study, Limburg, Belgium, 2010-2013

Exposure and Time window	Adjusted regression coefficients[a], %	95% CI	*P*-value
$PM_{2.5}$			
Entire pregnancy	35.0	13.9, 60.0	0.0006
Trimester 1	29.0	4.9, 58.6	0.02
Trimester 2	39.3	12.3, 72.7	0.003
Trimester 3	13.2	-9.4, 41.3	0.27
BC[b]			
Entire pregnancy	13.9	-0.21, 29.9	0.05
Trimester 1	23.6	4.4, 46.4	0.01
Trimester 2	8.7	-9.9, 31.0	0.38
Trimester 3	-4.3	-19.7, 14.1	0.62
NO_2			
Entire pregnancy	9.7	-3.8, 25.0	0.17
Trimester 1	14.7	-1.3, 33.4	0.07
Trimester 2	0.25	-21.9, 27.4	0.98
Trimester 3	3.1	-16.3, 27.0	0.77

[a] Estimates were adjusted for gestational age, maternal age, maternal education, pregestational body mass index, smoking status, newborn's sex, newborn's ethnicity, and seasonality.
[b] Data were available for 271 mother-newborn pairs.

Sensitivity analysis

Excluding newborns of non-European origin (n = 44), induced deliveries (n = 26), or mothers who smoked before and during pregnancy (n = 105) did not alter the results of the main model for placental 3-NTp and ambient air pollution.

Excluding cesarean births (n = 16) from the analysis did not change our findings, except for entire pregnancy BC exposure (the P value changed from 0.05 to 0.09). Models including parity as a covariate did not alter the observed associations. Furthermore, mutual adjustment for entire pregnancy nitrogen dioxide or BC exposure in the main entire pregnancy $PM_{2.5}$ exposure model did not change our main findings. Maternal education was not associated with any of the air pollutants (P's ≥ 0.33). In addition, we did not observe a significant association between socioeconomic status and $PM_{2.5}$ (P = 0.52) or BC (P = 0.14) exposure. For nitrogen dioxide exposure, the lowest socioeconomic status group showed higher average residential nitrogen dioxide exposure [21.6 (SD, 4.6) µg/m3 vs. 19.6 (SD, 4.6)

μg/m^3; P = 0.02]. However, socioeconomic status was not associated with 3-NTp and therefore did not fulfill the definition of a confounder. Nevertheless, our results did not alter if we substituted maternal socioeconomic status for maternal education in the model.

DISCUSSION

The key finding of our study was that 3-NTp levels in the placenta were positively associated with $PM_{2.5}$ levels during gestation and that the associations were driven by exposures incurred during the first and second trimesters of gestation. For BC, the association with 3-NTp was mainly driven by the first trimester exposure window, and no associations were found with nitrogen dioxide exposure. Furthermore, the results for $PM_{2.5}$ remained the same after additional adjustment for BC or nitrogen dioxide exposure. Hence, our findings highlight, on the one hand, the relevance of placental 3-NTp as a biomarker of cumulative $PM_{2.5}$-induced prenatal oxidative stress and emphasize, on the other hand, the importance of investigating critical exposure windows throughout gestation, because susceptibility to fine particle air pollution may fluctuate during pregnancy.

The placenta regulates transport of water, gases, nutrients, and waste products between the fetus and the mother. This maternal-fetal barrier may also allow transfer of environmental chemicals and fine particles.[108] The placenta also acts as a sensor of the maternal-fetal environment and adapts to both intrinsic and extrinsic factors.[109] In mice as well as in humans, environmental insults such as fine particle air pollution may affect placental functional morphology and fetal growth.[34, 110] These detrimental changes in fetal programming may predetermine the risk of disease later in life.[111] A putative underlying mechanism of particulate matter-mediated adverse health outcomes is oxidative and nitrosative stress, as shown by the nitration of oxidative stress proteins in RAW 264.7 macrophages exposed *in vitro* to diesel exhaust particles.[112] Among 50 bus drivers in one study, an average $PM_{2.5}$ exposure of 32.1 μg/m³ resulted in increased oxidative and nitrosative stress, as reflected by protein carbonyl and 3-nitroyrosine levels in blood plasma, in comparison with 50 matched controls.[87] In our study, a much lower gestational $PM_{2.5}$ exposure (16.1 μg/m³) resulted in a wider range of 3-NTp levels in placenta (212 – 23,682 nM/mg protein) compared with the blood plasma

(471 – 3,228 nmol/L) of bus drivers. This may be due to the less developed detoxifying mechanisms in the fetus as well as the heterogeneity of cells in the placenta.

The biomarker 3-NTp is the stable product of protein nitration with peroxynitrite. Although nitration of placental proteins is found in normal pregnancies, at higher levels it may produce pronounced perturbations of placental function, as has been found in placental vessels in pathological pregnancies, such as those with preeclampsia and gestational diabetes.[97, 98] The targets for protein nitration are also involved in trophoblast invasion and regulation of placental vascular reactivity, which subsequently may affect placental function.[113] Therefore, nitrosative stress may be one of the underlying mechanisms that links altered placental function to changes in fetal programming.[114]

Hence, we hypothesize that particulate matter-induced oxidative stress during pregnancy may alter placental vascular function with the potential to affect fetal development and growth. In an experimental study in which mice with adverse intrauterine conditions were exposed to diesel exhaust particles, Weldy *et al.*[115] observed elevated 3-NTp protein modification, predominantly in perivascular regions on the fetal side of the placenta, suggesting that exposure to diesel exhaust particles *in utero* promotes vascular oxidative stress. In a study of atherosclerotic mice, long-term exposure to cigarette smoke free from nicotine and tar particles (15 minutes/day, 6 days/week, for 16 weeks) accelerated the accumulation of total cholesterol in the aorta and increased 3-NTp levels.[116] However, in a study of humans with stable coronary heart disease, acute exposure to ambient particles for 2 hours did not induce significant alterations in 3-NTp levels in exhaled breath condensate, nor did it affect vascular function.[117]

In our study population, we did not find significant associations between placental 3-NTp and birth weight ($P = 0.26$) or birth length ($P = 0.30$). This is in line with a finding in the entire ENVIR*ON*AGE birth cohort showing the absence of a direct association between birth weight and gestational $PM_{2.5}$ exposure.[118] This observation, however, does not necessarily imply that the stimulated reactive oxygen species formation associated with increased gestational $PM_{2.5}$ exposure would not influence more subtle biological events during the gestational growth of the fetus, for example, neurodevelopment.[99] Furthermore, we found that

gestational exposure to $PM_{2.5}$ and BC was associated with placental 3-NTp, which was mainly driven by the exposure incurred during the first and/or second trimesters. Recently, early and midpregnancy exposure windows were shown to be the most critical for adverse newborn health outcomes such as preterm birth.[36, 119] Moreover, Robledo *et al.*[120] observed that the preconception period may also be a potential window of susceptibility for increased risk of gestational diabetes mellitus in association with ambient air pollution (nitrogen oxides, sulfur dioxide, and ozone), but they did not find associations with particulate matter exposure. In our study population, no association was found between placental 3-NTp and nitrogen dioxide exposure either during or prior to pregnancy (Supplemental Material, Table S2). Nevertheless, the nitrosative stress-mediated health outcomes of ambient air pollution exposure during gestation are yet to be better characterized.

We cannot exclude the possibility that the associations observed in our study may be a reflection of systemic consequences of induced inflammatory conditions both in the pulmonary system of the mother and in placental tissue and/or may be related to translocation of ultrafine particles directly from the lung to the blood stream, from where they can pass the maternal-placental-fetal barrier.[56, 108] Therefore, we can currently only speculate about the mechanism of action with regard to whether inhalation of particles by the mother can elicit placental inflammatory reactions.

We acknowledge some limitations of our study. Protein nitration may be residue-, protein-, and tissue-specific, depending on the cellular location of the protein and the peroxynitrite generating system. Despite the fact that the biomarker 3-NTp only covers a fraction of the oxidative damage because of a limited number of proteins with preferential targets to nitration, evidence exists for nitration of placental signal transduction enzymes and transporters, which may markedly influence placental cellular functions.[90] The generalizability of our findings may be limited with regard to the placenta as a whole, since we used only 1 tissue sample of the fetal side of the placenta. Nevertheless, we consider the fetal side of the placenta as the most representative of the developing fetus. In addition, we standardized our sampling method by taking biopsies at a fixed location using a sampling device orientated to the implantation of the umbilical cord. We cannot exclude the possibility of a certain amount of selection bias in

our study population, as we could only recruit participants on weekends, and elective deliveries performed for medical reasons are scheduled for weekdays. At any rate, this selection procedure resulted in a healthy-pregnancy effect, limiting confounding effects of complicated pregnancies on 3-NTp outcome. Notwithstanding the fact that observational studies only allow investigators to characterize associations, there is also experimental evidence supporting our findings.[112, 115, 116]

CONCLUSIONS

To conclude, we observed a positive association between placental 3-NTp levels and exposure to $PM_{2.5}$ and possibly BC during pregnancy, which is in line with experimental evidence on exposure to cigarette smoke and diesel exhaust. Further research is needed to elucidate the potential health consequences later in life operating through particle-mediated nitrosative stress experienced during fetal life.

SUPPLEMENTAL MATERIAL

Supplemental Material, Table S1: Characteristics of the Study Participants from the ENVIR*ON*AGE Birth Cohort Compared to the Entire ENVIR*ON*AGE Birth Cohort (Limburg, Belgium, 2010-2013), and the Source Population (northern part of Belgium, 2002-2011)

Characteristic	Study participants (n=330)	ENVIR*ON*AGE (n=502)	Source population† (n=606,877)
Maternal age, y	29.4 (24.0-35.0)	29.2 (23.0-35.0)	29.5 (23.5-35.8)
<25	13.9%	15.2%	16.2%
25-34	71.5%	71.5%	70.7%
35+	14.6%	13.3%	13.1%
Pregestational body mass index	24.3 (19.6-30.7)	24.3 (19.7-30.6)	N/A
Maternal education			
Low	11.5%	12.0%[a]	13.1%
Middle	33.3%	35.8%[a]	40.8%
High	55.2%	52.2%[a]	46.1%
Parity			
1	53.0%	53.0%	46.9%
2	37.6%	36.3%	34.7%
≥3	9.4%	10.7%	18.4%
Male sex	50.0%	50.4%	51.4%
European-origin ethnicity	86.7%	86.2%[b]	87.7%
Birth weight, g	3443 (2913-4005)	3442 (2910-4005)	3360(2740-3965)

Values are percentages or means (10–90th percentiles).
Data available for [a] 500, [b] 501 subjects.
† Cox *et al*. 2013.[101]

Supplemental Material, Table S2: Estimated increase (%) in placental 3-NTp content associated with an interquartile range increment of $PM_{2.5,}$ BC, and nitrogen dioxide air pollution during the preconception period (3 months prior to pregnancy) among 330 mother-newborn pairs from the ENVIR*ON*AGE Study (Limburg, Belgium, 2010-2013)

Preconception exposure, µg/m³	**Adjusted regression coefficient, %**	**95 % CI**	***P*-value**
Preconception $PM_{2.5}$	-13.5	-27.1, 2.8	0.10
Preconception BC[a]	-8.1	-22.7, 9.3	0.34
Preconception NO_2	-5.1	-18.3, 10.2	0.49

[a]BC: n = 271

CHAPTER 2

IN UTERO FINE PARTICLE AIR POLLUTION AND PLACENTAL EXPRESSION OF GENES IN THE BRAIN-DERIVED NEUROTROPHIC FACTOR SIGNALING PATHWAY: AN ENVIR*ON*AGE BIRTH COHORT STUDY*

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ABSTRACT

Background: Developmental processes in the placenta and the fetal brain are shaped by the same biological signals. Recent evidence suggests that adaptive responses of the placenta to the maternal environment may influence central nervous system development.

Objectives: We studied the association between *in utero* exposure to fine particle air pollution with an aerodynamic diameter ≤ 2.5 µm ($PM_{2.5}$) and placental expression of genes implicated in neural development.

Methods: Expression of 10 target genes in the brain-derived neurotrophic factor (*BDNF*) signaling pathway were quantified in placental tissue of 90 mother-infant pairs from the ENVIR*ON*AGE birth cohort using quantitative real-time polymerase chain reaction. Trimester specific $PM_{2.5}$ exposure levels were estimated for each mother's home address using a spatiotemporal model. Mixed-effects models were used to evaluate the association between the target genes and $PM_{2.5}$ exposure measured in different time windows of pregnancy.

Results: A 5 µg/m³ increase in residential $PM_{2.5}$ exposure during the first trimester of pregnancy was associated with a 15.9% decrease (95% confidence interval (CI): -28.7, -3.2%, p = 0.015) in expression of placental *BDNF* at birth. The corresponding estimate for synapsin 1 (*SYN1*) was a 24.3% decrease (95% CI: -42.8, -5.8%, p = 0.011).

Conclusions: Placental expression of *BDNF* and *SYN1*, two genes implicated in normal neurodevelopmental trajectories, decreased with increasing *in utero* exposure to $PM_{2.5}$. Future studies are needed to confirm our findings and evaluate the potential relevance of associations between $PM_{2.5}$ and placental expression of *BDNF* and *SYN1* on neurodevelopment. We provide the first molecular epidemiological evidence concerning associations between *in utero* fine particle air pollution exposure and the expression of genes that may influence neurodevelopmental processes.

INTRODUCTION

Ambient air pollution is a global public health threat.[4] Recent evidence suggests that *in utero* exposure to particulate matter with a diameter ≤ 2.5 μm ($PM_{2.5}$), affects placental functional morphology in mice,[110] as well as normal fetal development in humans because of suboptimal intra-uterine environment.[84]

David Barker introduced the concept that early life stress contributes to later illness [121]. Perturbations in the maternal environment can be transmitted to the fetus by changes in placental function. This might affect fetal programming and thereby increase the risk of cardiovascular disease later in life.[122] Furthermore, recent findings show increasing support for effects of environmental exposures on diseases of the central nervous system.[123]

The neurodevelopmental trajectories of the fetal brain are vulnerable processes that may be disturbed by toxic insults and potentially by *in utero* exposures to air pollution. Experimental evidence obtained in mice shows that prenatal diesel exposure affects behavior,[124] neurotransmitter levels and spontaneous locomotor activity.[125] A prospective cohort study reported that children with higher prenatal exposure to ambient polycyclic aromatic hydrocarbons had a lower IQ at 5 years of age.[126] Suglia *et al.*[127] reported that exposure to black carbon was associated with reduced cognitive function scores in 8 to 11-year old children. Although both experimental and epidemiological evidence suggests that exposure to fine particle air pollution affects the brain of offspring in the developmental period, potential mechanisms that may underlie such early life changes have not been characterized.

Two recent studies[78, 79] suggest that the placenta, aside from transport of maternal nutrients, growth factors, and hormones, also plays an important role in central nervous development through adaptive responses to the maternal environment. Neurotrophins are implicated in a host of brain cellular functions. Multiple experimental studies have shown that brain-derived neurotrophic factor (*BDNF*) plays a role in development and function of the nervous system which includes also the thyroid hormone – brain development axis.[128] Moreover, it has been suggested that maternal BDNF is able to reach the fetal brain through the utero-placental barrier in mice and may therefore contribute to the development of the fetal central nervous system.[129] A recent report showed that cord blood

BDNF levels were positively associated with scores on Gesell Development Schedules at 2 years of age among children enrolled before and after the closure of a coal-fired power plant in Tongliang County, China.[130] Inspired by these findings, we studied placental expression of genes in the *BDNF* signaling pathway (Figure 1).[131] *BDNF* is expressed in the central and peripheral nervous system and in tissues/organs where it regulates morphogenesis, proliferation, apoptosis and developmental processes.[132] An *in vitro* study showed that BDNF and its specific receptor tyrosine kinase (TRKB), are also involved in embryo implantation, subsequent placental development and fetal growth by stimulating trophoblast cell growth and survival. Moreover, BDNF promotes neuronal maturation and differentiation of the developing nervous system[133] and participates in synaptogenesis[134]. For example, BDNF modulations of neurotransmitter release in mice can alter the activity of synapsin 1 (SYN1). The latter protein promotes axonal growth and neuroplasticity, helps to maintain synaptic contacts, and influences synaptic vesicle exocytosis via a mitogen-activated protein kinase (MAPK)-dependent phosphorylation.[135]

Environmental factors may modulate placental gene expression in a way that the fetus' normal neurodevelopmental trajectory is affected. In the present study, we investigated whether *in utero* exposure to $PM_{2.5}$ during different periods of prenatal life is associated with placental expression of neurodevelopmental genes in the *BDNF* signaling pathway at birth.

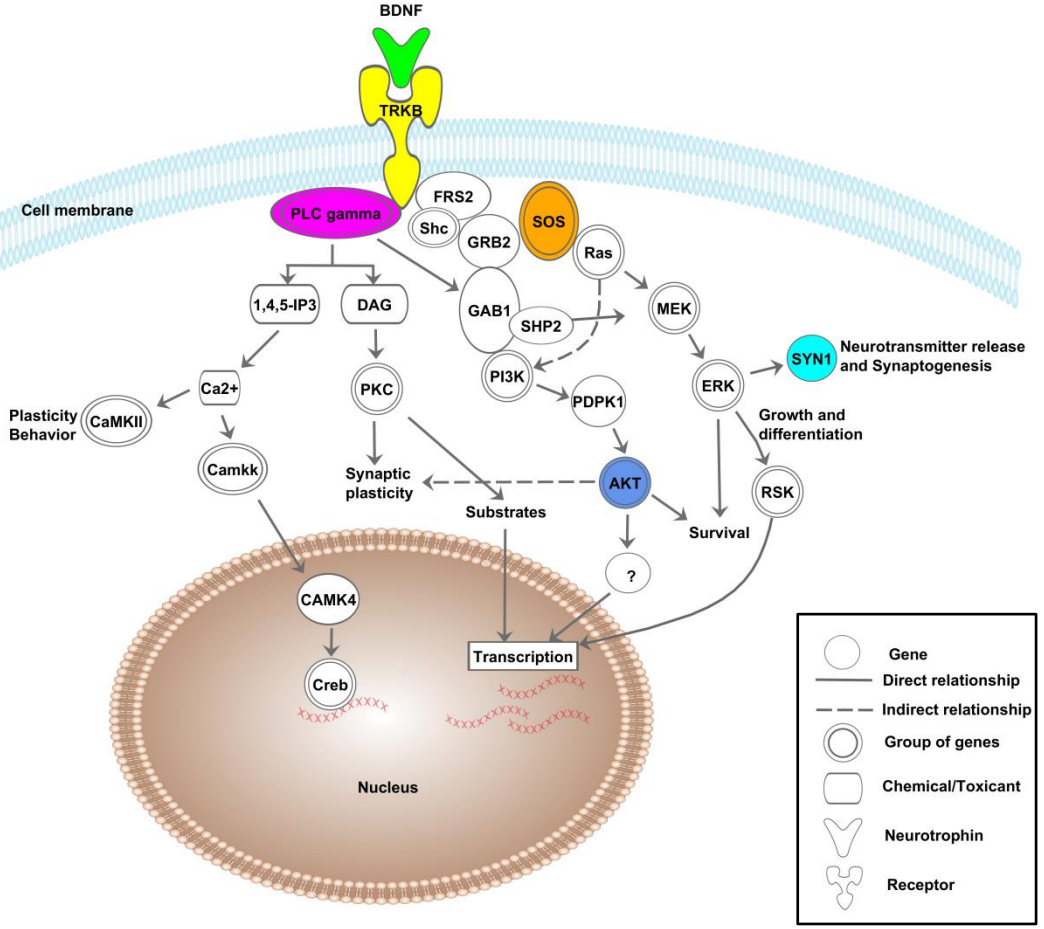


Figure 1. Overview of the genes within the BDNF signaling pathway [adapted with permission from Macmillan Publishers Ltd]. The binding of BDNF to its receptor TRKB initiates three main signaling cascades: PLC gamma cascade (*PLCG1*, and *PLCG2*), AKT cascade (*AKT1*, *AKT2*, and *AKT3*) and SOS cascade (*SOS1*, *SOS2* and *SYN1*). These cascades are involved in neuronal survival, growth, differentiation, and synaptic plasticity. The highlighted genes were explored in this study.

METHODS

Study population and measurements

The ongoing ENVIR*ON*AGE birth cohort enrolls mothers giving birth in the East-Limburg Hospital (ZOL, Genk, Belgium). The hospital has a catchment area of 2,422 km^2 and includes rural, suburban, and urban municipalities with population densities ranging from 82 to 743 inhabitants/km^2. From February 2010 through March 2012, we recruited mother-newborn pairs (only singletons) born between Friday 1200 hours and Monday 0700 hours. Enrollment was spread equally over all seasons of the year. The participation rate of eligible mothers (able to fill out a Dutch language questionnaire) was 56% (n=320). Most common reasons for nonparticipation of eligible mothers were recorded during the first month of the campaign: *a*) failure to ask for participation, *b*) communication problems, and *c*) complications during labor. In the present study, exclusion criteria based on exposure to active or passive tobacco smoking reduced the study population to 247 participants. From this smoke-free group, a random selection of 90 mother-newborn pairs was used for gene expression analysis. A comparison of our subsample with the full cohort and with the Flemish birth register[101] did not show significant differences in maternal age, pre-gestational body mass index (BMI), parity, ethnicity, birth weight and birth length. The study was approved by the Ethics Committee of Hasselt University and East-Limburg Hospital. Written informed consent was obtained from all participating mothers when they arrived at the hospital for delivery. Study questionnaires providing detailed information on place of residence, age, pre-gestational BMI, net weight gain during pregnancy, maternal education, occupation, smoking status, alcohol consumption, use of medication, parity and neonates' ethnicity were completed in the postnatal ward after delivery. Perinatal parameters such as neonates' sex, birth date, birth weight and length, gestational age, Apgar score, and ultrasonographic data were also collected after birth. Gestational age was estimated based on ultrasound data. Insulin levels were measured in cord blood using the E-modular 170 (Roche Diagnostics, Vilvoorde, Belgium).

Placental tissue

Placentas were collected within 10 minutes after birth and immediately frozen at -20°C. After thawing, placental tissue samples were taken at four standardized sites across the middle region of the fetal side of the placenta, approximately 4 cm away from the umbilical cord. Two samples were used in our analysis. The first was taken to the right of the main artery, the second in the third quadrant of the placenta. We sampled 1.0-1.5 cm below the chorio-amniotic membrane at a fixed location. Tissue samples were transferred to RNALater (Qiagen, KJ Venlo, the Netherlands) and incubated at 4 °C for 24 hours. Samples were archived at -20 °C.

RNA extraction

Samples were thawed and RNA was extracted from 20 to 25 mg placental tissue using the miRNeasy Mini Kit (Qiagen). Genomic DNA contamination was minimized with the Turbo DNA free kit (Ambion, Life Technologies, Foster City, CA, USA). The concentration of total RNA was measured with Nanodrop spectrophotometer (ND-1000, Isogen Life Science, De Meern, the Netherlands). The average yield ± SD of total RNA per placenta tissue was 8.8 ± 3.5 µg with $A_{260/280}$ ratio of 1.98 ± 0.05 and $A_{260/230}$ ratio of 1.75 ± 0.22. Extracted RNA was stored at -80 °C until further use.

Gene expression analysis

Expression of candidate genes (n = 10) within the *BDNF* signaling pathway was studied (see Supplemental Material, Table S1). Candidate genes were selected based on literature with regard to neurodevelopment (Figure 1). A maximum amount of 3 µg of total RNA was reverse transcribed into cDNA by means of the GoScript Reverse Transcription System (Promega, Madison, WI, USA) using a Veriti 96 well Thermal cycler (TC-5000, Techne, Burlington, NJ, USA). cDNA was stored at -20 °C until further measurements. A quantitative real-time polymerase chain reaction (qPCR) was set up by adding 2 µL of a 10 ng/µL dilution of cDNA together with TaqMan Fast Advanced Master Mix (Life Technologies) and PrimeTime™ assay (Integrated DNA Technologies, Coralville, IA, USA) in a final

reaction volume of 10 µL. Standard cycling conditions were used to analyze samples in a 7900HT Fast Real-Time PCR system (Life Technologies)). Cq values were collected with SDS2.3 software. MIQE (minimum information for publication of quantitative real-time PCR experiments) guidelines were taken into account.[136] Amplification efficiencies were between 90 and 110% for all assays (see Supplemental Material, Table S1) and amplification specificity was confirmed by gel electrophoresis (data not shown). Raw data were processed to normalized relative gene expression values with qBase plus software (Biogazelle, Zwijnaarde, Belgium) using *IPO8*, *POLR2A*, *UBC*, and *GAPDH* as reference genes for data normalization (see Supplemental Material, Table S1). Technical replicates were included when the difference in Cq value was < 0.75. The correlation coefficient of gene expression between the two samples varied between 0.29 for *SYN1* and 0.85 for *AKT1* (data not shown). Between-placenta variability was higher than within-placenta variability for all genes, except for *SYN1*, *AKT2* and *PLCG2* (see Supplemental Material, Table S2).

Exposure estimates

Regional background levels of $PM_{2.5}$ were interpolated for each mother's residential address using a spatiotemporal interpolation method (kriging) that uses land-cover data obtained from satellite images (CORINE land-cover data set) in combination with monitoring stations (n = 34).[102, 105] This model provides interpolated $PM_{2.5}$ values from the Belgian telemetric air quality networks in 4 × 4 km grids. Based on 34 different locations, validation statistics of the interpolation tool gave a temporal explained variance of > 0.8 for hourly $PM_{2.5}$ averages as well as for annual mean $PM_{2.5}$. Additionally, nitrogen dioxide (NO_2) exposures were interpolated using the same methods as $PM_{2.5}$ exposure. To explore potentially critical exposure windows during pregnancy, the daily interpolated $PM_{2.5}$ concentrations (micrograms per cubic meter) were averaged for various periods during pregnancy for which the date of conception was estimated based on ultrasound data[107] that is, the three trimesters (1–13 weeks, 14–26 weeks, and 27 weeks to delivery) and the early pregnancy stages: pre-implantation (1–5 days after estimated conception date), implantation (6–12 days), implantation range (6–21 days, imbedding of blastocyst in endometrium), post-implantation (22–28 days), and first month (1–30 days). Mean daily temperatures and relative

humidity for the study region were provided by the Royal Meteorological Institute (Brussels, Belgium).

Statistical analysis

Statistical analysis was carried out using SAS software (version 9.3, SAS Institute Inc., Cary, NC, USA). Continuous data were presented as mean ± SD and categorical data as frequencies and percentages.

In a first (single-gene) analysis, we examined the association between gene expression of two placenta samples and $PM_{2.5}$ exposure. The correlation between the two samples from a single placenta was accounted for by using mixed-effects models.[137] Models were adjusted for linear terms for maternal age, gestational age, cord blood insulin, delivery date, and NO_2 exposure, and indicator variables for newborn's sex, maternal education (low-middle-high), placental sampling site, and season at birth (winter, spring, summer and autumn). Because both air pollution[138] and BDNF[139] are related to glucose metabolism, cord blood insulin was added to the models. For each exposure window, estimates are calculated for a 5 $\mu g/m^3$ increment in $PM_{2.5}$ and results are presented as a percent change in gene expression relative to the mean gene expression.

In a second (multiple-gene) analysis, we explored the three different signaling cascades of the *BDNF* pathway. Gene expression values of genes belonging to the same cascade were treated as a single outcome and were entered into a mixed model. Within the *AKT* cascade, the response variable consisted of eight correlated gene expression values for each placenta that is, two samples per placenta and four target genes (*BDNF*, *AKT1*, *AKT2* and *AKT3*) measured in each sample. Similarly, the response variable consisted of eight gene expression values per placenta within the *SOS* cascade (*BDNF*, *SOS1*, *SOS2* and *SYN1*) and six gene expression values per placenta within the *PLCG* cascade (*BDNF*, *PLCG1* and *PLCG2*). *TRKB* was not significantly correlated with the other transcript levels within these cascades and therefore was excluded from this analysis (See Supplemental Material, Table S3). The mixed model adjusts for the correlation between the biopsies and for the correlation between the genes from a single placenta, whereas differences between genes are accounted for by entering them as a fixed effect into the model. Models were adjusted for the same confounders or covariates as in the single-gene analyses. The assumption that the effect of the

exposure was the same across all target genes within a cascade was assessed by including interaction terms between gene and exposure. Results are presented as a difference in gene expression for a 5 $\mu g/m^3$ increment in $PM_{2.5}$ for each exposure window.

RESULTS

Study population characteristics and exposure levels

Demographic characteristics of the 90 mother-infant pairs are presented in Table 1. Maternal age was on average ± SD 29.5 ± 4.6 years. Pre-pregnancy BMI averaged 24.1 ± 4.4 kg/m^2 with a mean net weight gain of 15.5 ± 7.2 kg during pregnancy. Fifty-eight (64.4%) of the mothers obtained a higher education degree. The total newborn population, comprising 47 boys (52.2%), had a mean gestational age of 39.1 weeks (range, 35 - 42), 92.2% were term-born infants and included a vast majority of primiparous (55.6%, n = 50) or secundiparous (32.2%, n = 29) newborns. Birth weight and length were 3450 ± 436 g and 50.5 ± 1.9 cm, respectively.

Mean outdoor $PM_{2.5}$ for the different time windows of pregnancy are reported in Table 2 and the values for NO_2 are presented in Supplemental Material, Table S4.

Table 1. Characteristics of mother-newborn pairs (*n* = 90) from the ENVIR*ON*AGE study, Limburg, Belgium, 2010-2012

Characteristics	Mean ± SD and number (%)
Maternal	
Age, years	29.5 ± 4.6
Pre-pregnancy body mass index	24.1 ± 4.4
Total weight gain, kg	15.5 ± 7.2
Educational level	
Low (less than high school)	11 (12.2)
Middle (high school diploma)	21 (23.3)
High (college or more)	58 (64.4)
Acetaminophen during pregnancy[a]	
No	45 (54.2)
Alcohol consumption during pregnancy[b]	
No	79 (89.8)
Parity	
1	50 (55.6)
2	29 (32.2)
≥ 3	11 (12.2)
Newborn	
Male sex	47 (52.2)
European-origin ethnicity[c]	74 (83.2)
Gestational age, weeks	39.1 ± 1.3
Born at term (≥ 37 w)	83 (92.2)
Season at birth	
Spring	22 (24.4)
Summer	19 (21.1)
Autumn	14 (15.6
Winter	35 (38.9)
Apgar score after 5 minutes	
6	1 (1.1)
7	0 (0)
8	6 (6.7)
9	25 (27.8)
10	58 (64.4)
Birth weight, g	3450 ± 436
Birth length, cm	50.5 ± 1.9
Cord blood Insulin, mU/L	7.3 ± 7.3

[a] Data available for 83, [b] 87 and [c] 89 subjects.

Table 2. $PM_{2.5}$ exposure (µg/m³) characteristics (n = 90)

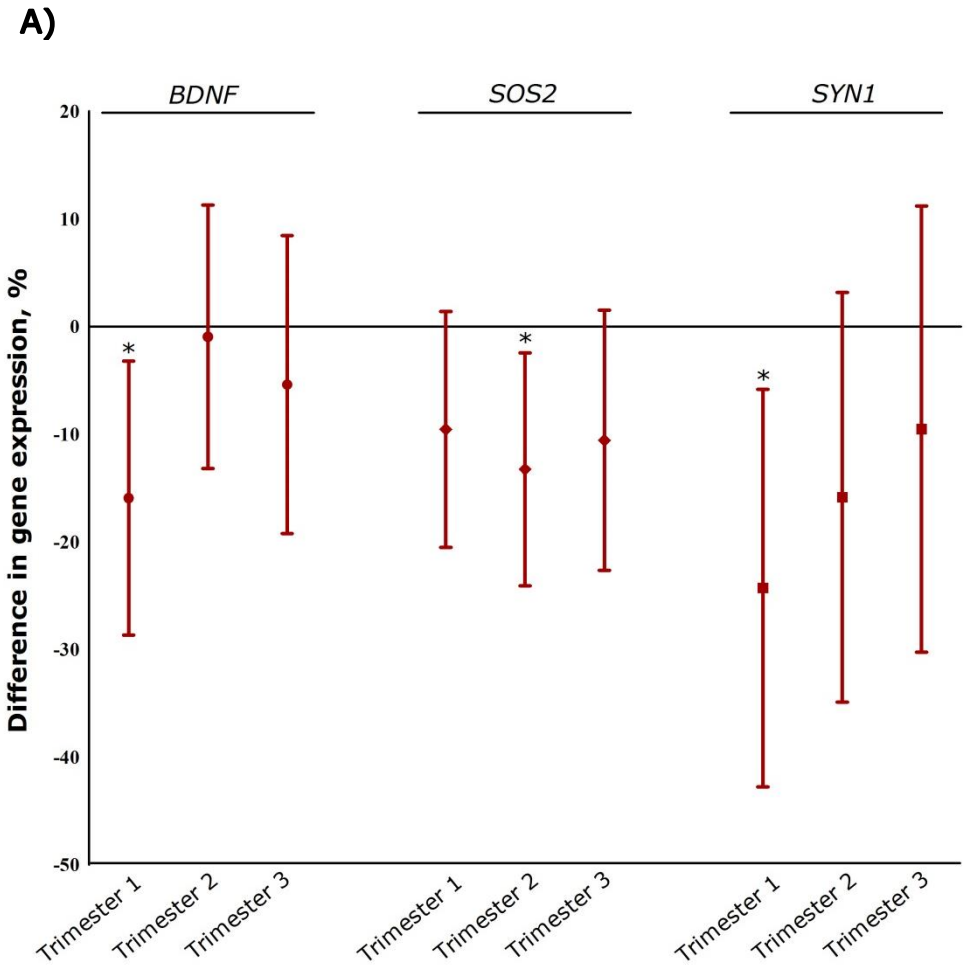
Time windows	Mean ± SD	25th percentile	75th percentile
Pre-implantation (1-5 days)	17.4 ± 10.5	10.7	20.6
Implantation (6-12 days)	17.0 ± 9.5	10.8	20.1
Implantation range[a] (6-21 days)	16.5 ± 7.5	11.3	18.9
Post-implantation (22-28 days)	15.0 ± 8.4	9.6	17.2
First month (1-30 days)	15.8 ± 6.6	11.6	17.9
Trimester 1 (1-13 weeks)	15.4 ± 5.4	11.7	18.0
Trimester 2 (14-26 weeks)	17.6 ± 7.0	12.0	22.8
Trimester 3 (27 weeks-delivery)	18.7 ± 6.0	14.9	23.0

[a] Data available for 79 subjects.

Gene expression of the BDNF signaling pathway in association with $PM_{2.5}$ exposure: Single-gene models

Placental *BDNF* gene expression was inversely associated with $PM_{2.5}$ exposures during the first trimester of pregnancy: placental *BDNF* expression decreased by 15.9% (95% confidence interval [CI]: -28.7, -3.2%, p = 0.015) for a 5 µg/m³ increment in $PM_{2.5}$ (Figure 2A). This association was adjusted for newborn's sex, maternal age, maternal education, gestational age, cord blood insulin, placental biopsy site, delivery date, season at birth, and NO_2 exposure. We observed no significant association between *BDNF* expression and $PM_{2.5}$ exposure in the second (p = 0.88) and third trimester (p = 0.44). In a second stage, we examined shorter time windows to target more specifically the critical stages of placental and fetal development, and estimated a significant negative association of placental *BDNF* gene expression with $PM_{2.5}$ exposure during the first month of pregnancy and during early implantation stages (Figure 2B). For the post-implantation window, the negative association weakens with loss of statistical significance. Significant inverse associations were found between *SYN1* and $PM_{2.5}$ during trimester 1 and between *SOS2* and $PM_{2.5}$ during trimester 2 [-24.3% (95% CI: -42.8, -5.8%, p = 0.011) and -13.3% (95% CI: -24.1, -2.4%, p = 0.017) for a 5 µg/m³ increment in $PM_{2.5}$ respectively] (Figure 2A). Within the shorter time windows, significant associations were observed between *SOS2* gene expression at birth and $PM_{2.5}$ exposure during several implantation stages and the first month of pregnancy

(Figure 2B). No significant associations were found between $PM_{2.5}$ exposure and other selected genes within the *BDNF* pathway (see Supplemental Material, Figure S1).



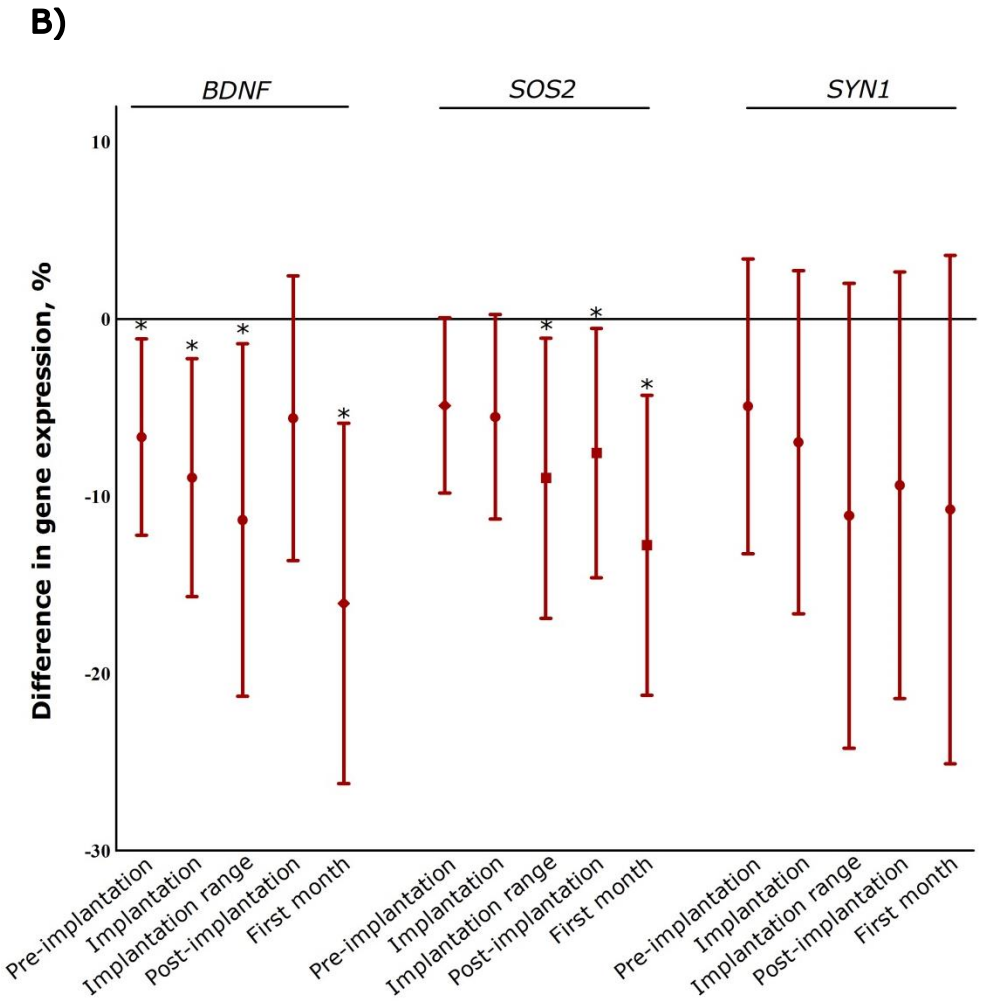


Figure 2. Difference in *BDNF, SOS2* and *SYN1* placental gene expression in association with *in utero* exposure to fine particle air pollution ($PM_{2.5}$) during various time windows (single-gene models; n=90). The effect estimate is the percent difference (95% CI) relative to mean gene expression for a 5 μg/m³ increase of $PM_{2.5}$ exposure (μg/m³). Time window-specific $PM_{2.5}$ exposures (μg/m³) were calculated by averaging the daily interpolated $PM_{2.5}$ concentrations for various periods during pregnancy: each of the three trimesters (A) and the early pregnancy stages (B). Estimates were adjusted for newborn's sex, maternal age, maternal education, gestational age, cord blood insulin, placental sampling site, delivery date, season at birth, and NO_2 exposure. * $p < 0.05$

BDNF signaling cascades in association with $PM_{2.5}$ exposure: Multiple-gene models

To test the assumption that the effect of the exposure was the same across all target genes within a cascade, we used an interaction term between the exposure and the variable identifying the gene. Because interaction terms were not significant, they were excluded from final models. We found for the *PLCG* cascade (*BDNF, PLCG1* and *PLCG2*) that $PM_{2.5}$ exposure during the first month (p = 0.001) and the first trimester (p = 0.009) of pregnancy was associated with significantly lower levels of placental gene expression at birth (Table 3). We also observed significant changes in gene expression in association with $PM_{2.5}$ exposure during the first month (p = 0.00002) and first trimester of pregnancy (p = 0.0001) for the *SOS* cascade (*BDNF*, *SOS1*, *SOS2* and *SYN1)*, whereas for the *AKT* cascade (*BDNF*, *AKT1*, *AKT2* and *AKT3*), associations between gene expression and $PM_{2.5}$ exposure were not significant (Table 3).

Table 3. Associations between cascade-specific placental gene expression and $PM_{2.5}$ exposure during pregnancy (multiple-gene models) (n = 90).

Time windows	*AKT* cascade *(BDNF, AKT1, AKT2, AKT3)*		*SOS* cascade *(BDNF, SOS1, SOS2, SYN1)*		*PLCG* cascade *(BDNF, PLCG1, PLCG2)*	
	β (95% CI)	*p*-value	β (95% CI)	*p*-value	β (95% CI)	*p*-value
First month of pregnancy	-0.03 (-0.07, 0.0009)	*p*=0.06	-0.1 (-0.2, -0.07)	*p*<0.001	-0.08 (-0.1, -0.03)	*p*=0.001
Trimester 1	-0.03 (-0.08, 0.02)	*p*=0.2	-0.15 (-0.2, -0.08)	*p*<0.001	-0.1 (-0.2, -0.03)	*p*=0.009
Trimester 2	0.02 (-0.03, 0.07)	*p*=0.4	-0.05 (-0.2, 0.04)	*p*=0.3	0.02 (-0.09, 0.1)	*p*=0.7
Trimester 3	-0.03 (-0.09, 0.02)	*p*=0.2	-0.09 (-0.2, 0.02)	*p*=0.1	-0.09 (-0.2, 0.01)	*p*=0.08

In three separate models, estimates (95% CI) express the multivariable adjusted change in gene expression for a 5 $\mu g/m^3$ increment in $PM_{2.5}$. Estimates were adjusted for newborn's sex, maternal age, maternal education, gestational age, cord blood insulin, placental sampling site, delivery date, season at birth, and NO_2 exposure. The models account for non-independence of placenta samples and genes within each cascade.

DISCUSSION

Both animal and epidemiologic studies indicate that nutrition and environmental stimuli influence *in utero* developmental pathways and may even induce permanent changes in metabolism and chronic disease susceptibility.[140] Transcriptional changes during the perinatal period are associated with morphological and functional development of the brain.[141] In this regard, recent studies suggest that aside from its traditional role in maternal-fetal exchange of nutrients, the placenta plays a role in neurodevelopmental processes through adaptive responses to the maternal environment.[77] A recent study provided evidence of significant measureable benefits of children's cognitive development and cord blood BDNF based on a comparison of two birth cohorts in Tongliang, China, with measurements before and after closure of the local power plant. The investigators found that after closure of the power plant, lower prenatal PAH-DNA adducts and higher concentrations of BDNF were found in cord blood, and that both were associated with improved developmental scores in children.[130] Here, we demonstrated that placental *BDNF* and *SYN1* gene expression levels at birth were inversely associated with $PM_{2.5}$ exposure levels in the first trimester of pregnancy. We surmise that an altered expression of these genetic targets could be part of a molecular mechanism through which fine particle air pollution exposure might affect placental processes.

The concept of the placental role in brain development is relatively new and in line with the groundbreaking observations of fetal programming and disease susceptibility later in life.[121] Critical developmental processes in the placenta and fetal brain are shaped by the same biological signals.[77] In mice, Broad and Keverne[78] observed a strong co-expression of imprinted genes in the hypothalamus and placenta at mid-gestation (embryonic day 11-13), an important period of neuronal proliferation and differentiation. Experimental evidence showed that both BDNF and SYN1 are involved in critical developmental processes of the nervous system, including proliferation, migration, differentiation and synaptogenesis.[142, 143] In mice, *Bdnf* signaling plays important paracrine roles during blastocyst outgrowth.[144] It might promote the development of pre-implantation embryos by suppressing apoptosis and stimulating trophoblast cell growth and survival.[145] Furthermore, *Bdnf* appears to play an important role in

ventricular progenitor cell migration in the developing mouse cerebral cortex.[146] In mice, the BDNF protein contributes to regulation of spontaneous correlated activity at early developmental stages by increasing synaptogenesis and expression of the K+/Cl- co-transporter KCC2.[147] In line with these experimental observations, we found that exposure to fine particle air pollution from the estimated day of conception up to embryo implantation was negatively associated with placental *BDNF* expression at birth. In the same cohort, total DNA hypo-methylation was associated with specific $PM_{2.5}$ exposure windows around implantation.[107] This type of epigenetic modifications could be a biologically plausible link between *in utero* exposures and altered gene expression at birth.

Multiple signaling cascades (Figure 1) implicated in several neurological processes are initiated once BDNF binds to its receptor.[148] In our study, we observed no significant correlation between the expression of *TRKB* and the expression of other selected genes in the *BDNF* signaling pathway. However, studies in mice showed that *Trkb* mRNA levels are already high during prenatal period and that expression does not significantly fluctuate throughout development.[149] The PLCG cascade, underlying BDNF, has been linked to synaptic plasticity.[150] Furthermore, mutations in the PLC gamma docking site alters hippocampal plasticity in mice by which learning was affected.[151] In the present study, we found an inverse association between gene expression within the *PLCG* cascade and $PM_{2.5}$ exposure during the first month and during the first trimester of pregnancy. We hypothesize that differences in gene expression of the *BDNF* pathway might alter signaling and thereby neurodevelopmental processes. We also observed differences in gene expression within the *SOS* cascade in association with $PM_{2.5}$ exposure during the first month and first trimester of pregnancy. In general, in response to upstream stimuli the SOS proteins function as enzymatic factors interacting with RAS proteins to promote guanine nucleotide exchange (GDP/GTP) followed by the formation of the active RAS-GTP complex.[152] In humans, the SOS family contains two different genes (*SOS1* and *SOS2*), located on different chromosomes. Although these genes are highly similar in structure and sequence, a study in mice demonstrated that the lack of SOS1 protein leads to embryonic death, whereas lack of SOS2 did not alter fetal growth and development.[153] Via the SOS cascade, BDNF increases Ras–MAPK dependent phosphorylation of SYN1[135], which promotes axonal growth and neuroplasticity.

In our study, placental *SYN1* gene expression was decreased with maternal exposure to fine particle air pollution during the first trimester of pregnancy. During development, the expression of *SYN1* correlates temporally and topographically with synaptogenic differentiation.[154] Animal studies revealed that during the development of the hippocampus the temporal onset and the peak expression of *Syn1* coincides with neuronal and synaptogenic differentiation of granule cell neurons.[155]

Biological mechanisms through which PM might affect the placenta and subsequent development of the fetus are uncertain. The formation of inflammatory and oxidative stressors is thought to be of importance.[56] Inflammation might contribute to inadequate placental perfusion affecting nutritional processes or oxygenation of maternal blood. In addition, activation of inflammatory cells, which are capable of forming reactive oxygen species, increases oxidative stress–induced DNA damage, which appears to be a particularly important mechanism of action of PM.[56] This suggests that depending on the chemicals present on the surface of PM, two different pathways might be considered to affect the transcriptional release and operation of genes, *a*) indirectly via systemic consequences of induced inflammatory conditions both in mother lungs as well as in placental tissue, or *b*) via translocation of inhaled fine particles from the lung into the blood stream leading to oxidative stress in blood cells and potentially in placental tissue. An *ex vivo* human placental perfusion model showed that particles up to 240 nm in diameter can cross the placental barrier.[108]

A common problem in molecular epidemiology studies is the need to adjust for the multiple comparisons in the analyses, which may be a first limitation of our study. We have done multiple statistical analyses to identify associations of different genes in the *BDNF* signaling pathway and the different exposure windows. However, overall our analysis find consistent results with the strongest effect for *BDNF*. A second limitation is that our small sample has an over-representation of higher educated women. Therefore the generalizability of our findings may be limited. However, this methodological consideration was deliberately applied to decrease the risk of potential residual confounding in smaller samples. A third limitation of the present study is the complexity of the placenta tissue. Because the placenta is composed of different cells including

syncytiotrophoblasts, mesenchymal cells and fibroblasts as well as maternal blood and cord blood, the within-placenta variability is high.[156] Tissue composition of each sample can differ considerably and this can influence gene expression patterns. To minimize sample to sample variation, we standardized our sampling method by taking two fetal-side samples. Observational population studies allow us to characterize only associations between exposure and biomarkers of effect using noninvasive methods and this may be a fourth limitation of our study. It might be that our observations are functionally not related to *in utero* neurodevelopment, but reflect placental function and development in general. However, recent experimental evidence suggests that the placenta might be a useful surrogate tissue to explore fetal brain development.[77]

CONCLUSIONS

In our study population, estimated *in utero* $PM_{2.5}$ exposure during the first trimester of pregnancy was negatively associated with the placental transcription of *BDNF* and *SYN1*, two genes implicated in neural development. Average estimated $PM_{2.5}$ exposures in our study population were below the European Union $PM_{2.5}$ limit (25 $\mu g/m^3$) but above the U.S. $PM_{2.5}$ limit (12 $\mu g/m^3$). Furthermore, the effects of $PM_{2.5}$ exposure are potentially transmitted through the *PLCG* and *SOS* signaling cascades. Our molecular epidemiological findings add to recent experimental research suggesting that developmental processes in the placenta and fetal brain are shaped by the same biological signals. However, it is necessary to replicate our results in other study populations. Furthermore, the long-term consequences of these observations remain to be elucidated.

SUPPLEMENTAL MATERIAL

Supplemental Material, Table S1: Primer assays for selected genes and their RefSeq number

Abbreviation	Gene	RefSeq number	Primetime® Std qPCR Assay	Efficiency (%)
Target genes				
BDNF	Brain-derived neurotrophic factor	NM_001709	Hs.PT.56a.27098180.g	100
TRKB	Neurotrophic tyrosine kinase receptor type 2	NM_001018065	Hs.PT.56a.39058236.g	112
AKT1	V-akt murine thymoma viral oncogene homolog 1	NM_005163	Hs.PT.56a.15697853.g	92
AKT2	V-akt murine thymoma viral oncogene homolog 2	NM_001243027	Hs.PT.56a.143554.g	99
AKT3	V-akt murine thymoma viral oncogene homolog 3	NM_005465	Hs.PT.56a.4178001	92
SOS1	Son of sevenless homolog 1	NM_005633	Hs.PT.56a.20852108	93
SOS2	Son of sevenless homolog 2	NM_006939	Hs.PT.56a.14433335	92
PLCG1	Phospholipase C gamma 1	NM_182811	Hs.PT.56a.2187691	99
PLCG2	Phospholipase C gamma 2	NM_002661	Hs.PT.56a.45287498	99
SYN1	Synapsin 1	NM_006950	Hs.PT.56a.4883078.g	107
Reference genes				
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	NM_001256799	Hs.PT.53a.24391631.gs	100
IPO8	Importine 8	NM_001190995	Hs.PT.56a.40532361	95
UBC	Ubiquitine C	NM_021009	Hs.PT.39a.22214853	95
POLR2A	Polymerase (RNA) II, polypeptide A	NM_000937	Hs.PT.56a.25515089	95

Supplemental Material, Table S2: Within-placenta and between-placenta variability of the two placental samples for each gene

Gene	Within-placenta variability (%)	Between-placenta variability (%)
BDNF	38.7	61.3
TRKB	42.3	57.7
AKT1	16.1	83.9
AKT2	52.7	47.3
AKT3	39.0	61.0
SOS1	43.7	56.3
SOS2	38.9	61.1
PLCG1	30.2	69.8
PLCG2	53.3	46.7
SYN1	69.3	30.7

Supplemental Material, Table S3: Correlations between genes in the *BDNF* signaling pathway

	BDNF	*TRKB*	*AKT1*	*AKT2*	*AKT3*	*SOS1*	*SOS2*	*PLCG1*	*PLCG2*	*SYN1*
BDNF	1	0.071 0.43	-0.48 <.0001	0.071 0.39	-0.23 0.004	0.18 0.03	0.43 <.0001	-0.43 <.0001	0.19 0.02	0.19 0.03
TRKB		1	-0.07 0.42	-0.04 0.63	-0.027 0.75	-0.0069 0.94	0.058 0.50	-0.034 0.70	-0.021 0.82	0.16 0.08
AKT1			1	0.33 <.0001	0.42 <.0001	-0.10 0.21	-0.40 <.0001	0.74 <.0001	-0.13 0.12	-0.02 0.77
AKT2				1	0.65 <.0001	0.48 <.0001	0.27 0.0004	0.28 0.0002	0.43 <.0001	0.28 0.0005
AKT3					1	0.39 <.0001	0.18 0.018	0.36 <.0001	0.049 0.54	0.16 0.05
SOS1						1	0.60 <.0001	0.13 0.096	0.54 <.0001	0.08 0.33
SOS2							1	-0.30 <.0001	0.26 0.0012	-0.04 0.67
PLCG1								1	0.15 0.068	0.07 0.39
PLCG2									1	0.31 0.0002
SYN1										1

Given is pearson correlation and p-value

Supplemental Material, Table S4: Exposure characteristics of nitrogen dioxide (NO_2) (n = 90)

	NO_2,ug/m³		
Time windows	**Mean ± SD**	**25th percentile**	**75th percentile**
Pre-implantation (1-5d)	21.6 ± 8.9	15.8	25.5
Implantation (6-12d)	21.1 ± 8.1	15.6	25.6
Implantation range[a] (6-21d)	21.0 ± 7.2	15.8	25.0
Post-implantation (22-28d)	19.9 ± 8.2	13.3	24.1
First month (1-30d)	20.4 ± 7.1	15.3	24.4
Trimester 1 (1-13w)	20.3 ± 6.6	15.9	24.3
Trimester 2 (14-26w)	22.3 ± 7.2	16.6	26.4
Trimester 3 (27w-delivery)	23.6 ± 7.3	18.3	28.1

[a] Data available for 79 subjects

CHAPTER 3

LOWER PLACENTAL LEPTIN PROMOTER METHYLATION IN ASSOCIATION WITH FINE PARTICULATE MATTER AIR POLLUTION DURING PREGNANCY AND PLACENTAL NITROSATIVE STRESS AT BIRTH IN THE ENVIR*ON*AGE COHORT*

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ABSTRACT

Background: Particulate matter with a diameter ≤ 2.5 μm ($PM_{2.5}$) affects human fetal development during pregnancy. Oxidative stress is a putative mechanism by which $PM_{2.5}$ may exert its effects. Leptin (LEP) is an energy regulating hormone involved in fetal growth and development. We investigated in placental tissue whether DNA methylation of the *LEP* promoter is associated with $PM_{2.5}$ and whether the oxidative/nitrosative stress biomarker 3-nitrotyrosine (3-NTp) is involved.

Methods: *LEP* DNA methylation status of 361 placentas from the ENVIR*ON*AGE birth cohort was assessed using bisulfite-PCR-pyrosequencing. Placental 3-NTp (n = 313) was determined with an ELISA assay. Daily $PM_{2.5}$ exposure levels were estimated for each mother's residence, accounted for residential mobility during pregnancy, using a spatiotemporal interpolation model.

Results: After adjustment for *a priori* chosen covariates, placental *LEP* methylation was 1.4% lower (95 % CI: -2.7, -0.19%) in association with an interquartile range increment (7.5 μg/m³) in second trimester $PM_{2.5}$ exposure and 0.43% lower (95% CI: -0.85, -0.02%) in association with a doubling of placental 3-NTp content.

Conclusions: *LEP* methylation status in the placenta was negatively associated with $PM_{2.5}$ exposure during the second trimester, and with placental 3-NTp, a marker of oxidative/nitrosative stress. Additional research is needed to confirm our findings and to assess whether oxidative/nitrosative stress might contribute to associations between $PM_{2.5}$ and placental epigenetic events. Potential consequences for health during the neonatal period and later in life warrant further exploration.

INTRODUCTION

The *"Developmental Origins of Health and Disease"* concept describes how the environment may affect intra-uterine development and early childhood, and how it induces developmental changes bearing long-term consequences for health and disease risk later in life.[157, 158] Factors like parental lifestyle, diet, obesity, chemical and environmental exposures have been shown to modulate disease risk.[159, 160] These factors do not simply disrupt development or induce disease themselves, but they can affect onset and progress of disease development. Epigenetic events, such as changes in DNA methylation, are believed to play an important role in this process[69] and may be plausible candidates through which early-life conditions contribute to disease susceptibility later in life.[161]

Exposure to ambient air pollution and particulate matter with a diameter ≤ 2.5 μm ($PM_{2.5}$) during pregnancy may affect fetal growth and development, thereby increasing the risk of low birth weight[84] and preterm birth[36]. Oxidative stress is one of the putative mechanisms by which $PM_{2.5}$ may disrupt biological pathways/systems.[87] In addition, it has been linked with altered DNA methylation levels.[162-164] In biological media, an excess amount of reactive oxygen species may interact with proteins and generate 3-nitrotyrosine residues (3-NTp), a product of tyrosine nitration and a biomarker of oxidative stress and inflammation.[89, 90] Preliminary evidence showed higher expression of 3-NTp, based on immuno-histochemical staining, in high-risk pregnancies such as pre-eclampsia[98] and insulin-dependent diabetes[97]. We have shown recently that the concentration of 3-NTp in the placenta is positively linked with $PM_{2.5}$ exposure during pregnancy.[165]

The placenta is the main interface for maternal-fetal exchange of nutrients and waste, and it responds to perturbations of the maternal environment through adaptive changes.[77, 166] Recently, we reported that PM is associated with global methylation and gene-specific mitochondrial methylation in the placenta[66, 107] and with mitochondrial oxidative DNA damage in cord blood and maternal blood[167] in the ENVIR*ON*AGE birth cohort.

Leptin (LEP) is a hormone that regulates hunger and energy homeostasis via actions on the hypothalamus. During pregnancy, placental LEP plays a functional role in embryo implantation, intra-uterine development, and fetal growth.[168]

Adverse physiological conditions during pregnancy such as maternal obesity and gestational diabetes have been associated with higher placental *LEP* methylation [169], whereas other studies have found lower placental *LEP* methylation in mothers with early-onset pre-eclampsia[170] or impaired glucose metabolism[171]. Furthermore, placental *LEP* methylation was associated with significant differences in infant neurobehaviour scores in boys, but there were no significant associations in girls (n = 223 and 221 term births, respectively).[172] A possible link between $PM_{2.5}$ exposure during pregnancy and placental *LEP* methylation has not been investigated so far. We hypothesize that gestational $PM_{2.5}$ exposure during critical periods of prenatal life is associated with changes in placental DNA methylation of *LEP.* We also explored whether the oxidative stress biomarker 3-NTp might be acting as a mediator of the association between $PM_{2.5}$ and LEP methylation by comparing the association with and without adjustment for 3-NTp.

METHODS

Study population

The on-going ENVIR*ON*AGE birth cohort (ENVIRonmental influence *ON* early AGEing) recruits mother-newborn pairs at the delivery ward of the East-Limburg Hospital (Genk, Belgium). The hospital has a catchment area of 2,422 km^2 and includes rural, suburban, and urban municipalities with population densities ranging from 82 to 743 inhabitants/km^2.[173] The participation rate of eligible mothers (mothers able to fill out a Dutch language questionnaire) in the birth cohort is approximately 61%. The questionnaire collects detailed information on maternal age, pre-pregnancy body mass index (BMI), maternal education and occupation, smoking status, alcohol consumption, place of residence, use of medication, parity, and ethnicity of the newborn.[66, 99] The study protocol was approved by the ethical committees of the Hasselt University and the East-Limburg Hospital, and complied with the Helsinki declaration. Written informed consent was obtained from all participants.

In the present study, 400 bio-banked placental tissue samples were randomly selected from 502 mother-newborn pairs recruited between February 2010 and May 2013. After exclusion of samples with missing data of $PM_{2.5}$

exposure (n = 3) or lifestyle characteristics (n = 4) and those not meeting the pyrosequencing quality control criteria (n = 32), statistical analyses were carried out for 361 subjects in the $PM_{2.5}$ exposure models. For the 3-nitrotyrosine models, we additionally missed 3-NTp values for 48 mother-newborn pairs resulting in 313 subjects for statistical analysis. Characteristics of these groups at enrolment were similar to those of the entire cohort (Supplemental Material, Table S1).

Placental sampling

Whole placentas were stored in a -20°C freezer within 10 minutes after delivery. After thawing, we sampled placental tissue 1-1.5 cm below the chorio-amniotic membrane to avoid membrane contamination. These samples were taken at a fixed location on the fetal site in the quadrant right from the main artery, approximately four cm away from the umbilical cord, as published previously.[174] Each sample was washed and rubbed thoroughly in a petridish filled with phosphate buffered saline to remove blood as much as possible, then snap-frozen in liquid nitrogen and archived at -80°C until DNA methylation and 3-NTp measurements.

DNA methylation analysis

Genomic DNA was isolated from placental tissue samples using the QIAamp DNA mini kit (Qiagen Inc., Venlo, Netherlands) and quantified with a ND-1000 spectrophotometer (Isogen Life Science, De Meern, Netherlands). The DNA samples had an average yield (SD) of 8.6 (6.4) µg with an $A_{260/280}$ ratio of 1.91 (0.08) and an $A_{260/230}$ ratio of 2.23 (0.35). An aliquot of 500 ng DNA from each sample was sodium-bisulfite-modified with the EZ-96 DNA methylation gold kit in a final elution volume of 40 µL M-elution buffer. The procedures were executed according to the manufacturer's instructions (Zymo Research, Irvine, CA, USA). DNA methylation analysis was carried out using highly quantitative bisulfite-PCR pyrosequencing. We investigated seven CpG dinucleotide sites within the promoter region of *LEP*. These sites were chosen from literature[172, 175] and data derived from the hg19 (GRCh37) UCSC Genome Browser (http://genome.ucsc.edu/)[176, 177] illustrating significant transcription factor binding by ChIP analysis to the CpG island promoter region of interest.

Supplemental Material, Figure S1 displays the chromosomal position of the *LEP* promoter region investigated. PCR and sequencing primers were designed with the Pyromark Assay Design software (forward primer: 5'-AGGTGTATATTGAGGGTTTAGGGTTAG-3'; biotinylated reverse primer: 5'-ACATCCCTCCTAACTCAATTTC-3', and sequencing primer: 5'-GGGAGTTGGAGTTAGAAATG-3'). The PCR product of the *LEP* region of interest was amplified from bisulfite-modified DNA with the Pyromark PCR kit (Qiagen, Inc.). Cycling conditions started with an initial PCR activation at 95°C for 15 min, followed by 45 cycles at 94°C for 30 s, 56°C for 30 s, and 72°C for 30 s, to end with a final extension for 10 min at 72°C. The PCR product was sequenced with a Pyromark Q24 Instrument (Qiagen Inc.). We excluded 32 samples that did not pass the standard quality control implemented in the Pyromark Q24 Advanced software (Qiagen Inc.) from further analysis. The percentage of methylation was determined with the Pyromark Q24 Advanced software. The software used different parameters for quality assessment including unsuccessful bisulfite treatment (allowed percentage), peak height threshold (required peak height), and stringency levels (pattern/sum deviation in variable positions). The efficiency of the bisulfite-conversion process was assessed using non-CpG cytosine residues within the sequence. Duplicates of the pyrosequencing runs (n=38) were highly correlated for the mean of the CpG sites (r^2=0.99) as well as for each CpG site separately (r^2 ranging from 0.90 to 0.99).

3-nitrotyrosine protein measurement

Thawed placental tissue samples with a wet weight of approximately 10 mg were manually homogenized on ice in lysis buffer [10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100 and Protease Inhibitor Cocktail, Complete, mini, (Roche, Basel, Switzerland)] and sonicated three times in bursts of 10 seconds. The samples were allowed to settle for 20 min on ice and then centrifuged at 16,000×*g* for 20 min at 4°C. The supernatants were aliquoted and frozen at -20°C until further measurements.

Total protein concentration of the placenta sample was determined with the Bio-rad protein assay according to the manufacturer's instructions (Bio-rad, Belgium). The amount of 3-nitrotyrosine in each sample was quantified with a competitive ELISA (Oxiselect nitrotyrosine ELISA kit, Cell Biolabs, CA, USA) and

absorbance measurements were performed at 450 nm using a FLUOstar Omega (BMG Labtech, Offenburg, Germany). Concentrations of 3-NTp were determined using a standard curve of predetermined nitrated BSA standards. Data were normalized to the amount of protein present in the sample and were presented as nM/mg protein.

Particulate matter air pollution exposure

$PM_{2.5}$ exposure (µg/m³) concentrations were modeled using a spatial temporal interpolation method (Kriging)[102] for each mothers' residential address in combination with a dispersion model. The interpolation method uses land-cover data obtained from satellite images (CORINE land-cover data set) and pollution data collected from a governmental stationary monitoring network. Coupled with a dispersion model[104, 105] that uses emissions from point sources and line sources, this model chain provides $PM_{2.5}$ values in a high-resolution receptor grid (average grids of 25 x 25m). Overall model performance was evaluated by leave-one-out cross-validation including 34 monitoring points for $PM_{2.5}$. Validation statistics of the interpolation tool explained more than 80% of the temporal and spatial variability in the Flemish Region of Belgium.[105] To explore potentially critical exposure windows, we averaged the daily interpolated $PM_{2.5}$ concentrations for each of the three pregnancy trimesters, i.e., first trimester (week 1 to 13), second trimester (week 14 to 26) and third trimester (week 27 to delivery). The date of conception was estimated on the basis of the first day of the mother's last menstrual period, combined with the first ultrasound exam. Complete information for the residential address during pregnancy was obtained by questionnaire and checked with hospital records. For those who moved during pregnancy, we calculated the trimester-specific exposures allowing for the changes in address during this period (based on the daily exposure levels at the different residential addresses).

Statistical analyses

Statistical analyses were carried out using SAS software (version 9.3, SAS Institute Inc., Cary, NC, USA). Continuous data were presented as mean ± SD and categorical data as frequencies and percentages. The 3-NTp content was

log_{10}-transformed to normalize the distribution. To avoid multiple testing, we evaluated the association between the placental methylation status of the *LEP* promoter region of interest and gestational $PM_{2.5}$ exposure or placental 3-nitrotyrosine content using mixed-effects models. In these models, the seven studied CpG sites were integrated into a single factor (individual CpG sites treated as repeated measures using an unstructured covariance structure model) [66]. For each trimester-specific $PM_{2.5}$ exposure model, we adjusted for *a priori* chosen covariates including continuous variables, i.e. maternal age, gestational age, and pre-pregnancy BMI, and categorical variables, i.e. newborn sex (boy – girl), maternal education (low – middle – high), smoking status (never-smoker – former-smoker – smoker), ethnicity of the newborn (non-European – European origin), and trimester-specific season (season at gestational exposure window: autumn – winter – spring – summer). Socioeconomic status was based upon the mothers' education and coded as "low" (no diploma or primary school), "middle" (high school) or "high" (college or university degree). Smoking status was defined as never-smoker, former-smoker (quit smoking before pregnancy), and smoker (continued smoking during pregnancy). The ethnicity of the newborn was defined on the basis of the native country of the newborn's grandparents and was classified "of European origin" when two or more grandparents were European. In addition, because placental *LEP* methylation was measured at birth, we mutually adjusted each model for the other gestational exposure windows to estimate the independent effect of each trimester of exposure. The results are presented for each gestational exposure window as an absolute percentage change in placental *LEP* methylation for a trimester-specific interquartile range (IQR) increment in $PM_{2.5}$ ($\mu g/m^3$). The 3-NTp models were adjusted for the aforementioned covariates, except for trimester-specific season which was replaced by season at delivery, and the estimated effect sizes are presented for a doubling in placental 3-NTp content (nM/mg protein). P-value < 0.05 was used to define statistical significance.

In a sensitivity analysis we examined the associations between placental *LEP* methylation and $PM_{2.5}$ exposure or placental 3-NTp content while excluding mothers with gestational diabetes, gestational hypertension, pre-eclampsia or preterm births. Furthermore, additional adjustment of the main model for mother's total weight gain was evaluated. We also examined the associations

between the methylation at individual CpG sites and $PM_{2.5}$ exposure or placental 3-NTp using multiple linear regressions (Supplemental Material, Figure S2 and Table S2). Finally, we included placental 3-NTp as a covariate in the mixed-effects model of the association between placental *LEP* methylation and trimester-specific $PM_{2.5}$ to determine whether estimated associations changed with adjustment for this potential mediator.

RESULTS

Study population characteristics and measurements in placenta

Demographic, lifestyle, and other characteristics of the total group of 361 mother-newborn pairs (maternal age 29.4 ± 4.7 years) are presented in Table 1. Pre-gestational BMI averaged 24.1 ± 4.3 and 52.4% of the mothers obtained a higher education degree. Fifty mothers (13.9%) reported to have smoked during pregnancy, whereas the majority (67.3%) never smoked cigarettes. The newborn population, comprising 189 boys (52.3%), had a mean gestational age of 39.3 weeks (range: 35 - 42). Most of the newborns were term-born infants (96.1%) and the majority were primiparous (51.2%) or secundiparous (37.7%) births. Mean birth weight and length were 3426 ± 450 g and 50.5 ± 2.1 cm respectively. The population characteristics of the 3-nitrotyrosine group (n = 313) were consistent with those from the total group (Table 1). The 3-nitrotyrosine levels averaged (range) 3,703 (100 – 23,681) nM/mg protein and the mean (range) methylation levels of the seven CpG sites investigated in the placental *LEP* promoter region are shown in Table 2. The mean methylation level of CpG4 (61.5%) was substantially higher than the other six CpG sites (<22.3%).

Table 1. Characteristics of mother-newborn pairs

Characteristics	Total group (n = 361)	3-nitrotyrosine group (n = 313)
Mother		
Age, y	29.4 ± 4.7	29.5 ± 4.6
Pre-pregnancy BMI, kg/m²	24.1 ± 4.3	24.1 ± 4.5
Total weight gain, kg [a]	14.8 ± 6.9	14.6 ± 7.1
Education		
Low	47 (13.0%)	38 (12.1%)
Middle	125 (34.6%)	105 (33.6%)
High	189 (52.4%)	170 (54.3%)
Self-reported smoking status		
Never-smoker	243 (67.3%)	212 (67.7%)
Past-smoker	68 (18.8%)	59 (18.9%)
Smoker	50 (13.9%)	42 (13.4%)
Parity		
1	185 (51.2%)	164 (52.4%)
2	136 (37.7%)	116 (36.1%)
≥ 3	40 (11.1%)	33 (10.5%)
Pregnancy complications		
Gestational diabetes	13 (3.6%)	13 (4.2%)
Gestational hypertension	7 (1.9%)	6 (1.9%)
Pre-eclampsia	2 (0.6%)	2 (0.6%)
Preterm birth	14 (3.9%)	12 (3.8%)
Newborn		
Male sex	189 (52.3%)	164 (52.4%)
European-origin ethnicity	310 (85.9%)	271 (86.6%)
Gestational age, w	39.3 ± 1.3	39.3 ± 1.3
Born at term (≥ 37 w)	347 (96.1%)	301 (96.2%)
Season of delivery		
Spring	100 (27.7%)	88 (28.1%)
Summer	51 (14.1%)	41 (13.1%)
Autumn	102 (28.3%)	86 (27.5%)
Winter	108 (29.9%)	98 (31.3%)
Apgar score after 5 min		
6	1 (0.3%)	0 (0%)
7	6 (1.7%)	6 (1.9%)
8	16 (4.4%)	15 (4.8%)
9	102 (28.2%)	86 (27.5%)
10	236 (65.4%)	206 (65.8%)
Birth weight, g	3426 ± 450	3424 ± 450
Birth length, cm [a]	50.5 ± 2.1	50.5 ± 2.1

Continuous data are presented as mean ± SD; categorical variables as number (%).
[a]Data available for 360 and 312 subjects respectively.

Table 2. Molecular measurements on placental tissue samples (n = 361).

Measurement	**Mean (range)**
3-NTp, nM/mg protein[a]	3,703 (100 – 23,681**)**
LEP methylation, %	
CpG1	10.0 (0.53 – 42.9)
CpG2	12.7 (0.66 – 38.0)
CpG3	8.5 (0.91 – 34.9)
CpG4	61.5 (33.5 – 88.6)
CpG5	13.6 (2.0 – 34.9)
CpG6	13.5 (1.1 – 38.2)
CpG7	22.3 (0.52 – 47.6)

[a] 3-Nitrotyrosine, geometric mean (range), n = 313

$PM_{2.5}$ exposure

The distribution of the outdoor $PM_{2.5}$ levels for the different time windows of pregnancy are shown in Table 3. The average (25th-75th percentile) trimester-specific $PM_{2.5}$ exposure was 15.7 (11.5-19.7) μg/m^3 for the first trimester, 15.5 (11.4-18.9) μg/m^3 for the second trimester, and 17.2 (12.0-21.9) μg/m^3 for the third trimester of pregnancy.

Table 3. Exposure characteristics of airborne particulate matter ≤ 2.5 ($PM_{2.5}$) (n = 361)

Time windows $PM_{2.5}$, μg/m^3	**Mean ± SD**	**25th percentile**	**Median (IQR)**	**75th percentile**
Trimester 1	15.7 ± 5.3	11.5	13.9 (8.2)-14.6 (7.5)	19.7
Trimester 2	15.5 ± 4.9	11.4		18.9
Trimester 3	17.2 ± 5.8	12.0	16.9 (9.9)	21.9

Placental LEP *promoter methylation at birth and its association with $PM_{2.5}$ exposure or placental 3-nitrotyrosine*

The seven CpG sites investigated in the placental *LEP* promoter region were highly correlated with each other (r: 0.47 – 0.88). In male neonate placenta the *LEP* promoter methylation was higher compared to placenta of female neonates [1.33%, 95 % confidence interval (CI): 0.40, 2.27%, p = 0.005 for the male neonate placenta versus the female neonate placenta]. *LEP* promoter methylation was not associated with mother's pre-pregnancy BMI (0.003%; 95% CI: –0.10, 0.11%; p = 0.96 for a one-unit increase in BMI based on the adjusted mixed-effects model of *LEP* methylation and trimester-specific $PM_{2.5}$) (Supplemental Material, Table S2) or total weight gain (-0.042%; 95% CI: –0.11, 0.03%; p = 0.24 for a one-unit increase in total weight gain based on the same model, but without adjustment for pre-pregnancy BMI) (Supplemental Material, Table S2). We fitted a mixed-effects model to evaluate the association between the methylation levels in the *LEP* promoter region of interest (individual CpG sites treated as repeated measures) and $PM_{2.5}$ exposure. After adjustment for newborn sex, maternal age, maternal education, smoking status, gestational age, pre-pregnancy BMI, ethnicity, and gestational trimester-specific season, we estimated that overall *LEP* methylation in the placenta was 1.4% lower (95 % CI: -2.7, -0.19%, p = 0.02) with an IQR increment in second trimester $PM_{2.5}$ exposure (7.5 μg/m³) (Figure 1). No associations were observed between overall *LEP* methylation and an IQR increment in first trimester $PM_{2.5}$ exposure (8.2 μg/m³) (+0.49%; 95% CI: -0.97, 1.95%; p = 0.51) or third trimester $PM_{2.5}$ exposure (9.9 μg/m³) (-0.14%, 95% CI: -1.58, 1.30%, p = 0.13).

Both before (data not shown) and after adjustment for covariates (newborn sex, maternal age, maternal education, smoking status, gestational age, pre-pregnancy BMI, ethnicity, and season of delivery) a doubling in placental 3-NTp content at birth was associated with a significantly lower overall methylation level of the *LEP* region evaluated (-0.43%, 95% CI: -0.85, -0.02%, p = 0.04) (Figure 1).

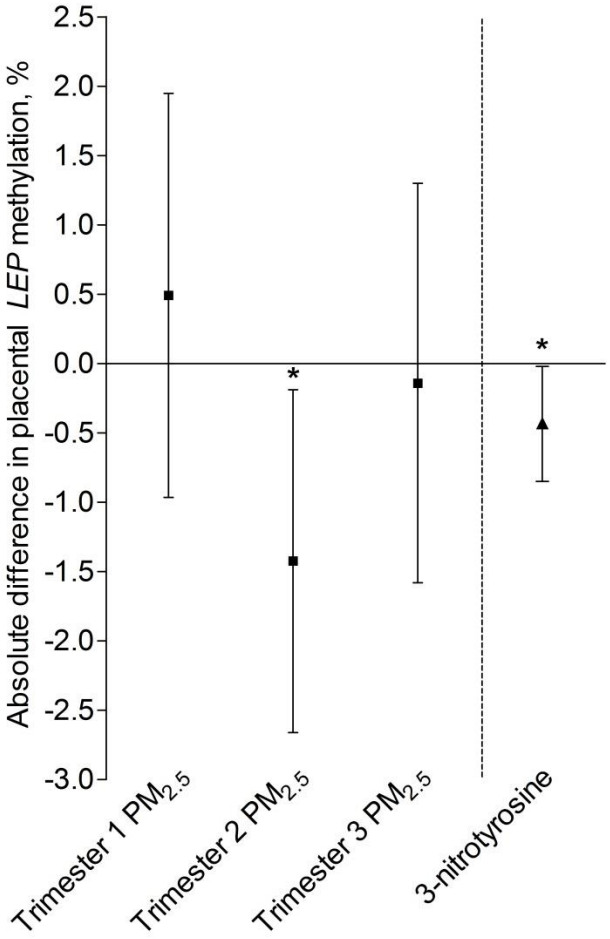


Figure 1 Placental *LEP* promoter DNA methylation in association with $PM_{2.5}$ exposure for different time windows of pregnancy (n = 361) or placental 3-nitrotyrosine (3-NTp) at birth (n = 313). Models were adjusted for newborn sex, maternal age, maternal education, maternal smoking status, gestational age, pre-pregnancy BMI, ethnicity, and season; i.e. gestational trimester-specific season in the $PM_{2.5}$ exposure models and season of delivery in the 3-NTp model. The trimester-specific $PM_{2.5}$ exposure models were mutually adjusted for the other gestational exposure windows to estimate the independent effect of each trimester of exposure. Estimates are presented as an absolute percentage difference in placental *LEP* promoter DNA methylation for a trimester-specific interquartile range increment in $PM_{2.5}$ exposure (trimester 1: 8.2 μg/m³; trimester 2: 7.5 μg/m³; trimester 3: 9.9 μg/m³) or a doubling in 3-NTp content (nM/mg protein). * $p < 0.05$

Sensitivity analysis

A sensitivity analysis in which preterm births (n = 14 for total group; n = 12 for 3-nitrotyrosine group), mothers with gestational diabetes/hypertension (n = 20 for total group; n = 19 for 3-nitrotyrosine group), or mothers with pre-eclampsia (n = 2 for both groups) were excluded, showed very little change in the estimated associations between the overall placental methylation of the *LEP* promoter region and second trimester $PM_{2.5}$ exposure or placental 3-NTp content (Supplemental Material, Table S3). Additional adjustment of the main mixed-effects model for mother's total weight gain during pregnancy did not change statistical significance (Supplemental Material, Table S3). Evaluation of the individual CpG sites based on multiple linear regression models suggested that associations were strongest with four of the seven individual CpG sites (CpG1: -1.5%, 95% CI: -3.1, 0.10%; p = 0.06; CpG2: -1.4%, 95% CI: -2.8, -0.03%, p = 0.05; CpG3: -1.3%, 95% CI: -2.7, -0.008%, p = 0.05; and CpG5: -1.8%, 95% CI: -3.4, -0.09%, p = 0.04) (Figure 2). For a doubling in placental 3-nitrotyrosine, the results of *LEP* methylation suggested solid associations with two individual CpG sites (CpG2: -0.50%, 95% CI: -0.97, -0.03%, p = 0.04 and CpG5: -0.53%, 95% CI: -1.10, -0.05%, p = 0.07) (Supplemental Material, Figure S2). Finally, adjustment of the main mixed-effects model for placental 3-NTp content (n = 313) resulted in a weakening of the association between placental *LEP* promoter methylation and $PM_{2.5}$ exposure of the second gestational window (-1.1%, 95% CI: -2.4, 0.22%; p = 0.10 versus -1.33%, 95% CI: -2.63, -0.03%, p = 0.04).

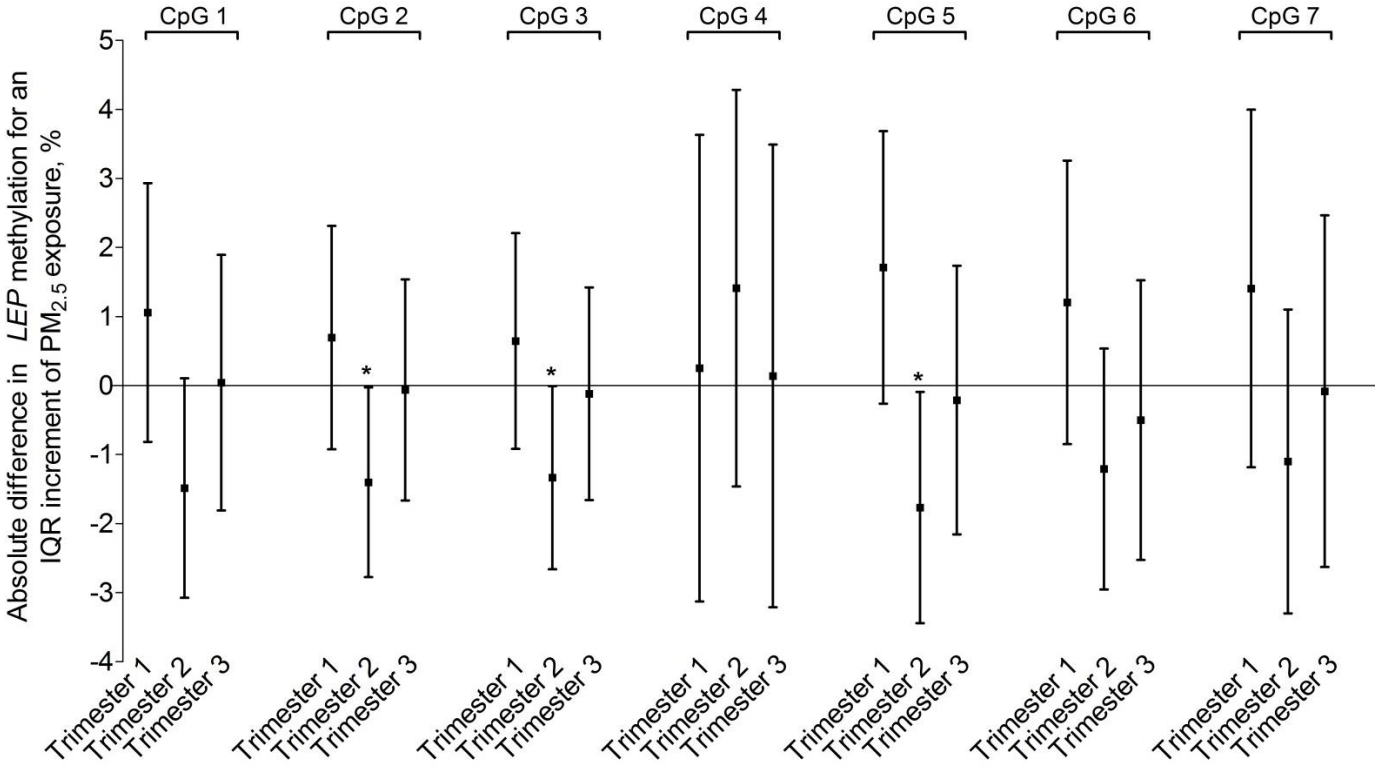


Figure 2 Placental CpG-specific *LEP* promoter DNA methylation in association with $PM_{2.5}$ exposure for different time windows of pregnancy (n = 361). Models were adjusted for newborn sex, maternal age, maternal education, maternal smoking status, gestational age, pre-pregnancy BMI, ethnicity, and gestational trimester-specific season. The trimester-specific $PM_{2.5}$ exposure models were mutually adjusted for the other gestational exposure windows to estimate the independent effect of each trimester of exposure. Estimates are presented as absolute percentage difference in *LEP* promoter DNA methylation for a trimester-specific interquartile range increment in $PM_{2.5}$ exposure (trimester 1: 8.2 µg/m³; trimester 2: 7.5 µg/m³; trimester 3: 9.9 µg/m³). * $p < 0.05$.

DISCUSSION

The human placenta is the anatomo-physiological barrier between mother and fetus. External factors may interfere with placental functions and alter signaling pathways, hormone production, nutrient and waste transfer, embryo implantation and cellular growth.[178] Literature suggests that epigenetic mechanisms play a role in the complex interplay between environment and genes, and may predispose to disease phenotypes.[69, 161] In a previous study on the ENVIR*ON*AGE birth cohort we showed a positive association between $PM_{2.5}$ exposure and placental 3-NTp.[165] The key findings of the present study are the significant inverse associations of both second trimester $PM_{2.5}$ exposure and placental 3-NTp concentrations at birth with DNA methylation of the *LEP* promoter region in the placenta. Associations varied among the individual CpG sites.

During pregnancy, LEP is thought to play a functional role in embryo implantation, intra-uterine development, and fetal growth.[168, 179] It has been shown that umbilical cord blood LEP concentrations were positively correlated with term birth weight in a study population that included 70 newborns with intrauterine growth retardation and 62 newborns classified as having normal growth.[180] In the placenta, LEP is synthesized by trophoblasts and mostly secreted in the maternal blood circulation.[168] Studies indicated that the contribution of placental LEP secretion to circulating fetal leptin is minimal [181, 182], and that fetal adipose tissue is most likely the main source of fetal LEP [180, 182, 183].

Reproductive events involving leptin are crucial for adequate functional development of the placenta, including regulation of nutrient transport, placental angiogenesis, trophoblast mitogenesis, and immunomodulation.[184] When LEP binds to its receptor, it stimulates angiogenic factors such as the vascular endothelial growth factor, thereby activating p38, MAPK and Akt pathways that induce proliferation, motility, and angiogenesis.[185] These processes are critical in placental development, angiogenesis in villi, and fetal-derived vascularization.[186] Furthermore, in-situ hybridization and immunohistochemistry of placental tissue showed that placental leptin in humans is expressed in syncytiotrophoblast cells (facing maternal circulation) and villous vascular endothelial cells (facing fetal circulation) [187].

For the second trimester exposure window, we found a decreased *LEP* methylation in placental tissue at the fetal side in association with an IQR increment (7.5 µg/m³) in $PM_{2.5}$ exposure. The negative association with placental *LEP* methylation is in line with evidence of *LEP* hypomethylation in placenta of complicated pregnancies such as early-onset pre-eclampsia[170] and impaired glucose metabolism[171], both known to adversely influence placental growth and vascularization. This is consistent with earlier observations of increased placental LEP expression reported in other studies of complicated pregnancies.[188, 189] Placental LEP is believed to exert a local protective immunomodulating response.[190] As successful pregnancies are associated with downregulation of pro-inflammatory cytokines such as tumor necrosis factor alpha, LEP may have a local protective response at the maternal-fetal interface.[191, 192] In the context of this literature evidence, future studies should address the consequences of hypomethylation of the placental *LEP* status and its possible involvement in placental immunomodulation and vascularization.

In addition to the negative association between placental *LEP* promoter methylation and mid-gestation $PM_{2.5}$ exposure, we found also a negative association between *LEP* promoter methylation and placental 3-NTp content, which was independent of maternal smoking and other factors. The prevalence of 3-NTp, based on immuno-histochemical staining, has been observed in two small studies with different high-risk pregnancies, including pre-eclampsia and gestational diabetes.[97, 98] In these complicated pregnancies, the higher presence and level of 3-NTp residues in placental tissue may indicate vascular damage.[193] An experimental study investigating diesel exhaust particle (DEP) exposure in mice, suggested that *in utero* DEP promotes vascular oxidative stress as shown by elevated 3-nitrotyrosine protein modification.[115] The presence of 3-NTp in placenta and its association with $PM_{2.5}$ exposure[165] may be indicative of a PM-linked inflammation.

It is important to mention that a TATA box and a potential binding site for the C/EBP transcription factor are present in the studied promotor region. CpG4 is situated in the recognition sequence of C/EBP.[194] An experimental study investigating methylation-dependent transcriptional activity of a human *LEP* promoter fragment in Lisa-2 cultured cells (a liposarcoma cell line) showed that methylation of the CpG4 site (corresponding to CpG position -51 in fig. 6 of

Melzner *et al.* 2002) was important for down-regulation of promoter activity of *LEP*.[195] Demethylation of the CpG sites, which are proximal to the TATA box, was found essential for *LEP* expression in primary fibroblasts and HeLa cells.[194] $PM_{2.5}$ air pollution was not significantly associated with methylation of the CpG4 site in our study. We observed that the individual CpG sites varied in average methylation, especially at the CpG4 site, which was substantially higher methylated than the other CpG sites. Methylation at the CpG2, 3, and 5 sites, which flank the transcription factor sequence as well as the TATA box region, was significantly lower in association with an IQR increase in second trimester $PM_{2.5}$ air pollution.

We acknowledge some study limitations. First, pyrosequencing assays can capture only a small region of 80 base pairs in the *LEP* promoter region and it is possible that we missed additional methylation changes in the promoter region. On the other hand, bisulfite-PCR-pyrosequencing has the advantage of being a highly standardized quantitative procedure that allowed us to obtain accurate results.[196, 197] Second, the placenta is a tissue of different cell types with the presence of maternal and cord blood. As the composition of placenta samples can differ and might influence DNA methylation and gene expression patterns, a standardized methodological protocol was used for sampling each placenta at an almost identical position. Furthermore, maternal and cord blood was removed as much as possible, and the placental 3-NTp content was expressed per mg of placental protein. Third, we cannot exclude any residual confounding by other environmental factors or characteristics associated with the exposures and outcome. Finally, we used a high-resolution receptor grid to estimate $PM_{2.5}$ exposure, but there is a possibility for exposure misclassification.

CONCLUSIONS

We estimated significant negative associations of placental *LEP* promoter region methylation with $PM_{2.5}$ exposure during the second gestational trimester, and with placental 3-NTp, a marker of oxidative/nitrosative stress, at birth. The associated CpG methylation sites are flanking a nucleotide sequence with a regulatory function.[194] Additional research is needed to confirm our findings in other study populations and evaluate the potential impact of placenta *LEP* methylation on health during the neonatal period and later in life.

SUPPLEMENTAL MATERIAL

Supplemental Material, Table S1: Characteristics of the groups compared to the entire cohort at the moment of selection (ENVIR*ON*AGE birth cohort).

Characteristics	Total group (n = 361)	3-nitrotyrosine group (n = 313)	Entire cohort (n=502)
Mother			
Age, y	29.4 ± 4.7	29.5 ± 4.6	29.2 ± 4.6
Pre-pregnancy BMI	24.1 ± 4.3	24.1 ± 4.5	24.3 ± 4.6
Education			
Low	47 (13.0%)	38 (12.1%)	60 (12.0%)
Middle	125 (34.6%)	105 (33.6%)	179 (35.8%)
High	189 (52.4%)	170 (54.3%)	261 (52.2%)
Self-reported smoking status			
Never-smoker	243 (67.3%)	212 (67.7%)	326 (64.9%)
Past-smoker	68 (18.8%)	59 (18.9%)	99 (19.7%)
Smoker	50 (13.9%)	42 (13.4%)	77 (15.3%)
Parity			
1	185 (51.2%)	164 (52.4%)	266 (53.0%)
2	136 (37.7%)	116 (36.1%)	182 (36.3%)
≥ 3	40 (11.1%)	33 (10.5%)	54 (10.7%)
Newborn			
Male sex	189 (52.3%)	164 (52.4%)	253 (50.4%)
European-origin ethnicity	310 (85.9%)	271 (86.6%)	432 (86.2%)
Gestational age, w	39.3 ± 1.3	39.3 ± 1.3	39.3 ± 1.3
Born at term (≥ 37 w)	347 (96.1%)	301 (96.2%)	487 (97.0%)
Season of delivery			
Spring	100 (27.7%)	88 (28.1%)	135 (26.9%)
Summer	51 (14.1%)	41 (13.1%)	86 (17.1%)
Autumn	102 (28.3%)	86 (27.5%)	135 (26.9%)
Winter	108 (29.9%)	98 (31.3%)	146 (29.1%)
Apgar score after 5 min			
6	1 (0.3%)	0 (0%)	1 (0.2%)
7	6 (1.7%)	6 (1.9%)	7 (1.4%)
8	16 (4.4%)	15 (4.8%)	20 (4.0%)
9	102 (28.2%)	86 (27.5%)	148 (29.5%)
10	236 (65.4%)	206 (65.8%)	326 (64.9%)
Birth weight, g	3426 ± 450	3424 ± 450	3443 ± 438
Birth length, cm[a]	50.5 ± 2.1	50.5 ± 2.1	50.4 ± 1.9

Continuous data are presented as mean ± SD; categorical variables as number (%).

[a] Data available for 360, 312, and 501 subjects respectively.

Supplemental Material, Table S2: Effect estimates of the covariates in the models associating placental *LEP* methylation with gestational trimester-specific $PM_{2.5}$ exposure or placental 3-nitrotyrosine at birth.

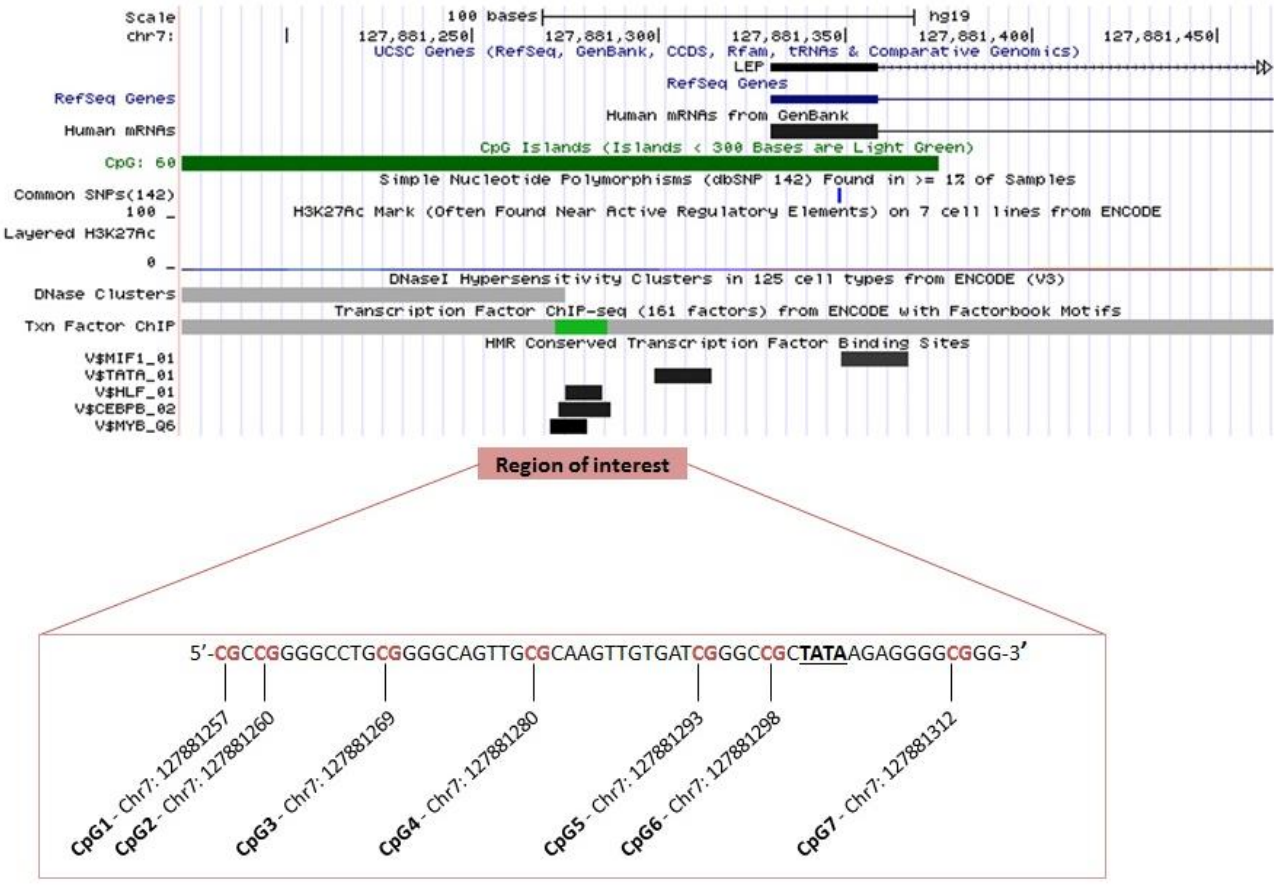
Co-variates	Trimester-specific $PM_{2.5}$-exposure model		3-nitrotyrosine model	
	β (95% CI)	p-value	β (95% CI)	p-value
Mother				
Age, y	-0.077 (-0.18, 0.03)	0.14	-0.092 (-0.20, 0.02)	0.10
Pre-pregnancy BMI	0.003 (-0.10, 0.11)	0.96	-0.015 (-0.12, 0.09	0.78
Total weight gain	-0.042 (-0.11, 0.03)	0.24	-0.043 (-0.11, 0.03)	0.22
Gestational age, d	-0.032 (-0.08, 0.02)	0.22	-0.032 (-1.03, 1.22)	0.26
Education				
Low	0.015 (-1.52, 1.55)	0.98	0.59 (-1.04, 2.22)	0.47
Middle	-0.12 (-1.20, 0.96)	0.83	0.09 (-1.03, 1.22)	0.87
High	Ref.			
Self-reported smoking status				
Never-smoker	0.67 (-0.76, 2.10)	0.36	1.06 (-0.45, 2.57)	0.17
Past-smoker	0.57 (-1.11, 2.25)	0.51	1.51 (-0.26, 3.28)	0.09
Smoker	Ref.		Ref.	
Newborn				
Sex				
Male	1.33 (0.40, 2.27)	0.005	1.30 (0.32, 2.27)	0.01
Female	Ref.		Ref.	
Ethnicity				
European	0.81 (-0.57, 2.19)	0.25	1.23 (-0.23, 2.70)	0.10
non-European	Ref.		Ref.	
Season				
Autumn	1.31 (-0.62, 3.24)	0.18	-1.37 (-2.67, -0.077)	0.04
Winter	-1.15 (-4.03, 1.74)	0.43	-0.49 (-1.96, 0.99)	0.52
Spring	0.35 (-1.57, 2.26)	0.72	-1.26 (-2.73, 0.22)	0.09
Summer	Ref.		Ref.	

Effect estimates are presented as an absolute percentage difference in *LEP* methylation for a unit increase in the corresponding covariate. The trimester-specific $PM_{2.5}$-exposure model included all covariates shown in the table including all trimester-specific $PM_{2.5}$ exposures and trimester-specific season, except for total weight gain. The same covariates were used in the 3-nitrotyrosine model including $\log_{10}$ 3-NTp and season at delivery. Similar models were used to estimate total weight gain, excluding pre-pregnancy BMI.

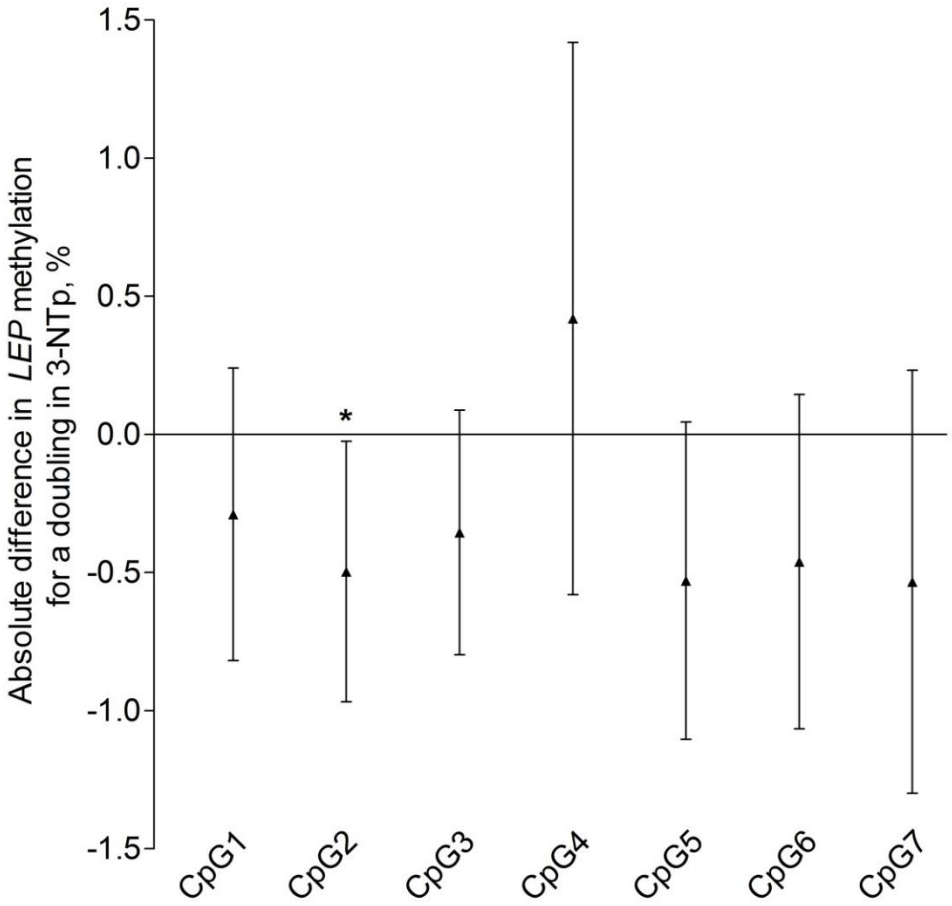
Supplemental Material, Table S3. Sensitivity analysis: associations between placental *LEP* methylation and trimester-specific $PM_{2.5}$ exposure or 3-NTp at birth with exclusion of pregnancy complications or additional adjustment for total weight gain.

	Trimester 1 $PM_{2.5}$ exposure		**Trimester 2 $PM_{2.5}$ exposure**		**Trimester 3 $PM_{2.5}$ exposure**		**3-NTp**	
	%	**95% CI**	**%**	**95% CI**	**%**	**95% CI**	**%**	**95% CI**
Main analysis	0.49	(-0.97, 1.95)	-1.42	(-2.66, -0.19)	-0.14	-1.58, 1.30	-0.48	(-0.88, -0.09)
- preterm births	0.43	(-0.97, 1.87)	-1.51	(-2.74, -0.27)	-0.58	-2.03, 0.87	-0.50	(-0.91, -0.09)
- gestational diabetes	0.54	(-0.97, 2.04)	-1.37	(-2.64, -0.11)	-0.15	-1.62, 1.33	-0.38	(-0.80, 0.04)
- hypertension	0.44	(-0.97, 1.90)	-1.43	(-2.67, -0.18)	0.06	-1.40, 1.52	-0.40	(-0.82, 0.03)
- pre-eclamspia	0.55	(-0.97, 2.00)	-1.41	(-2.65, -0.18)	-0.12	-1.55, 1.32	-0.42	(-0.83, -0.002)
+ total weight gain	0.54	(-0.97,2.00)	-1.40	(-2.64, 0.16)	-0.13	-1.57, 1.31	-0.43	(-0.84, -0.02)

Models were adjusted for newborn sex, maternal age, maternal education, maternal smoking status, gestational age, pre-pregnancy BMI, ethnicity, and season; i.e. gestational trimester-specific season in the $PM_{2.5}$ exposure models and season of delivery in the 3-NTp model. The trimester-specific $PM_{2.5}$ exposure models were mutually adjusted for the other gestational exposure windows to estimate the independent effect of each trimester of exposure. Estimates are presented as an absolute percentage difference in placental *LEP* promoter DNA methylation for a trimester-specific interquartile range increment in $PM_{2.5}$ exposure (trimester 1: 8.2 μg/m³; trimester 2: 7.5 μg/m³; trimester 3: 9.9 μg/m³) or a doubling in 3-NTp content (nM/mg protein).



Supplemental Material, Figure S1: Region of interest in the *LEP* promoter based upon Assembly GRCh37/hg19 of the UCSC genome browser.[176, 177]



Supplemental Material, Figure S2: Associations between placental CpG-specific *LEP* methylation and placental 3-nitrotyrosine at birth. Estimates are adjusted for newborn sex, maternal age, maternal education, smoking status, pre-pregnancy BMI, gestational age, ethnicity, and season of delivery. Results are presented as an absolute percentage difference in *LEP* promoter DNA methylation for a doubling in placental 3-nitrotyrosine content (nM/mg protein). * $p<0.05$

PART 2: CHILDREN, COGNITION AND AIR POLLUTION

CHAPTER 4

RECENT *VERSUS* CHRONIC EXPOSURE TO PARTICULATE MATTER AIR POLLUTION IN ASSOCIATION WITH NEUROBEHAVIORAL PERFORMANCE IN A PANEL STUDY OF PRIMARY SCHOOLCHILDREN*

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ABSTRACT

Background: Children's neuropsychological abilities are in a developmental stage. Recent air pollution exposure and neurobehavioral performance are scarcely studied.

Methods: In a panel study, we repeatedly administered to each child the following neurobehavioral tests: Stroop Test (selective attention) and Continuous Performance Test (sustained attention), Digit Span Forward and Backward Tests (short-term memory), and Digit-Symbol and Pattern Comparison Tests (visual information processing speed). At school, recent inside classroom particulate matter ≤ 2.5 or 10 µm exposure ($PM_{2.5,}$ PM_{10}) was monitored on each examination day. At the child's residence, recent (same day up to 2 days before) and chronic (365 days before examination) exposures to $PM_{2.5}$, PM_{10} and black carbon (BC) were modeled.

Results: Repeated neurobehavioral test performances (n=894) of the children (n=310) reflected slower Stroop Test (p=0.05) and Digit-Symbol Test (p=0.01) performances with increasing recent inside classroom $PM_{2.5}$ exposure. An interquartile range (IQR) increment in residential outdoor $PM_{2.5}$ exposure was associated with a total latency of 0.087 seconds (SE: ± 0.034; p=0.01) in the Pattern Comparison Test. Regarding chronic exposure at residence, an IQR increment of $PM_{2.5}$ exposure was associated with slower performances in the Continuous Performance (9.45 ± 3.47 msec; p=0.007) and Stroop Tests (59.9 ± 26.5 msec; p=0.02). Similar results were obtained for PM_{10} exposure.

Conclusions: In essence, we showed differential neurobehavioral changes robustly and inversely associated with recent or chronic ambient exposure to PM air pollution at residence, i.e., with recent exposure for visual information processing speed (Pattern Comparison Test) and with chronic exposure for sustained and selective attention.

INTRODUCTION

Polluted air is a complex mixture of water vapor, gases, and solid particles. Evidence is growing that ambient air pollution exposure may be neurotoxic.[123] When small particles (particulate matter with a diameter < 10 μm, PM_{10}) deposit in the lungs, they may trigger the release of inflammatory mediators in the systemic circulation.[198, 199] Fine particles (PM < 2.5 μm, $PM_{2.5}$) can also translocate into the circulation leading to increased systemic inflammation,[200] which may adversely affect the central nervous system (CNS).[201, 202] Besides the link with systemic inflammation, particles < 0.1 μm might also cause harm to the CNS in a more direct way by crossing the blood-brain-barrier or by retro-axonal translocation via the olfactory nerve.[203, 204] Experimental studies in rodents demonstrated a wide range of biological CNS effects of air pollution exposure including a pro-inflammatory cytokine response, glial activation, oxidative stress, changes in gene expression, and perturbations of levels and turnover of neurotransmitters.[40, 41, 45, 46, 125, 205] Epidemiological studies in adults showed that long-term exposure to traffic-related air pollution may contribute to neurodegenerative diseases, such as Parkinson's and Alzheimer's disease.[206, 207]

Studies in children suggested that neurotoxic effects of air pollution may translate into observable deterioration of neurobehavioral performance. In children from Boston of approximately 10 years old, average lifetime residential levels of black carbon (BC) were inversely associated with attention, memory, learning, and intelligence.[208, 209] In another prospective cohort study, prenatal air pollution exposure as assessed by personal monitoring of polycyclic aromatic hydrocarbons was inversely associated with neurodevelopmental characteristics (intelligence, behavior) in early childhood.[210-212] Furthermore, cross-sectional studies also reported inverse associations between neurobehavioral performance of children and indicators of chronic air pollution exposure.[213, 214] Recently, it has been shown that children exposed to high traffic-related air pollution have a smaller enhancement in neurobehavioral development after one year in comparison to children exposed to low air pollution.[215] We found that traffic exposure in adolescents, as reflected by a composite factor combining information about traffic density, time spent in traffic, and urinary concentration of *trans,trans*-muconic acid, was negatively associated with sustained attention.[216]

Despite these suggestive studies, there is still insufficient evidence on the consistency of the associations between fine particle air pollution and neurbehavioral performance deficit.[55] Neurobehavioral changes associated with recent air pollution exposure (i.e., exposure on the day and a few days before the neurobehavioral examination) have been scarcely studied. The aim of this study was to investigate with repeated measures whether neurobehavioral performance was differently associated with recent *versus* chronic air pollution exposure in a panel of primary schoolchildren.

METHODS

Study population

This investigation was part of the COGNAC (COGNition and Air pollution in Children) study. Between 2011 and 2013, we invited children (grades three to six) from three primary schools in Flanders (Belgium) to participate. These schools were located in urban areas with a substantial amount of traffic (Figure 1). Typical particulate matter air pollution ($PM_{2.5}$) in the recruitment area was mainly characterized by the following components: elemental carbon (3%), organic mass (20%), sea salt (5%), ammonium (12%), nitrate (21%), ammonium sulfate (18%) and mineral dust (3%).[217]

The parents of the participants filled out a questionnaire to collect information about the current and previous residential addresses, the socioeconomic status of the family, the smoking behavior of the family members, and they provided informed consent for participation. Socioeconomic status was based on the mother's education (up to high school diploma; college or university diploma) and the highest rank of occupation of either parents (unemployed or unqualified worker; qualified worker, white collar assistant, or teaching staff; self-employed, specialist or member of management). The out-of-school sport activities were defined as "none" (no out-of-school sport activities), "low" (≤ 3 hours per week), "middle' (> 3 to < 6 hours per week) and "high" (≥ 6 hours per week). The study protocol was approved by the medical ethics commissions of Hasselt University and the East-Limburg Hospital.

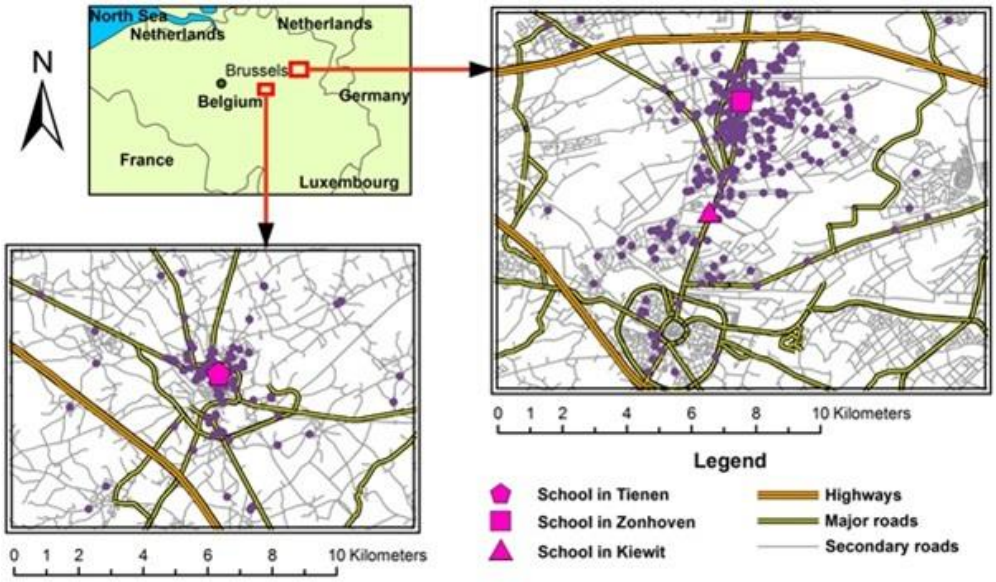

Figure 1. Study area with indication of the school locations in the three municipalities and the road system. Dots represent the residential addresses of the schoolchildren.

In total, 334 children agreed to participate in the study, however 24 had to be removed from the database because of missing data on mother's education and/or occupation of the parents, passive smoking exposure, or residential outdoor air pollution exposure. Of the 310 children, 277 (89.3%) were examined three times, 30 (9.7%) two times, and 3 (1%) once, amounting to a total number of 894 examinations. The examinations took place between December 2011 and February 2014 on Monday, Tuesday, Thursday, and Friday between 9:00 a.m. and 2:00 p.m. The mean (SD) period of time between two consecutive examinations was 41 (23) days. Each neurobehavioral examination was in principle scheduled for the same time of the day for the same child, but in some cases it was not possible due to school activities. For the same child, the time of the day at which the neurobehavioral examinations took place differed on average (SD) 24 (48) min.

Assessment of PM air pollution exposure at the schools

At the schools, we used portable devices (AEROCET 531; MetOne Instruments Inc., Grants Pass, OR, USA) to carry out area measurements of particulate matter [PM with a diameter ≤ 2.5 µm ($PM_{2.5}$) and ≤ 10 µm (PM_{10})] inside the classroom on the examination day (Table 1). Continuous air monitoring was carried out from 9 to 12 a.m. as 2 min interval measurements which were averaged and expressed as µg/m³.

Modeled outdoor air pollution and traffic indicators at residence

For the child's residence, we used a spatial temporal interpolation method to model the daily residential exposure levels (µg/m³) of $PM_{2.5}$, PM_{10}, and BC (Table 1). This method takes into account land-cover data obtained from satellite images (CORINE land-cover data set)[102] and pollution data of fixed monitoring stations in combination with a dispersion model.[104, 105] The model calculates the daily interpolated exposure concentrations in a high resolution receptor grid based on information from the Belgian telemetric air quality networks, point sources, and line sources. Overall model performance was evaluated by leave-one-out cross-validation and was based on 34 monitoring points for $PM_{2.5}$, 58 for PM_{10}, and 14 for BC. Validation statistics of the interpolation tool gave a spatial temporal explained variance of more than 0.80 for $PM_{2.5}$[105], 0.70 for PM_{10}[105], and 0.74 for BC[218]. We used this model to estimate the recent exposure at residence up to 48 hours before the neurobehavioral examination as well as the chronic exposure at residence reflected by the annual mean concentration of the year before the examination. When a child had more than one residential address at the moment of the study, we calculated a weighted average using the proportion of time spent at each location. We calculated also the residential proximity to major roads (RPMR), defined as highways and other national roads, using geographic information system functions (ArcGIS 9.3).

Table 1. Overview of the PM air pollution exposure indicator measurements or estimates used for the panel study in schoolchildren.

	$PM_{2.5}$ (μg/m³)	**PM_{10}** (μg/m³)	**BC** (μg/m³)	**RPMR** (m)
Recent exposure:				
At schools on day of examination[a]				
Inside the classroom	×	×		
At residential address[b]				
Lag 0 (day of examination)	×	×	×	
Lag 1 (1 day before examination)	×	×	×	
Lag 2 (2 days before examination)	×	×	×	
Chronic exposure:				
At residential address[b]	×	×	×	×

PM, particulate matter with aerodynamic diameter < 2.5 μm ($PM_{2.5}$) or < 10 μm (PM_{10}); BC, black carbon; RPMR, residential proximity to major road.
[a] Actual air pollution measurements by area sampling in the classrooms.
[b] Estimates of outdoor air pollution by spatial temporal interpolation modeling.

Assessment of traffic noise

A GIS-based noise model including the Flemish street and railway networks was used to estimate traffic noise levels in 5 dB(A)-intervals according to the European Noise Directive (2002/49/EC)[219]. The modeling of road noise level included road traffic intensity, vehicle-type-specific traffic density, type of street surface, small-scale topography of the area, and the presence or dimensions of buildings and reflecting objects. Railway noise modeling included the amount of passing trains, type of trains, speed, small-scale topography of the area, and the presence or dimensions of buildings and reflecting objects. Weighted equivalent noise levels in dB(A) for traffic over day-time (based on the weighted yearly average noise level between 7 a.m. to 7 p.m., and 7 p.m. to 11 p.m.,) and at night (yearly average noise level between 11 p.m. and 7 a.m) were modeled. Exposure to traffic noise was categorized as ≤ 55 dB(A), > 55 to ≤ 60 dB(A), and > 60 dB(A).

Neurobehavioral tests

The neurobehavioral examination lasted approximately 20 min. The room where the examinations took place was quiet, appropriately lighted, and ventilated. We administered a computer version of the Stroop Test[220] and the following four tests from the Neurobehavioral Evaluation System 3 (NES3) battery: Continuous Performance, Digit Span, Digit-Symbol, and Pattern Comparison.[221, 222]

In the Stroop Test (selective attention domain), four buttons are displayed on the screen (yellow, red, blue, and green). During the test, the name of one of these colors appears on the screen printed in a different color than the name. The task is to touch as fast as possible the button that has the same color as the name, ignoring the color of the printed name. Before the test, eight practice trials take place followed by 48 test trials. The mean reaction time is the average time that passed between the appearance of the name and touching the correct button. This performance indicator was only calculated when the total number of test trails with wrong responses was smaller than or equal to 16.

In the Continuous Performance Test (sustained attention domain), silhouettes of animals (e.g., a cat) are displayed on the screen, one at the time and each for approximately 200 msec. The task is to immediately respond to the cat's silhouette in this case by pressing the spacebar, but not the silhouette of another animal. A new silhouette is displayed each 1000 msec.

The Digit Span Test (short-term memory domain) consists of two parts. In the first part, the task is to reproduce a series of digits after an auditory presentation in the order of the presentation. The test starts with a sequence of three digits. In case of a correct answer, a one digit longer sequence is presented. The test continues until two consecutive incorrect answers are given. In the second part of the test, the task is to reproduce the digits in the reverse order of the presentation.

In the Digit-Symbol Test (visual information processing speed domain), a row of 9 symbols paired with 9 digits is shown at the top of the screen. The same 9 symbols but in a different order are displayed at the bottom of the screen. During the test 27 digits appear consecutively on the screen. When a digit is shown, the task is to indicate as fast as possible the symbol which is paired with this digit in the row of symbols at the bottom of the screen. A new digit appears only after the correct symbol has been indicated.

In the Pattern Comparison Test (visual information processing speed domain), three matrices consisting of 10 × 10 blocks are shown. Two of them are identical. The task is to indicate which pattern is different from the other two patterns. The test includes 25 items.

We used as performance parameters, the mean reaction time in the Continuous Performance Test and the Stroop Test, the maximum span forward and backward in the Digit Span Test, and the total latency or the average latency in the Digit-Symbol Test and Pattern Comparison Test respectively.

Statistical analysis

We performed recent and chronic PM exposure-response analyses using mixed effects models that included random effects for each participant across the neurobehavioral examinations (SAS, version 9.2; SAS Institute Inc., Cary, NC, USA). This method allows each participant to serve as his/her own control over time and eliminates within-subject confounding by personal characteristics that do not change over time. We express the effect estimates for an interquartile range (IQR) increment in recent ($PM_{2.5}$, PM_{10}) and chronic ($PM_{2.5}$, PM_{10}, BC) exposures or living twice as close to major roads (residential proximity to major roads: RPMR). The effect estimates are presented as change in msec for reaction time of the Continuous Performance Test and the Stroop Test, change in number of digits for the Digit Span Forward and Backward Tests, and change in seconds for the latency of the Digit-Symbol Test and Pattern Comparison Test. All analyses were adjusted for *a priori* chosen covariates including sex, age (linear and quadratic term), education of the mother, highest rank of occupation of either parents, passive smoking, out-of-school sport activities, traffic noise (weighted noise during day), hours of computer screen time per week, and day of the week. To capture the non-linear effect of age, we included a quadratic term. Furthermore, a time-varying covariate was included for the measurement occasion (relatedness of examination periods) which is an important predictor of neurobehavioral performance due to the learning effect. In the chronic exposure models, we additionally adjusted for the month of examination to account for seasonality. Since differences are possible for between- and within-subject air pollution effects, we fitted explicit models for recent exposure which included terms for between- and within-subject exposure effects. We reported the within

effects. Finally, we tested in a sensitivity analysis the robustness of the findings and replaced residential weighted day-time noise levels by weighted night-time levels.

RESULTS

Study population characteristics, neurobehavioral performances, and exposure to PM air pollution

Characteristics and neurobehavioral test performances of the study group are summarized in Table 2. The number of boys and girls for the three schools combined was approximately equal. The mean (SD) age was 10.2 (1.3) years. The majority (60.9%) of the children's mothers had a college or university diploma and 41 participants were exposed to passive smoking. 41.3% of the children participated up to three hours per week in out-of-school sport activities. For residential traffic noise during the day, 78.4% were exposed to ≤ 55 dB, 13.2% to >55 to ≤ 60 dB, and 8.4% to >60 dB. During the night, 96.8% were exposed to ≤ 55dB and 3.2% to > 55 dB residential traffic noise.

Over the examination days, the neurobehavioral test performances averaged ± SD for sustained attention 593 ± 51.2 msec in the Continuous Performance Test and 1417 ± 377 msec for selective attention in the Stroop Test, for short-term memory 5.26 ± 0.94 and 4.03 ± 0.97 digits in the Digit Span Forward and Backward Tests respectively, and for visual information processing speed 123 ± 23.5 sec and 4.18 ± 1.01 sec for total latency and average latency in the Digit-Symbol Test and Pattern Comparison Test respectively.

Table 2. Demographic characteristics of the participants.

	n=310
Schools	
Kiewit	69 (22.3%)
Tienen	62 (20.0%)
Zonhoven	179 (57.7%)
Demographic characteristics	
Boys	158 (50.9%)
Age	10.2 ± 1.3
Level of education of the mother	
Up to high school diploma	121 (39.1%)
College or university diploma	189 (60.9%)
Most prestigious category of occupation of either parents,	
Unemployed or not qualified worker	20 (6.4%)
Qualified worker, white-collar assistant, or teaching staff	131 (42.3%)
Self-employed, specialist, or member of management	159 (51.3%)
Passive smoking,	41 (13.2%)
Out-of-school sport activities	
None	36 (11.6%)
≤ 3 hours/week	128 (41.3%)
> 3 to < 6 hours/week	87 (28.1%)
≥ 6 hours/week	59 (19.0%)
Computer screen use, hours per week	4.3 ± 3.8

Values represent number (%) or arithmetic mean ± SD.

The median (interquartile range; IQR) concentrations of PM air pollution inside the classrooms at the schools were, for $PM_{2.5}$ 5.14 (8.85) μg/m^3, and for PM_{10} 33.5 (55.2) μg/m^3 (Table 3). Table 3 also shows the modeled estimates of recent residential ambient air exposure to $PM_{2.5}$, PM_{10}, and BC from Lag 0 to Lag 2. Chronic ambient PM exposure was characterized by the median residential exposure (IQR) over the year before the examination [15.7 (1.16) μg/m^3 for $PM_{2.5}$, 21.3 (1.61) μg/m^3 for PM_{10}, and 1.54 (0.20) μg/m³ for BC] and median distance (IQR) from residence to major roads [RPMR, 333 (669) m].

Table 3. Recent and chronic exposure characteristics (N=310).

	Median	25th percentile	75th percentile	IQR
Recent (at schools)[a]				
$PM_{2.5}$, μg/m³	5.14	2.80	11.6	8.85
PM_{10}, μg/m³	33.5	20.9	76.1	55.2
Recent (at residence)[b]				
$PM_{2.5}$, μg/m³				
Lag 0	16.5	9.10	28.0	18.9
Lag 1	15.2	8.85	27.5	18.7
Lag 2	15.5	8.90	31.8	22.9
PM_{10}, μg/m³				
Lag 0	21.2	13.0	32.2	19.2
Lag 1	19.5	13.0	30.9	17.9
Lag 2	18.9	13.0	36.2	23.2
BC, μg/m³				
Lag 0	1.55	1.00	2.25	1.25
Lag 1	1.36	1.03	2.03	1.00
Lag 2	1.52	0.93	2.30	1.37
Chronic (at residence)[b]				
$PM_{2.5}$, μg/m³	15.7	15.2	16.4	1.16
PM_{10}, μg/m³	21.3	20.7	22.3	1.61
BC, μg/m³	1.54	1.43	1.63	0.20
RPMR, m	333	133	832	699

PM, particulate matter with aerodynamic diameter < 2.5 μm ($PM_{2.5}$) or < 10 μm (PM_{10}); BC, black carbon; RPMR, residential proximity to major roads.
[a] Air pollution at school is obtained by area sampling in the classrooms and averaged over the examination days.
[b] Average ambient air pollution at the residential address over different periods before examination is obtained by spatial temporal interpolation modeling.

Associations between recent PM exposure and neurobehavioral performance

For the sustained attention (Continuous Performance Test) and short-term memory (Digit Span Forward and Backward Tests) domains, the repeated neurobehavioral test performances within the same child were not associated with recent inside classroom exposure ($PM_{2.5}$, PM_{10}) at school on the examination day (Table 4). Similarly, no associations were shown for recent exposure at residence

($PM_{2.5}$, PM_{10}, BC) on the examination day (Lag 0), one day before (Lag 1), and two days (Lag 2) before (Table 5). For selective attention, recent inside classroom $PM_{2.5}$ and PM_{10} exposures were significantly associated with the Stroop Test showing a 42.7 msec longer mean reaction time [95% confidence interval (CI): -0.40 to 85.8, p=0.05] for an IQR increment in $PM_{2.5}$ exposure (Table 4). The corresponding estimate for recent inside PM_{10} exposure was 50.2 msec (95% CI: 8.55 to 91.8, p=0.02).

For the visual information processing speed domain, significant associations were found between the Digit-Symbol Test performance and recent inside classroom $PM_{2.5}$ or PM_{10} exposure (Table 4). An IQR increment in $PM_{2.5}$ exposure showed a total latency increase of 2.05 seconds (95% CI: 0.43 to 3.66; p=0.01). The corresponding result for PM_{10} was 1.9 seconds (p=0.02). The results of the Pattern Comparison Test were adversely associated with recent residential $PM_{2.5}$ and PM_{10} exposure the day of the examination (Lag 0) (Table 5). For an IQR increment of $PM_{2.5}$ exposure, the average latency increased by 0.087 seconds (95% CI: 0.02 to 0.15; p=0.01), while the corresponding estimate for PM_{10} was 0.081 seconds (p=0.01). The days before the examination, i.e., at Lag 1 and Lag 2, an IQR increment of $PM_{2.5}$ exposure also showed an increase in average latency of the Pattern Comparison Test with 0.066 seconds (p=0.04) and 0.079 seconds (p=0.03) respectively. For BC, exposure the day before examination (Lag 1) was also associated with the Pattern Comparison Test (0.051 seconds in average latency, p = 0.04). For the other recent inside classroom and residential outdoor exposure indicators, no associations were found with the Digit-Symbol or Pattern Comparison Tests (Table 4 and 5).

The associations described above were independent of the covariates sex, age (linear and quadratic), education of the mother, highest rank of occupation of either parents, passive smoking, out-of-school sport activities, traffic noise during day, hours per week spent behind a computer, day of the week, and relatedness of the different examination periods. Additional adjustment for chronic residential exposure as well as replacement of the traffic noise during day by traffic noise during night did not change the results.

Table 4. Associations between neurobehavioral test performances and recent inside classroom PM exposures.

Neurobehavioral Test	Recent indoor $PM_{2.5}$			Recent indoor PM_{10}		
	β	95% CI	p-value	β	95% CI	p-value
Attention						
Continuous Performance	-1.29	-5.51 to 2.93	0.55	-2.16	-6.26 to 1.94	0.30
Stroop	42.7	-0.40 to 85.8	0.05	50.2	8.55 to 91.8	0.02
Short-term Memory						
Digit Span Forward	-0.05	-0.16 to 0.05	0.32	-0.002	-0.10 to 0.10	0.97
Digit Span Backward	-0.06	-0.18 to 0.06	0.35	-0.03	-0.15 to 0.08	0.57
Visual Information Processing Speed						
Digit-Symbol	2.05	0.43 to 3.66	0.01	1.9	0.34 to 3.42	0.02
Pattern Comparison	-0.01	-0.11 to 0.09	0.79	-0.03	-0.13 to 0.07	0.54

PM, particulate matter with aerodynamic diameter < 2.5 μm ($PM_{2.5}$) or < 10 μm (PM_{10}); the metric of $PM_{2.5}$ and PM_{10} is μg/m³. For an interquartile increment in recent indoor exposure to $PM_{2.5}$ (8.85 μg/m³) or PM_{10} (55.2 μg/m³), the effect estimates are represented as msec change for the Continuous Performance Test and the Stroop Test, change in number of digits for the Digit Span Forward and Backward Tests, and change in sec of latency for the Digit-Symbol and Pattern Comparison Tests. All analyses were adjusted for sex, age (linear and quadratic), education of the mother, occupation of the parents, passive smoking, out-of-school physical activity, traffic noise, hours spent after computers, day of the week, and relatedness of the examination periods.

Table 5. Associations between neurobehavioral test performances and recent ambient PM exposures at residence.

	Attention						
Recent residential exposure	**Continuous performance**				**Stroop**		
	β	95% CI		p-value	β	95% CI	p-value
$PM_{2.5}$, lag 0	-1.57	-4.45	to 1.32	0.29	-16.7	-46.4 to 12.9	0.27
$PM_{2.5}$, lag 1	-0.59	-3.31	to 2.14	0.67	-20.3	-48.3 to 7.66	0.15
$PM_{2.5}$, lag 2	-0.11	-3.11	to 2.89	0.94	-26.0	-56.4 to 4.46	0.09
PM_{10}, lag 0	-1.27	-3.99	to 1.45	0.36	-13.8	-41.8 to 14.1	0.33
PM_{10}, lag 1	-0.47	-2.82	to 1.89	0.70	-12.2	-36.4 to 12.1	0.33
PM_{10}, lag 2	-0.10	-2.94	to 2.74	0.94	-22.4	-51.1 to 6.33	0.13
BC, lag 0	-0.48	-3.17	to 2.20	0.72	-4.48	-32.0 to 23.1	0.75
BC, lag 1	0.27	-1.83	to 2.37	0.80	-3.96	-25.7 to 17.8	0.72
BC, lag 2	0.66	-1.84	to 3.16	0.61	-3.80	-29.6 to 22.0	0.77
	Memory						
	Digit Span forward				**Digit Span backward**		
	β	95% CI		p-value	β	95% CI	p-value
$PM_{2.5}$, lag 0	-0.050	-0.124	to 0.024	0.18	0.008	-0.075 to 0.092	0.84
$PM_{2.5}$, lag 1	-0.056	-0.126	to 0.015	0.12	-0.028	-0.11 to 0.051	0.49
$PM_{2.5}$, lag 2	-0.071	-0.149	to 0.007	0.07	0.006	-0.081 to 0.093	0.89
PM_{10}, lag 0	-0.046	-0.116	to 0.024	0.20	-0.0031	-0.082 to 0.075	0.94
PM_{10}, lag 1	-0.045	-0.106	to 0.016	0.15	-0.034	-0.103 to 0.034	0.33
PM_{10}, lag 2	-0.067	-0.140	to 0.006	0.07	0.000	-0.082 to 0.082	0.99
BC, lag 0	-0.065	-0.134	to 0.003	0.06	0.019	-0.059 to 0.096	0.64
BC, lag 1	-0.025	-0.080	to 0.029	0.36	-0.025	-0.086 to 0.036	0.42
BC, lag 2	-0.051	-0.116	to 0.014	0.12	0.013	-0.060 to 0.086	0.72

	Visual processing speed							
	Digit Symbol				Pattern comparison			
	β	95% CI		p-value	β	95% CI		p-value
$PM_{2.5}$, lag 0	-0.77	-1.88	to 0.33	0.17	0.087	0.020	to 0.15	0.01
$PM_{2.5}$, lag 1	-0.95	-2.00	to 0.10	0.08	0.066	0.004	to 0.13	0.04
$PM_{2.5}$, lag 2	-0.80	-1.95	to 0.35	0.17	0.079	0.010	to 0.15	0.03
PM_{10}, lag 0	-0.50	-1.54	to 0.54	0.35	0.081	0.019	to 0.14	0.01
PM_{10}, lag 1	-0.69	-1.60	to 0.21	0.13	0.046	-0.009	to 0.10	0.10
PM_{10}, lag 2	-0.73	-1.82	to 0.36	0.19	0.057	-0.009	to 0.12	0.09
BC, lag 0	-0.61	-1.64	to 0.42	0.24	0.041	-0.022	to 0.103	0.20
BC, lag 1	-0.78	-1.59	to 0.03	0.06	0.051	0.003	to 0.099	0.04
BC, lag 2	-0.50	-1.47	to 0.47	0.31	0.006	-0.053	to 0.064	0.85

PM, particulate matter with aerodynamic diameter < 2.5 μm ($PM_{2.5}$) or < 10 μm (PM_{10}); BC, Black carbon; the metric of $PM_{2.5}$, PM_{10}, and BC is μg/m³.

For an interquartile increment in recent (Lag 0, 1, and 2) indoor exposure to $PM_{2.5}$ (18.9, 18.7, and 22.9 μg/m³), PM_{10} (19.2, 17.9, and 23.2 μg/m³) or BC (1.25, 1.00, and 1.37 μg/m³), the effect estimates are represented as msec change for the Continuous Performance Test and the Stroop Test, change in number of digits for the Digit Span Forward and Backward Tests, and change in sec of latency for the Digit-Symbol and Pattern Comparison Tests. All analyses were adjusted for sex, age (linear and quadratic), education of the mother, occupation of the parents, passive smoking, out-of-school physical activity, traffic noise, hours of computer screen time per week, day of the week, and relatedness of the examination periods.

Associations between chronic PM exposure and neurobehavioral performance

In the models studying chronic exposure, we accounted for the following covariates: sex, age (linear and squared term), education of the mother, occupation of the parents, passive smoking, out-of-school sport activities, traffic noise during day, hours per week spent behind a computer, day of the week, month of examination and relatedness of the examination periods. Independent of these covariates, chronic exposure to $PM_{2.5}$ was adversely associated with the attention domain (Continuous Performance and Stroop Tests). For an IQR increment of chronic $PM_{2.5}$ exposure, the reaction time increased by 9.45 msec (95% CI: 2.59 to 16.3; p=0.007) for the Continuous Performance Test and by 59.9 msec (95% CI: 8.1 to 111.6; p=0.02) for the Stroop Test. For PM_{10}, the estimates were in the same direction (Table 6). For BC exposure, we only observed a tendency towards significance for the Continuous Performance Test (5.72 msec; 95% CI: -0.34 to 11.8; p = 0.06) and for residential proximity to major roads (RPMR) no significant associations were found with both attention tests. A sensitivity analysis in which traffic noise during day was replaced by traffic noise during night did not change the main results.

For the short-term memory domain, chronic exposures to $PM_{2.5}$, PM_{10}, BC and residential proximity to major roads did not show significant associations with both short-term memory tests (Table 6). For the visual information processing speed domain, none of the chronic exposure indicators ($PM_{2.5}$, PM_{10}, BC, and RPMR) was associated with the performances of the Digit-Symbol and Pattern Comparison Tests (Table 6).

Table 6: Associations between neurobehavioral test performances and chronic ambient PM exposures at residence.

	Attention							
Chronic	**Continuous Performance Test**				**Stroop Test**			
exposure	β	95% CI		p-value	β	95% CI		p-value
$PM_{2.5}$	9.45	2.59	to 16.3	0.007	59.9	8.1	to 111.6	0.02
PM_{10}	8.66	2.50	to 14.8	0.006	76.5	29.3	to 123.6	0.002
BC	5.72	-0.34	to 11.8	0.06	6.7	-38.4	to 51.9	0.77
RPMR	1.92	-1.0	to 4.85	0.20	0.90	-20.6	to 22.4	0.93
	Short-term memory							
	Digit Span Forward Test				**Digit Span Backward Test**			
	β	95% CI		p-value	β	95% CI		p-value
$PM_{2.5}$	-0.025	-0.15	to 0.10	0.70	0.057	-0.071	to 0.18	0.38
PM_{10}	-0.063	-0.18	to 0.057	0.30	0.057	-0.064	to 0.18	0.35
BC	0.025	-0.09	to 0.14	0.66	0.10	-0.011	to 0.20	0.08
RPMR	-0.047	-0.10	to 0.005	0.08	-0.043	-0.095	to 0.009	0.11
	Visual information processing speed							
	Digit-Symbol Test				**Pattern Comparison Test**			
	β	95% CI		p-value	β	95% CI		p-value
$PM_{2.5}$	2.1	-0.65	to 4.91	0.13	0.050	-0.089	to 0.19	0.48
PM_{10}	2.09	-0.39	to 4.57	0.10	0.023	-0.103	to 0.15	0.72
BC	0.50	-1.99	to 2.99	0.69	0.065	-0.06	to 0.19	0.30
RPMR	0.39	-0.79	to 1.58	0.51	0.0002	-0.057	to 0.057	1.00

PM, particulate matter with aerodynamic diameter < 2.5 μm ($PM_{2.5}$) or < 10 μm (PM_{10}); BC, black carbon; RPMR, residential proximity to major roads. The metric of $PM_{2.5}$, PM_{10}, and BC is μg/m³ and residential proximity to major roads (RPMR) is expressed in m.
For an interquartile increment in chronic exposure to $PM_{2.5}$ (1.16 μg/m³), PM_{10} (1.61 μg/m³), and BC (0.20 μg/m³) or for living twice as close to major roads, the effect estimates are represented as msec change for the Continuous Performance Test and the Stroop Test, change in number of digits for the Digit Span Forward and Backward Tests, and change in sec of latency for the Digit-Symbol and Pattern Comparison Tests. All analyses were adjusted for sex, age (linear and quadratic), education of the mother, occupation of the parents, passive smoking, out-of-school physical activity, traffic noise, hours of computer screen time per week, day of the week, and relatedness of the examination periods.

DISCUSSION

The CNS of schoolchildren and thus also their neurobehavioral performances are still in a stage of development and may be vulnerable to both recent and chronic PM air pollution. In our panel study of children with repeated measures of neurobehavioral performances, neither recent nor chronic PM exposure affected short-term memory. However, other findings indicated consistent negative associations of selective attention (Stroop Test) with both recent classroom and chronic ambient residential PM exposure, while decreased sustained attention was associated only with chronic ambient PM exposure at residence. Visual information processing speed seemed to decrease only in conditions of recent PM exposure, either in the classroom (Digit-Symbol Test) or at residence (Pattern Comparison Test). These associations persisted by taking into account the learning effect over the various examination days and by allowing for sex, age, familial socioeconomic position, out-of-school sport activities, and residential traffic noise (day and night). Some of these results highlighted changes in neurobehavioral test performances in association with PM air pollution exposures well below the current EU standards (annual mean $PM_{2.5}$: 25 µg/m³ and PM_{10}: 40 µg/m³) and just above the current US standard ($PM_{2.5}$: 12 µg/m³).

Recent PM exposure

In contrast to chronic exposure, the neurotoxic potential of recent exposure to PM air pollution in children has not been thoroughly investigated so far. Our panel study of schoolchildren revealed adverse changes in neurobehavioral test performances associated with an IQR increment of recent PM air pollution exposure, i.e., selective attention (Stroop Test) and visual information processing speed (Digit-Symbol and Pattern Comparison Tests).

These findings may be interpreted in the context of experimental studies which emphasized the involvement of inflammatory events on neurobehavioral functioning. Administration of bacterial lipopolysaccharide showed in rats a rapid induction of systemic inflammation which strongly impaired memory retrieval in a task thought to require hippocampal pattern separation and context-object discrimination, whereas it did not impair mere memory retrieval in hippocampal

dependent tasks.[223] This finding supports the contention that acute neuro-inflammation may impair context discrimination memory via disruption of pattern separation processes in the hippocampus. Another experimental study on short-term (4 h) air pollution exposures showed rapid modulation of genes in the vaso-regulatory pathway of the brain.[224] In a randomized cross-over study of humans exposed to diesel motor exhaust (1 h), a significantly lower brain activity (EEG) has been detected in the left frontal cortex as reflected by increased median power frequency within 30 min of exposure, an effect still detectable until 1 h after exposure stopped.[225] Our observational findings are in line with the hypothesis of PM-induced cerebral inflammation and suggest a prompt neurobehavioral response.

Chronic PM exposure

As to chronic PM exposure, the two tests of the attention domain (Continuous Performance Test and Stroop Test) were adversely associated with an IQR increment of chronic ambient $PM_{2.5}$ and PM_{10} air pollution at residence.

Several recent studies reported an inverse association between chronic air pollution exposure and attention-related outcomes. Recently, we observed in adolescents a negative association between sustained attention, assessed using the Continuous Performance Test, and traffic exposure as reflected by a composite indicator comprising traffic density, time spent in traffic, and urinary *trans,trans* muconic-acid.[216] In a Boston study of 174 children (boys and girls) between 7 and 14 years of age, attention measured by the Continuous Performance Test was negatively associated with residential concentrations of lifetime BC exposure in boys only.[209] Though, their findings did not remain comparing the highest BC exposure quartile with the lowest quartile. In our study, we found strong associations between PM air pollution and the Continuous Performance Test, and a negative tendency was observed with chronic BC exposure ($p=0.06$). A possible explanation for the less pronounced effects of BC compared with particulate matter air pollution might be a potential higher exposure misclassification for BC as these models were built using less measuring points (14 for BC compared to 58 for PM_{10}). A Chinese study reported that children whose school was located in an area with low traffic density performed better in the Continuous Performance Test than those from a school with higher traffic-related air pollution.[214] In an

Indian study, the prevalence of ADHD was higher in urban children than in controls living in an area with low air pollution.[226] Another study indicated that the average residential elemental carbon concentrations during the first year of life were associated with a higher risk of hyperactivity at 7 years of age as assessed by Parent Rating Scale of the Behavioral Assessment System for Children.[227] A large study in Barcelona including more than 2,700 children showed that those exposed to high traffic-related air pollution levels at school had over a one-year school period a lower development of their working memory.[215]

A plausible explanation of our neurotoxic findings with chronic PM exposure may be linked to alterations in several CNS sub-structures, white matter lesions in cortical areas of the left cerebral hemisphere, and vascular changes.[54, 228] Calderón-Garcidueñas and colleagues[25] autopsied highly exposed children and young adults who suddenly died in Mexico City (average annual ambient air $PM_{2.5}$: 35.9 μg/m^3) and compared their findings with those of "control autopsies" of age-matched persons exposed to lower $PM_{2.5}$ concentrations (< 15 μg/m^3). The authors demonstrated in the highly exposed group an up-regulation of cyclo-oxygenase 2 (COX2), interleukin 1β (IL1β), and CD14 gene expression in the olfactory bulb, frontal cortex, substantia nigra and vagus nerves as well as disruption of the blood-brain-barrier, endothelial activation, and inflammatory cell trafficking. These adverse outcomes may be directly linked to deposition of ultrafine particles, a sub-fraction of $PM_{2.5}$, in cortical areas or to retro-axonal transport via the olfactory nerve, or alternatively by neuro-inflammation following a systemic inflammatory response to air pollution.[203, 204]

Strengths and limitations

Our study has several strengths. First, the risk of bias in the analysis of short-term CNS effects of air pollution was small because of the panel study design and the statistical approach used. More specifically, they allowed to eliminate the risk of reverse causality and confounding by person-related characteristics. Second, recent PM exposure at school was characterized by area sampling in the classroom on the days of the neurobehavioral examinations. Third, recent and chronic ambient exposures to $PM_{2.5}$, PM_{10}, and BC at residence were estimated for each participant using a high-resolution spatial temporal model. This may explain why significant associations were found with attention tests despite the low spatial

contrast in chronic $PM_{2.5}$ exposure (IQR difference 1.16 µg/m³). This observation is in line with other studies such as the Worcester Heart Attack Study which showed a link with acute myocardial infarction for an IQR $PM_{2.5}$ exposure contrast of 0.59 µg/m³.[229] Fourth, we used three estimates for recent ambient exposure at residence to cover the time period of exposure and potential lagged effects up to two days (Lag 0 to 2).

We acknowledge some limitations of the study. First, we cannot exclude residual confounding by other neurotoxic substances, such as lead, if these were strongly associated with the air pollution indexes. However, in a recent study we showed in adolescents that the associations between neurobehavioral performance and traffic-related exposure were independent of blood lead.[230] The present study, based on repeated measures, tested an *a priori* hypothesis involving interrelated neurobehavioral outcomes as well as strongly correlated PM exposures (e.g. correlation coefficient for chronic PM_{10} and $PM_{2.5}$ was r=0.87). Therefore, the neurobehavioral test performances or exposure indicators did not provide a completely independent opportunity for a type I error. For these reasons we did not adjust for multiple testing. Nevertheless, it seems unlikely that the consistency of negative phenotype-exposure associations would merely occur by chance as reflected by the significantly diminished performances, not only in the two Attention Tests (chronic ambient exposures to $PM_{2.5}$ and PM_{10} at residence) but also in the Pattern Comparison Test (recent ambient exposures to $PM_{2.5}$, PM_{10}, or BC on Lag 0-2 at residence). Further study is needed to find out why the inverse associations between the Stroop or Digit-Symbol test results and recent inside classroom PM exposure could not be corroborated for recent ambient PM exposure at residence. It is plausible that recent PM exposure characterized by 3-hour monitoring of classroom air displays different features in comparison to modeled recent PM exposure. Furthermore, the robustness and consistency of prompt neurobehavioral responses to recent exposure might be less outspoken than in the case of chronic ambient PM exposure at residence.

CONCLUSIONS

This is the first panel study comparing neurobehavioral changes of recent and chronic PM air pollution exposure. The repeated measurement study design in primary schoolchildren showed differential neurobehavioral changes robustly and inversely associated with recent or chronic ambient exposure to PM air pollution at residence, i.e., with recent exposure for visual information processing speed (Pattern Comparison Test) and with chronic exposure for sustained and selective attention. These neurotoxic findings on behavioral performances in schoolchildren associated with PM air pollution as found in Belgium in the years 2012-2014, strongly support the current tendency to bring the EU limits for ambient air PM-exposure as low as the WHO guidelines.

CHAPTER 5

CHILDREN'S URINARY ENVIRONMENTAL CARBON LOAD: A NOVEL MARKER REFLECTING RESIDENTIAL AMBIENT AIR POLLUTION EXPOSURE?*

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ABSTRACT

Background: Ambient air pollution, including black carbon, entails a serious public health risk because of its carcinogenic potential and as climate pollutant. To date, an internal exposure marker to black carbon particles having cleared from the systemic circulation into the urine does not exist. We developed and validated a novel method to measure black carbon particles in a label-free way in urine.

Methods: We detected and quantified urinary carbon load in 289 children (aged 9-12 years) using white-light generation under femtosecond pulsed laser illumination. Children's residential black carbon exposure was estimated based on a high-resolution spatial temporal interpolation method.

Results: We were able to detect urinary black carbon in all children, with an overall average (SD) of 98.2 x 10^5 (29.8 x 10^5) particles/mL . The urinary black carbon load was positively associated with medium-term up to chronic (one month or more) residential black carbon exposure, *i.e.* +5.33 x 10^5 particles/mL higher carbon load (95% CI: 1.56 x 10^5 to 9.10 x 10^5 particles/mL) for an interquartile range (IQR) increment in annual residential black carbon exposure. Consistently, children who lived closer to a major road (≤ 160 m) had higher urinary black carbon load (6.93 x 10^5 particles/mL; 95% CI: 0.77 x 10^5 to 13.1 x 10^5).

Conclusions: Urinary black carbon mirrors the accumulation of medium-term up to chronic exposure to combustion-related air pollution. This specific biomarker reflects internal systemic black carbon particles, cleared from the circulation into the urine, allowing its utility in unraveling particulate-related health effects.

INTRODUCTION

Current ambient outdoor air pollution is responsible for 4.2 million premature deaths worldwide (based on data between 1990 and 2015),[3] and ranked within the top ten of important risk factors for public health. Children are especially vulnerable to the detrimental effects of air pollution and have for the same ambient concentrations a higher internal dose compared to adults due to their higher respiratory rate. Combustion-related particulate matter (PM) air pollution, including black carbon (BC), is associated in early life with lower birth weight,[34] decreased cognitive function in children,[231, 232] impaired cognitive aging,[233] increased cardiovascular morbidity and mortality[4, 5] as well as respiratory diseases[234] and lung cancer[26] in adult life. Three hypotheses are formulated to rationalize these findings. First, particles produce pulmonary oxidative stress and inflammation with a systemic release of cytokines.[5] Second, the smallest particles translocate from the lungs into the circulation with effects in different organ systems. Third, particles interact with pulmonary receptors or nerves with effects *via* the autonomic nervous system.[235] Experimental studies on animals have shown that a substantial fraction of intratracheally introduced ultrafine particles could translocate into the systemic circulation,[22] and even may translocate via the olfactory nerve to the brain when deposited in the nose.[23] However, in humans the subject of particle translocation is still under debate as to date studies failed to find a considerable fraction of inhaled particles translocated into the circulation.[236, 237] Furthermore, the clinical significance how these ultrafine particles contribute to health remains unclear.

Although some air pollution associations are established as causal,[5] risks might be considerably underestimated due to exposure misclassification. In most epidemiological studies, the exposure to PM air pollution, including black carbon, is not measured at the individual or time-activity pattern level. Instead, spatial temporal models are used, which basically use land cover data that is based on multiple primary sources (*i.e.,* road networks, line and point locations of potential sources, building density, etc.) to estimate the daily residential exposure values [104, 238] which results in incomplete information about individual mobility. In these studies, the bulk of exposure measurement error, which is typically "Berkson-like", leads to unbiased but more imprecise health effect estimates.[239] Despite

increased understanding of the health consequences of combustion-related air pollution; a critical barrier to progress in the field is our limited ability to monitor adequately personalized exposure over the life course. To overcome these shortcomings, we postulated the translocating nature of black carbon particles from the circulation into the urine so that these particles in urine form a biomarker reflecting exposure. Within this framework, we detected and quantified black carbon particles in urine of 289 children living in the northern part of Belgium with relatively low annual ambient black carbon concentrations in the study area ranging from 1.07 to 1.96 μg/m³.

METHODS

Calibration experiments for black carbon detection in urine

In this research, we employed a label-free and biocompatible imaging technique based on the non-incandescence related white-light generation potential of carbonaceous particles under femtosecond illumination.[240] Summarized, the heterogeneous and absorptive (dark color) nature of carbonaceous particles gives rise to the white-light phenomenon. For the signal to occur, these two conditions should explicitly be fulfilled. Therefore, (i) the possible contribution of any non-carbonaceous material (except for noble metals; they are able to generate plasmons) is excluded, since they do not comply with the aforementioned conditions; (ii) carbon-containing materials such as endogenous structures with carbon backbones will not generate the white-light since they do not contain multiple absorbing sites; and (iii) the material generating this signal should be in particle form for exhibiting the heterogeneity and absorptive character.

Here, several experiments were conducted to assess the potential for cross-reactivity or to assess specificity more generally. First, we validated and calibrated the application method for urine imaging using artificial urine. This artificial urine solution was imaged under identical imaging conditions as for analyzing the 'real' urine samples; to check for any signal coming from, for example, urinary salt crystals. Second, background signals from naturally present carbonaceous particles in the air and detection chambers were checked by measuring empty Ibidi wells. Third, cross-reactivity from the most structurally and chemically

resembling particles available, named silica particles[241], was checked under identical imaging conditions. Fourth, cross-reactivity from other carbonaceous materials was checked by measuring carbon nanotubes. Fifth, Raman spectroscopy measurements were executed on dried urine samples. For details see supplementary methods.

Optimized experimental protocol for black carbon detection in urine

The carbonaceous particles in the urine samples were analyzed and images collected using a Zeiss LSM510 META NLO (Carl Zeiss, Jena, Germany) mounted on an Axiovert 200 M equipped with a two-photon femtosecond pulsed laser (MaiTai DeepSee, Spectra-Physics, USA). A detailed description of the set-up and imaging protocol can be found in the supplementary methods.
From optimization measurements we found that 120 images obtained from 10-frame time lapses at three different positions in four different aliquots of one urine sample are necessary to gain highly reproducible results (<5 % coefficient of variation of three repeated measurements for 19 individuals, data not shown). Urine samples were aliquoted at 200 µL/well in Ibidi µ-slide 8 well plates (Ibidi GmbH, Germany). All images were taken 300 µm above the bottom glass.

To count the number of black carbon particles in the time frames of each urine sample, a peak-find algorithm in Matlab (Matlab 2010, MathWorks, The Netherlands) was used. A detailed description of the function of the customized Matlab routine can be found in supplementary methods.

Validation experiments of optimized urinary carbon load technique

The optimized technique for measuring carbon load in urine was validated by spiking urine with known concentrations of carbon black nanopowder (US Research Nanomaterials, USA) and by analysing the repeatability of urinary carbon load (see supplementary methods for the details).

Repeatability of urinary carbon load was assessed by calculating the coefficient of variation of urine samples taken at 3 different time points (± one

month from each other) (n=19) and analyzed using the optimized experimental protocol.

Urinary carbon load and real-life exposure to combustion-related air pollution in children

i) Study population and sample collection

We conducted this study in the framework of the COGNAC (COGNition and Air pollution in Children) study, which enrolled children (aged 9 – 12 years) from three different primary schools (Tienen, Zonhoven, Hasselt) in Flanders, Belgium.[232] In total, we recruited 334 children between January 2012 and February 2014. Parents were asked to fill out a questionnaire in order to get additional lifestyle information, the mother's education (low – no diploma or primary school, middle – high school degree, high – college or university degree), passive exposure to tobacco smoke and child's ethnicity, residence, transportation from and to the school, general health, state of mind and physical activity (times/week). Written informed consent was obtained from the parents and oral consent from the children. The study protocol was approved by the ethical committees of Hasselt University and East-Limburg Hospital, Belgium. Urine samples (available for 289 children) were collected on the first examination day using designated metal- and black carbon-free sample jars (Yvsolab, Belgium) and placed at 4°C until chronic storage at -80°C. To avoid external contamination from carbon particles we aliquoted urine samples in a clean room with filtered air (Genano®310, Genano OY, Espoo, Finland). Osmolality of urine was measured by the advanced Cryptomatic Osmometer.

ii) Residential exposure estimates.

We constructed estimates of ambient exposure [black carbon, nitrogen dioxide (NO_2) and particulate matter ≤ 2.5 μm ($PM_{2.5}$),], based on their residential address(es), using a spatial temporal interpolation method.[102] The interpolation method uses land-cover data obtained from satellite images (CORINE land-cover data set) and pollution data of fixed monitoring stations. Coupled with a dispersion model[104] that uses emissions from point sources and line sources, this model chain provides daily exposure values in a high-resolution receptor grid. Overall model

performance was evaluated by leave-one-out cross-validation including 14 monitoring points for black carbon, 44 for NO_2, and 34 for $PM_{2.5}$. Validation statistics of the interpolation tool gave a spatiotemporal explained variance of more than 0.74 for black carbon[106], 0.78 for NO_2[105], and 0.80 for $PM_{2.5}$[105]. We calculated different exposure windows by averaging daily concentrations over a period preceding the examination day, *i.e.* recent exposure (one day and one week before urine sampling), medium-term exposure (one month before sampling) and chronic exposure (one year and two years before sampling). When a child had more than one residential address at the moment of the study, we calculated a weighted average using the proportion of time spent at each location as weights. In addition, we also calculated the residential proximity to major roads, defined as highways and other national roads (a road with more than 10,000 motor vehicles/day), using geographic information system functions (ArcGIS 9.3).

iii) Statistical analyses.

Statistics were carried out using SAS software (version 9.3, SAS Institute Inc., Cary, NC, USA). Partial Pearson correlation coefficients were calculated to evaluate the correlations between urinary carbon load and recent, medium-term or chronic exposure to black carbon, NO_2 and $PM_{2.5}$ as well as residential proximity to major roads (living twice as close to major roads). To improve normality of the distributions we log-transformed residential proximity to major roads. Multiple linear regressions were performed to assess the independent associations between urinary carbon load and recent, medium-term, chronic exposure, or residential proximity to major roads, while accounting for person-related factors, including age, sex, and body mass index (BMI) of the child, mother's education, and urinary osmolality as well as a time-related factor, including month of examination. Results were presented as a change in urinary carbon load (particles per mL urine) for an interquartile range (IQR) increment in recent, medium-term or chronic exposure, living twice as close to the nearest major road.

In a sensitivity analysis, we evaluated whether osmolality, creatinine, education of father (up to high school diploma – college or university degree), occupation of either parents (unemployed or not qualified worker – qualified worker, white-collar assistant, or teaching staff – self-employed, specialist, or member of management), exposure to passive smoke (none – ≤ 10

cigarettes/day – > 10 cigarettes/day), physical activity (hours/week), or vegetable/fruit intake from own garden (percentage) affects the association between urinary carbon load and residential black carbon exposure. Additionally, we also checked the independence of recent and chronic residential black carbon exposure on urinary carbon load.

Finally, we calculated the ability to predict child's residential black carbon exposure based on the urinary carbon load. For this purpose, we estimated sensitivity and specificity of the prediction using receiver-operating characteristic (ROC) plots. Children were stratified according to their chronic residential black carbon exposure with the 75th percentile as cutoff point (1.64 µg/m³).

RESULTS

Calibration, optimization and validation of the label-free optical detection of carbon particles in urine

From the calibration and optimization experiments, we arrived at the following results: 1/ The employed detection technique is very specific for the detection of carbon particles in urine and does not detect other types of carbonaceous or non-carbonaceous particles by cross-reactivity, as shown by silica nanoparticles and carbon nanotubes (for a detailed description, see Supplementary notes, Note 1). Furthermore, no background signal could be observed from the artificial urine solution. 2/ Raman fingerprints of aggregates found in dried urine samples are identical to the fingerprint of carbon-based reference particles (see Supplemental Material Figure S1). 3/ Protocol parameters such as the optimal detection plane and measurement repetition rate were optimized to minimize the intra-sample variation. A flowchart of the optimized protocol is depicted in Figure 1.

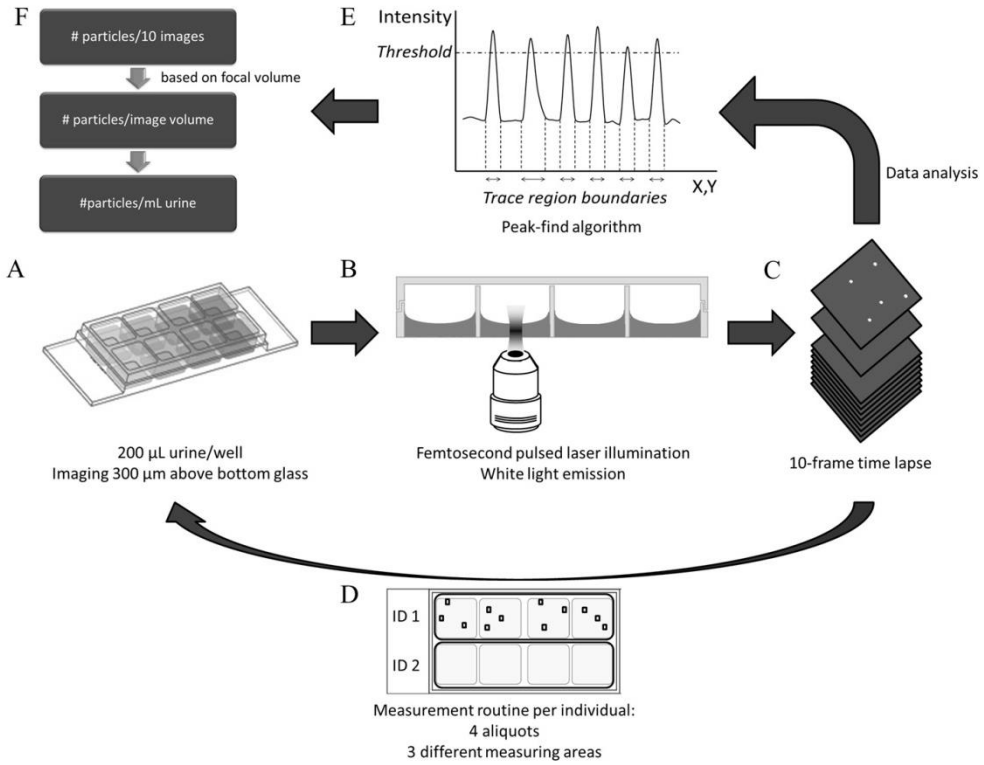

Figure 1. Flowchart of the optimized experimental protocol for black carbon detection in urine. Each urine sample is aliquoted at 200 µL/well in a Ibidi µ-slide 8 well and images are taken 300 µm above the bottom glass of the well plate (**A**). The samples are illuminated using a two-photon femtosecond pulsed laser tuned to a central wavelength of 810 nm (~ 9.7 mW radiant power at the sample position) and the white light generated by the black carbon particles naturally present in the urine is detected via analogue photomultiplier detection in epi-configuration in non-descanned mode using a 40x/1.1 water immersion objective at room temperature (**B**). 10 consecutive images are taken on one identical location in the same well. The resulting images have a field of view of 225 x 225 µm² with a 512x512 pixel resolution (0.44 x 0.44 µm² pixel size) and a pixel dwell time of 3.2 µs (**C**). In total, 120 images are obtained by recording 10-frame time lapses at three different locations in four different aliquots of one individual (ID) resulting in highly reproducible results (<5% coefficient of variation) (**D**). To determine the number of black carbon particles in the images, a peak-find algorithm counting connected pixels above a threshold value (15% lower than the highest intensity value) was used (**E**). The average amount of particles detected in the different time lapses is normalized to the image volume using the focal volume estimated from the point spread function of the optical system. Finally, the result is expressed as the total relative number, *i.e.* the number of detected black carbon particles per milliliter urine. All images of each individual are analyzed in this way to retrieve a number of detected black carbon particles per milliliter urine sample (**F**).

The optimized experimental protocol was validated by measuring and analyzing artificial urine spiked with increasing concentrations (0 to 120 µg/mL) of carbon black nanopowder. A linear relation was observed (R^2=0.98) between the amount of added and detected carbonaceous particles (see Supplemental Material Figure S2). Black carbon particle detection in urine by femtosecond pulsed laser microscopy is visualized in Figure 2. Repeatability of spot urine samples taken at three different time points (± one month from each other) (n=19) showed an average coefficient of variation of 20%.

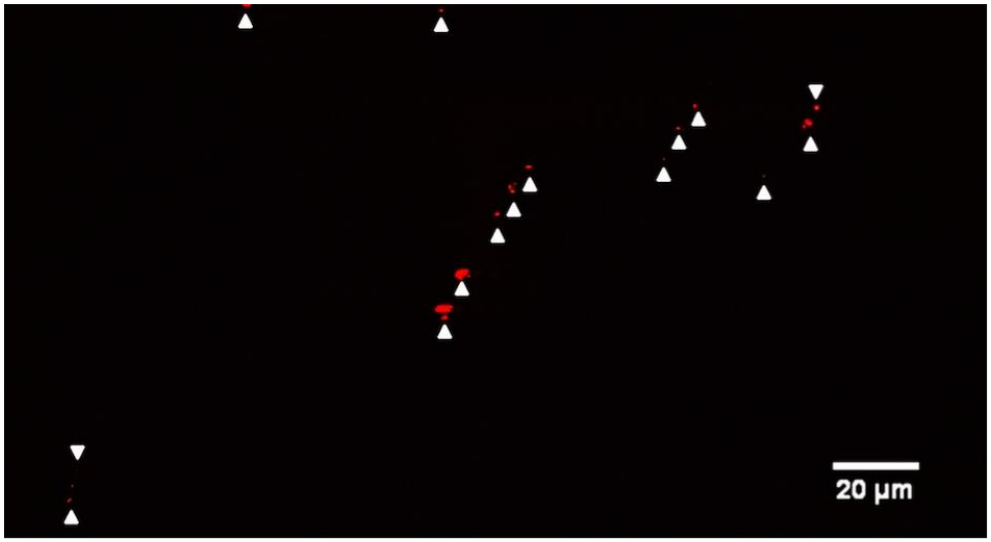

Figure 2. Black carbon particles in urine. Black carbon particles and aggregates visualized by femtosecond pulsed laser excitation at 810 nm and observation at 400 - 410 nm (red points indicated by arrowheads).

Urinary carbon load and residential black carbon exposure.

Demographic and lifestyle characteristics are presented in Table 1. Children (50.5% boys) were on average (SD) 10.3 (1.2) years old. The distribution over mother's low, and high educational class was 116 (40.1%), and 173 (59.9%), respectively. The children's BMI was 17.4 (2.9). Median (25^{th}-75^{th} percentile) modeled exposures of black carbon, particulate matter with an aerodynamic diameter ≤ 2.5 µm ($PM_{2.5}$) and nitrogen dioxide (NO_2) over various time windows of exposure ranging from recent exposure (one day and one week before urine sampling), medium-term exposure (one month before urine sampling), and chronic exposure (one and two years before urine sampling) as well as median

distance (25^{th}-75^{th} percentile) from residence to major roads are given in Table 2 and Supplemental Material, Table S1. The Pearson correlation coefficient between chronic (one year) and recent (one day and one week) residential black carbon exposure was 0.15 (95% CI: 0.04 to 0.26) and 0.09 (95% CI: -0.03 to 0.20), respectively. The correlation between chronic and medium-term (one month) residential black carbon exposure was 0.36 (95% CI: 0.26 to 0.46).

Table 1. Characteristics of the participants (n=289)

Anthropometric characteristics	
Boys	143 (50.5%)
Age, years	10.3 (1.2)
Body Mass Index (BMI)	17.4 (2.9)
Weight, kg	37.0 (9.6)
Length, cm	145 (1.0)
Lifestyle characteristics	
Mother's education	
Up to high school diploma	116 (40.1%)
College or university diploma	173 (59.9%)
Father's education[a]	
Up to high school diploma	132 (47.3%)
College or university diploma	147 (52.7%)
Most prestigious category of occupation of either parents	
Unemployed or not qualified worker	23 (7.9%)
Qualified worker, white-collar assistant, or teaching staff	118 (40.8%)
Self-employed, specialist, or member of management	148 (51.2%)
Exposure to passive tobacco smoke	
None	263 (78.3%)
≤ 10 Cigarettes/day	44 (13.1%
> 10 Cigarettes/day	29 (8.6%)
Urinary characteristics	
Osmolality, mOsm/kg	927.9 (212.3)
Creatinine, mg/dL[b]	127.0 (48.9)
Carbon load, particles/mL[#]	98.2×10^5 (29.8×10^5)

Arithmetic mean (SD) is given for the continuous variables. Number (%) is given for the categorical variables. Data available for [a] 279 participants, [b] 276 participants. [#] Mean (IQR).

Table 2. Residential black carbon exposure characteristics (n=289)

Residential exposure	Median	25th percentile	75th percentile
Recent black carbon, µg/m³			
1-day	1.45	1.19	2.23
1-week	1.68	1.27	2.27
Medium-term black carbon, µg/m³			
1-month	1.63	1.38	1.83
Chronic black carbon, µg/m³			
1-year	1.54	1.43	1.64
2-years	1.53	1.43	1.65
Distance to major roads, m	330.6	126.7	820.4

Urinary black carbon load averaged (IQR) 98.2 x 10^5 (29.8 x 10^5) particles per mL urine (Table 1) and did not differ between boys and girls (*P*=0.55). There was no association between urinary carbon load and child's age (*P*=0.74), weight (*P*=0.81), height (*P*=0.99), BMI (*P*=0.90), education of the mother (low versus high: +2.25 x 10^6, 95% confidence intervals (CI): -3.38 x 10^5 to 7.89 x 10^5, *P*=0.14), education of the father (low versus high: +1.61 x 10^5, 95% CI: -3.87 x 10^5 to 7.10 x 10^5, *P*=0.10), highest occupation of either parents (low versus high: +8.83 x 10^5, 95% CI: -1.28 x 10^5 to 1.89 x 10^5, *P*=0.14), physical activity (*P*=0.48), exposure to passive smoking (*P*>0.43), vegetable or fruit from own garden, (*P*=0.77). Osmolality was significantly associated with urinary carbon load, *i.e.*, +3.1 x 10^5 particles/mL (95% CI: 0.22 x 10^4 to 6.1 x 10^5, *P*=0.04) for an interquartile range (IQR) increment in osmolality (229 mOsm/kg), while urinary creatinine concentration was not a predictor of the urinary carbon load (*P*=0.82).

Both before (Figure 3A-B) and after adjustment (Figure 3B) for *a priori* chosen covariates including sex, age, BMI, mother's education, month of examination, and urinary osmolality, carbonaceous particles in urine were positively correlated to medium-term black carbon exposure (*partial r*=0.12, 95% CI: -0.002 to 0.23), chronic annual residential black carbon (*partial r*=0.17, 95% CI: 0.05 to 0.28) as well as residential proximity to the nearest major road (*partial r*=0.15, 95% CI: 0.03 to 0.26). The corresponding results for an IQR increment of medium-term black carbon and chronic annual residential black carbon

exposure were +5.90 x 10^5 particles/mL (95% CI: -0.81 x 10^4 to 11.9 x 10^5) and +5.33 x 10^5 particles/mL (95% CI: 1.56 x 10^5 to 9.10 x 10^5) more urinary carbonaceous particles, respectively. Furthermore, children living close to a major road (first tertile: ≤160 m or median: ≤330.6 m) had 7.29 x 10^5 particles/mL (95% CI: 1.12 x 10^5 to 13.5 x 10^5; *P*=0.02) or 8.78 x 10^5 particles/mL (95% CI: 3.14 x 10^5 to 14.4 x 10^5, *P*=0.0024) higher urinary carbon load respectively compared with those living further away (> 160 m or >330.6 m). Correspondingly, living twice as close to the nearest major road was associated with +2.02 x 10^5 particles/mL (95% CI: 4.09 x 10^4 to 3.64 x 10^5) higher urinary carbon load. In addition, similar patterns were found for medium-term to chronic annual residential NO_2 as well as chronic annual $PM_{2.5}$ exposure (Supplemental material, Figure S3). All recent exposure time windows (one day and one week) of black carbon, NO_2 and $PM_{2.5}$ were not correlated with the carbonaceous particles in urine.

Sensitivity analysis still showed an association between urinary carbon load and residential, annual BC exposure with and without adjustment for osmolality, with replacing osmolality by creatinine or by adjusting for both osmolality and creatinine as measures of urine concentration. (Supplemental material, Table S2). Furthermore, no changes were observed when the main model with *a priori* chosen covariates was additionally adjusted for education of the father, highest occupation of either parents, exposure to passive smoking, physical activity, creatinine, or vegetable/fruit intake from own garden (Supplemental material, Table S2). Furthermore, we also showed that the association between chronic residential exposure and internal urinary carbon load is independent of recent exposure (Supplemental material, Table S2).

Finally, figure 4 shows the ROC curve analysis with sensitivity and 1 minus specificity (false positive ratio) of chronic residential black carbon exposure (75^{th} percentile as cut off point – 1.64 µg/m³) in association with children's urinary carbon load. The model adjusted for aforementioned *a priori* chosen covariates had an area under the curve (AUC) value of 0.74 (95% CI: 0.67 to 0.81).

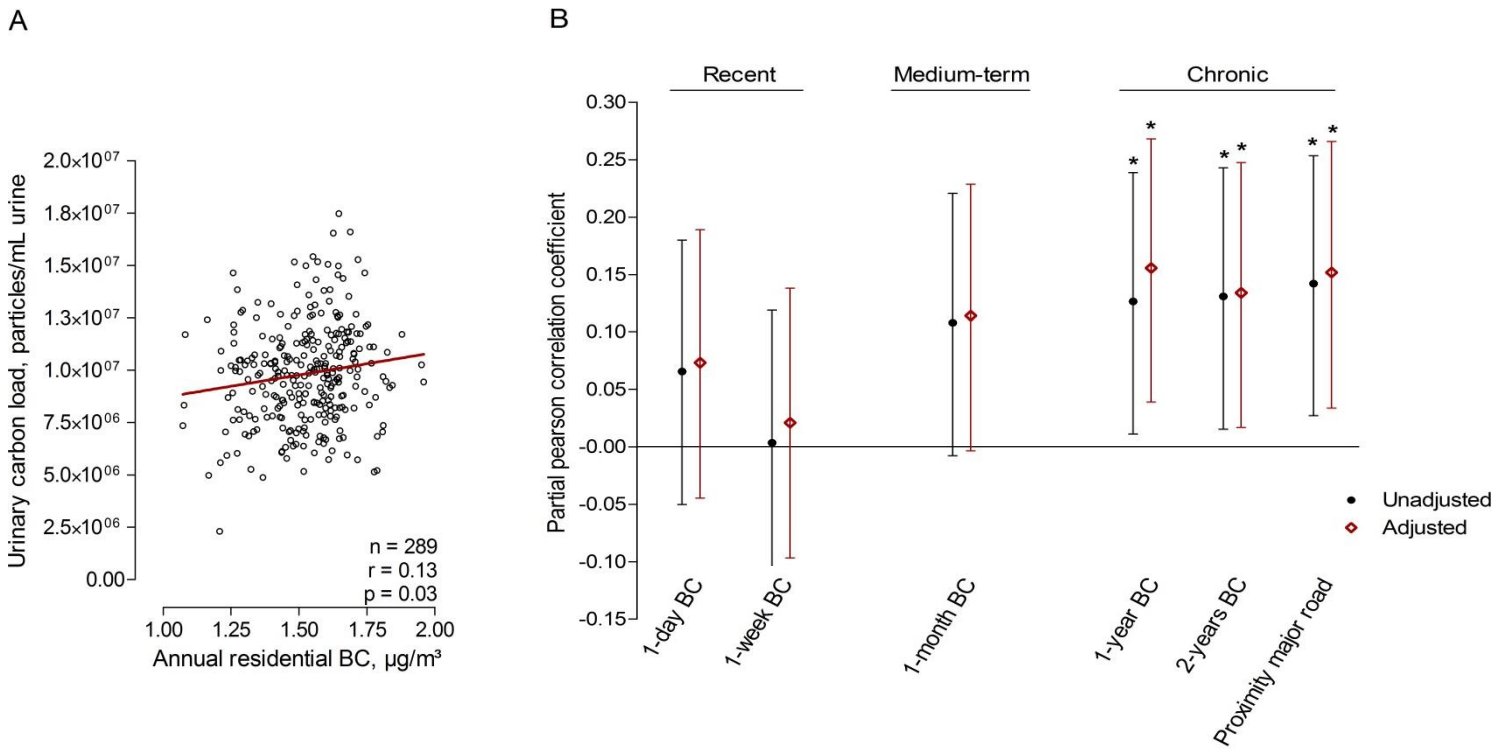

Figure 3. Correlation between urinary black carbon load and residential exposure to recent up to chronic black carbon or proximity to major roads. Dose-response relation between urinary black carbon load and annual residential black carbon exposure, adjusted for urinary osmolality (solid red line = regression line) (**A**). Unadjusted and adjusted (partial) Pearson correlation coefficient and 95% confidence intervals (**B**) between urinary black carbon load and residential recent (1-day and 1-week before urine sampling), medium-term (1-month before urine sampling) or chronic black carbon exposure (1-year and 2-years before urine sampling) or residential proximity to major roads (log_{10}). Partial correlation coefficients were adjusted for *a priori* chosen covariates including sex, age, BMI, mother's education, month of examination, and urinary osmolality. * $P<0{\cdot}05$.

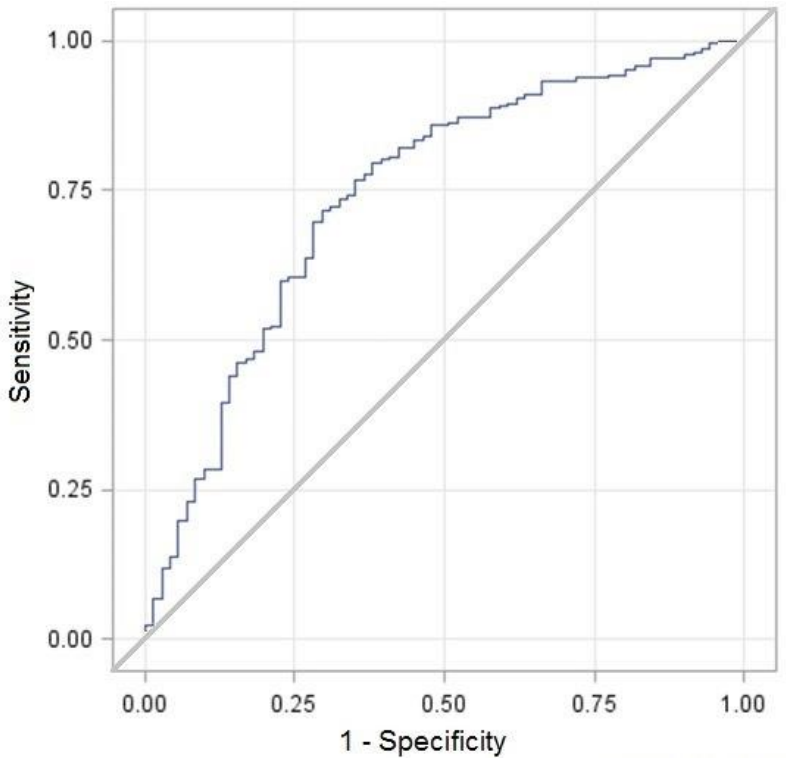

Figure 4. Receiver operating characteristics (ROC) curve for urinary carbon load distinguishing between high and low residential black carbon exposure. Performance of urinary carbon load to differentiate between high (> 75th percentile: 1.64 µg/m³) and low (≤ 1.64 µg/m³) exposure to chronic residential black carbon exposure. The model was adjusted for gender, age, BMI, mother's education, month of examination, and urinary osmolality.

DISCUSSION

In the prospect that ultrafine particles may translocate to the circulation and may be cleared into the urine, we developed a method to measure urinary black carbon load as an exposure measure of carbonaceous particles in a population at fairly low exposure levels, more specifically in children as a susceptible subgroup for the detrimental effects of air pollution. Overall, our results demonstrate the use of white-light generation of black carbon particles in urine under femtosecond pulsed laser illumination as a measure for exposure to combustion-related black carbon air pollution. The novel information from our work comprises the following: 1/

urinary black carbon load mirrors medium-term to chronic ambient exposure, even at low environmental concentrations; 2/ our method requires no additional labeling or preparation procedure and the raw data can be analyzed using a simple peak-find algorithm; 3/ detection of black carbon particles in urine reflects the passage of black carbon particles from the circulation into the urine.

Different experimental rat studies have demonstrated that ultrafine particles can translocate from the lung into the circulation.[242, 243] These particles can accumulate at sites of vascular inflammation[244] and have direct access to intracellular components such as proteins, organelles and DNA [243]. Furthermore, Oberdörster *et al.*[23] demonstrated that intranasally instilled solid ultrafine particles translocate along axons of the olfactory nerve into the central nervous system. In this regard, Maher *et al.*[245] recently identified the presence of magnetite nanoparticles, formed by combustion and/or friction derived heating, in the human brain. Suggested pathways for particle translocation into the circulation are either across the alveolar epithelium or across the intestinal epithelium from particles that have been cleared *via* mucous ingested into the gastrointestinal tract.[23] Nevertheless, the issue of particle translocation in humans is still controversial. Nemmar *et al.*[246] showed that inhaled technetium-99m labeled ultrafine carbon particles could rapidly pass into the systemic circulation. Other studies, however, using short-term inhalation (up to two days) of technetium-99m labeled ultrafine carbon particles found that most of the inhaled particles were retained in the lung periphery and in the conducting airways without substantial translocation to the systemic circulation.[11, 236, 237] Mills *et al*.[236] reported that the radioactive moiety of the label, rather than the particle itself, was detected in the blood. Wiebert and colleagues found no significant translocation of inhaled 35 nm carbon particles to the circulation in humans.[237] Another study[247] on pulmonary deposition and retention of indium-111 labeled ultrafine carbon particles in healthy individuals only found marginal translocation of particles from lungs to blood (0.3%). Moreover, there was no observable elimination of particles from the body via urine one week post-administration. These studies, which are based on labeling techniques and short-term exposure conditions, contrast with our current findings using label-free white light detection of urinary black carbon in children continuously exposed to low levels of air pollution in real life. Because of the stability of inhaled ultrafine carbon particles,

long-term retention in the human lung is expected and may accumulate to a chronic particle load.[11] In this regard, Churg and Brauer[248] and Brauer *et al.*[249] observed that large quantities of fine and ultrafine aggregates retain in the human lung parenchyma whereby ultrafine particles make up only a small fraction of the retained total. Furthermore, a recent study showed that circulation levels of 5 nm gold particles were greater compared to inhaled 30 nm particles.[244] The smallest particles are in steady state as they are retained longer with potential translocation mechanisms from the lungs to the system.[11, 23] This is in line with our observations that residential medium-term to chronic ambient black carbon concentrations are significantly associated with urinary black carbon load. In contrast to our method, labelling studies are not able to detect the background load of particles in urine.

In the past, efforts have been made to identify a reliable and effective biomarker for combustion-related exposure. Oxidative stress is considered as one of the mechanisms through which traffic-related air pollution exerts its effects on human health. The urinary excretion of 8-oxo-7,8-dihydro-2-deoxyguanosine is used as a biomarker of response to evaluate the pro-oxidant effects of vehicle exhaust emissions on DNA.[250] Another example is the metabolite of benzene, urinary trans, trans-muconic acid (*t,t*-MA), which has been considered as a proxy-biomarker for traffic.[251, 252] However, the aforementioned biomarkers are not specifically related to combustion-associated air pollution. Furthermore, these biomarkers do not reflect chronic exposure. More recently, carbon load in alveolar macrophages has been used as a biomarker of exposure to traffic exhaust pollution and biomass smoke exposure.[253, 254] However, this technique requires semi-invasive sampling procedures (sputum induction) with success rates of approximately 60%,[254, 255] and identified black carbon particles using light microscopy, thereby underestimating the total amount of carbon load.[253] Our current technique to detect carbon load in urine does not require invasive sampling procedures and uses label-free white-light generation detection to determine the amount of black carbon particles in urine, a technique specifically to detect carbonaceous particles. Aside from continuous analysis, we established ROC curves to distinguish residential chronic low (75th percentile) exposed from higher exposed children for black carbon and showed an area under the curve of

0.74. Therefore, our novel exposure biomarker has the ability to distinguish between true- and false positives.

We acknowledge some limitations of our study. First, ambient black carbon particles in the air could have contaminated the urine samples. By using a clean room with filtered air to handle the urine samples and using sterile metal-free collection tubes, we avoid potential external contamination of carbon particles. Furthermore, no background signals from naturally present carbonaceous particles in the air were observed in the detection chambers or in the sterile metal-free collection tubes. Second, we cannot exclude that black carbon particles detected in urine might mirror particles entered through food or drinks, or even uptake through skin instead of translocation from the lung to the system. However, in our sensitivity analysis, consuming vegetables or fruit from own garden did not predict the carbon load in urine. Third, urban air consists of particles with a size between 0.02 and 100 μm, with primary particle size ranging from 6 to 100 nm.[256] Diesel exhaust particles usually consist of aggregates with a diameter of 10 to 40 nm.[257] Particles with a diameter up to 75 nm may diffuse and accumulate in the mesangium of the glomerulus.[258] The glomerular filtration instigates renal clearance of particles with a size of 10 nm and smaller.[258] While it is possible to detect the smallest particles present in the urine it is not possible to determine their size and distribution due to the diffraction limit in optical microscopy. Fourth, our external exposure estimates of black carbon relates on modelled residential exposure, and not on personal monitoring. Measurement *via* personal exposure samplers is not practical to assess long-term exposure of large population samples. Validation statistics of the exposure model showed an explained spatiotemporal variance of >0.74 for black carbon.

CONCLUSIONS

In conclusion, we showed for the first time that urinary black carbon load in children is associated with medium-term to chronic exposure to ambient combustion related pollution. This specific biomarker reflects internal systemic black carbon particles allowing its utility in unraveling particulate-related health effects, and can be used in different study populations over the entire life course.

SUPPLEMENTARY METHODS

Calibration experiments for black carbon detection in urine.

The employed artificial urine contained urea and inorganic salts. It was prepared by dissolving 2.43 g urea (Janssen Chimica, Belgium), 0.30 g sodium citrate dehydrate (Janssen Chimica), 0.63 g sodium chloride, 0.45 g potassium chloride, 0.16 g ammonium chloride (Merck Chemicals, Belgium), 0.09 g calcium chloride dehydrate, 0.10 g magnesium sulfate heptahydrate (Acros Organics, Belgium), 0.03 g sodium hydrogen carbonate (Sigma-Aldrich, Belgium), 0.26 g sodium sulfate (UCB, Belgium), 0.10 g sodium phosphate monobasic monohydrate (Sigma-Aldrich, Belgium), and 0.01 g sodium hydrogen phosphate in 200 mL ultrapure water (composition adapted from Chutipongtanate *et al.*[259]).
The employed silica and carbon nanotubes were retrieved from Sigma Aldrich (S5130, fumed, 7 nm), and Joint Research Centrum (multi-walled carbon nanotubes, reference material NM400) and National Institute of Health (single-walled carbon nanotubes, reference material 2483), respectively. All materials were checked using identical imaging conditions.
For the Raman spectroscopy measurements, a drop of urine was dried on a cleaned microscopy slide and sealed using a cover slip. Raman spectra were collected with a CCD camera (Newton, Andor, UK) equipped with a blazed grating monochromator (IHR320, Horiba, Japan). A 633 nm Helium Neon Laser (Research and Electro-Optics INC, USA) with an average power at the samples of 1 mW was used. The Raman signal passed a long pass filter of 645 nm. The integration time was 10 s and averages of 6 scans are shown. Data were collected in air at room temperature.

Optimized experimental protocol for black carbon detection in urine.

A 40x/1.1 water immersion objective (LD C-Apochromat 40x/1.1 W Korr UV-Vis-IR, Carl Zeiss) was used and the laser was tuned to a central wavelength of 810 nm with a ~ 9.7 mW radiant power at the sample position. Black carbon particle emission was detected *via* analogue photomultiplier detection in epi-configuration

in non-descanned mode after the signal passed through a 400 – 410 nm band pass filter. The resulting images had a field view of 225 x 225 μm² with 512x512 pixel resolution (0.44 x 0.44 μm² pixel size) and a 3.2 μs pixel dwell time. The point spread function in X- and Y-direction is about 350 nm. All data were recorded at room temperature (22°C).

The Matlab routine, for determining the number of particles in the acquired images, counts connected pixels above a threshold value. A threshold value of 15% lower than the highest intensity value was chosen, which gave highly reproducible values. The average amount of black carbon particles obtained in this way was normalized using the focal volume estimated from the point spread function of the optical system to obtain results expressing the total amount of detected black carbon in the imaged volume. Finally, the results are expressed as the number of detected black carbon particles per milliliter urine.

Validation experiments of optimized urinary carbon load technique.

To spike the urine samples for validation experiments, carbon black nanopowder was employed. However, it should be noted that carbon black powder in solution tends to form larger aggregates. Therefore the following precautions were taken: (i) diluting in urine helps to gain better dispersity, since the proteins are beneficial, (ii) the stock solution was ultrasonicated in a water bath for a sufficient amount of time, (iii) the dilution series was additionally sonicated, (iv) all solutions and dilutions were freshly prepared, just before measuring them, (v) all solutions and dilutions were checked visually and by dynamic light scattering for aggregates, (vi) all solutions and dilutions appeared to be stable up to two hours after preparation, which is a sufficient amount of time to measure the calibration curve.

From the spiked urine samples seven 10-frame time lapses were acquired using the optimized experimental protocol as described below. The number along the vertical axis was determined by counting the number of detected particles in each collected time series and by subsequently normalizing this number to the amount of particles per millilitre urine using the focal volume estimated from the point spread function. The amount of detected particles is expressed as a mean

with standard deviation and plotted against the known concentration of added carbon black. The curve was linearly fitted.

SUPPLEMENTARY NOTES

Note 1: specificity and cross-reactivity of detection technique

It is important to realize that the described signal, namely the white-light generation under femtosecond pulsed laser illumination, is a very distinct and specific signal for carbonaceous particles. A detailed description of the discussed signal can be found in the following paper: Bové *et al.* "Biocompatible Label-Free Detection of Carbon Black Particles by Femtosecond Pulsed Laser Microscopy." *Nano letters* 16.5 (2016): 3173-3178. Summarized, the heterogeneous and absorptive (dark color) nature of carbonaceous particles gives rise to the white-light phenomenon. This means that for the signal to occur, these two conditions should be met. Therefore, (i) excluding the possible contribution of any other non-carbonaceous material (except for nobel metals, they are able to generate plasmons); (ii) carbon-containing materials such as endogenous structures with carbon backbones; (iii) the material generating this signal should be in particle form for exhibiting the heterogeneity and absorptive character. Different experiments were conducted to assess the potential cross-reactivity or general specificity:

1) **Control experiment using artificial urine:** to check for background signals from, for example, salt crystals or urea in the urine, an artificial urine solution was imaged using identical imaging conditions. No background signals were detected.
2) **Cross-reactivity with similar non-carbonaceous particles:** no signals are expected from non-carbonaceous or other type of particles, since they do not comply with the minimal requirements for generating the white-light signal. To further proof the independence from other particles, silica particles – which are the most resembling (structurally and chemically) particles available – were imaged under identical imaging conditions. As expected, no white-light signal can be observed from this type of particles.
3) **Cross-reactivity with other carbonaceous materials:** no other carbonaceous materials are expected to be found in urine, except for possibly carbon nanotubes. Therefore, we performed additional measurements on

carbon nanotubes and some signal is generated by this type of carbonaceous materials. However, the signal was further analyzed and it was found that: (i) this signal is much weaker than the signal from carbon black or black carbon particles, meaning that at the laser powers described in the paper the contribution of the carbon nanotubes is essentially zero; (ii) there is a lifetime of the signal observable meaning that mainly fluorescence is probed instead of white-light generation which is instantaneous (autofluorescence was excluded from the signal detection by choosing appropriate optical filters); (iii) the majority of the carbon nanotube signal is present at other wavelengths than probed in this paper so that this signal essentially does not contribute to the signal in the white light channel.

4) Additionally, **Raman spectroscopy measurements** on dried urine samples were executed (Supplemental Material, Figure S1). It is clear from these results that black aggregates found in dried urine generate a fingerprint identical to the fingerprint from carbon particles. This is clear evidence that the white light signal is: (i) generated by carbon in particle form, (ii) the signal is very specific, (iii) the technique also works in extremely challenging conditions, since the autofluorescence from dried urine is extremely bright and omnipresent. We like to emphasize that the large aggregates/agglomerates of carbon are formed by drying the urine, when we inspect the urine in fluid state no such aggregates/agglomerates are detectable. We also have to mention that, as the Raman and white light measurements were performed on different set-ups and even in different institutes, it was not possible to retrieve the exact location of one carbon aggregates (not all set-ups are equipped with a motorized stage to determine coordinates from samples). As a result, we performed white-light and Raman spectroscopy on two different aggregates/agglomerates but in the same dried urine samples to provide all necessary convincing evidence.

To conclude, the white-light generation by carbonaceous particles is a very distinct and specific signal. The necessary tests were conducted to prove this statement. We believe that this method is very specific and even more specific than the absorption-based air measurements, which are currently performed.

SUPPLEMENTARY MATERIAL

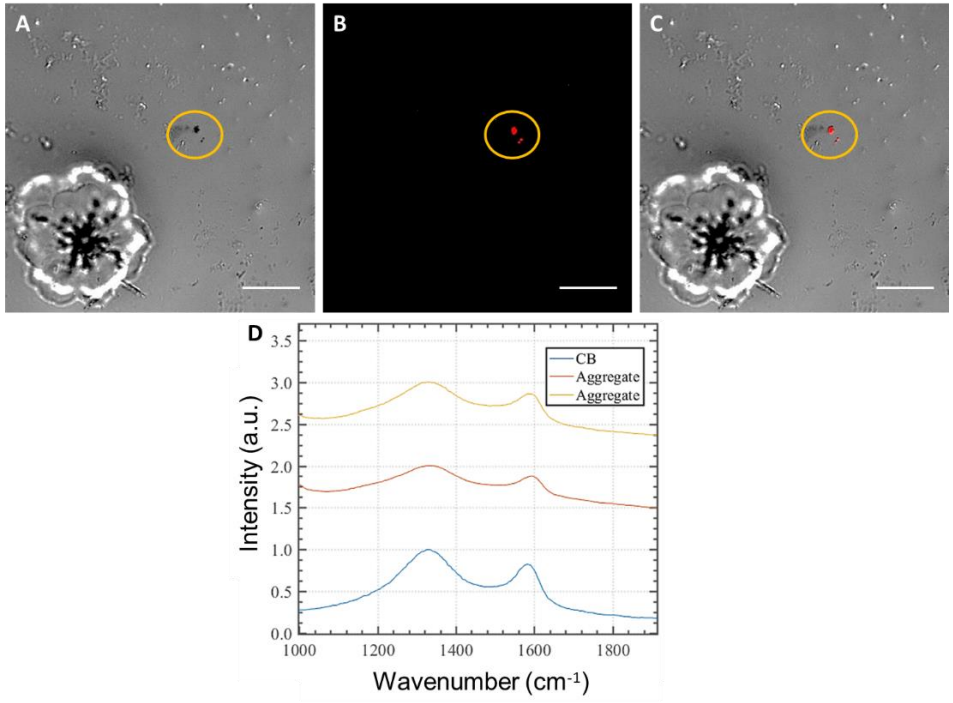

Figure S1. Evidence of carbon aggregates in dried urine samples. (A) Light microscopy image of some carbon aggregates (orange circle) in a dried urine sample, (B) white-light detection of the corresponding aggregates, (C) overlay image of images A and B, (D) Raman fingerprints of the aggregates compared to the fingerprint of carbon black particles (reference material). Raman spectra of all data collected display very broad D- peaks (left peak) and G- peaks (right peak) typical of amorphous carbon. Scale bars: 15 μm

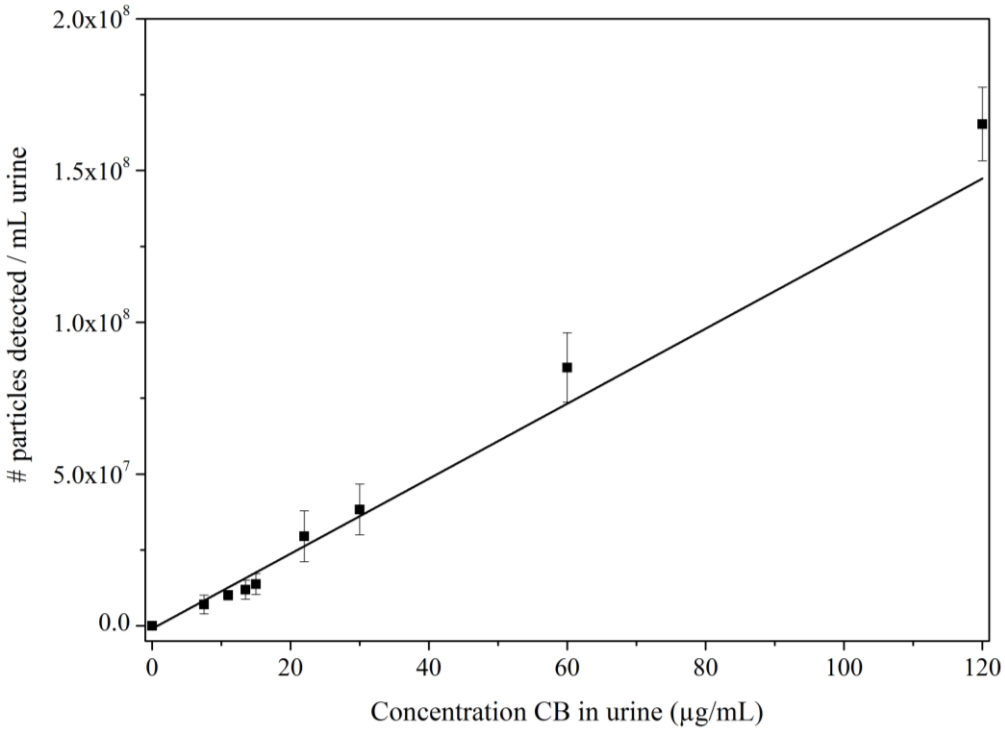

Figure S2. Relation between the added concentration CB in artificial urine and the amount of particles detected per mL urine. The data are average ± standard deviation (n=7) and fitted linearly (R^2=0.98).

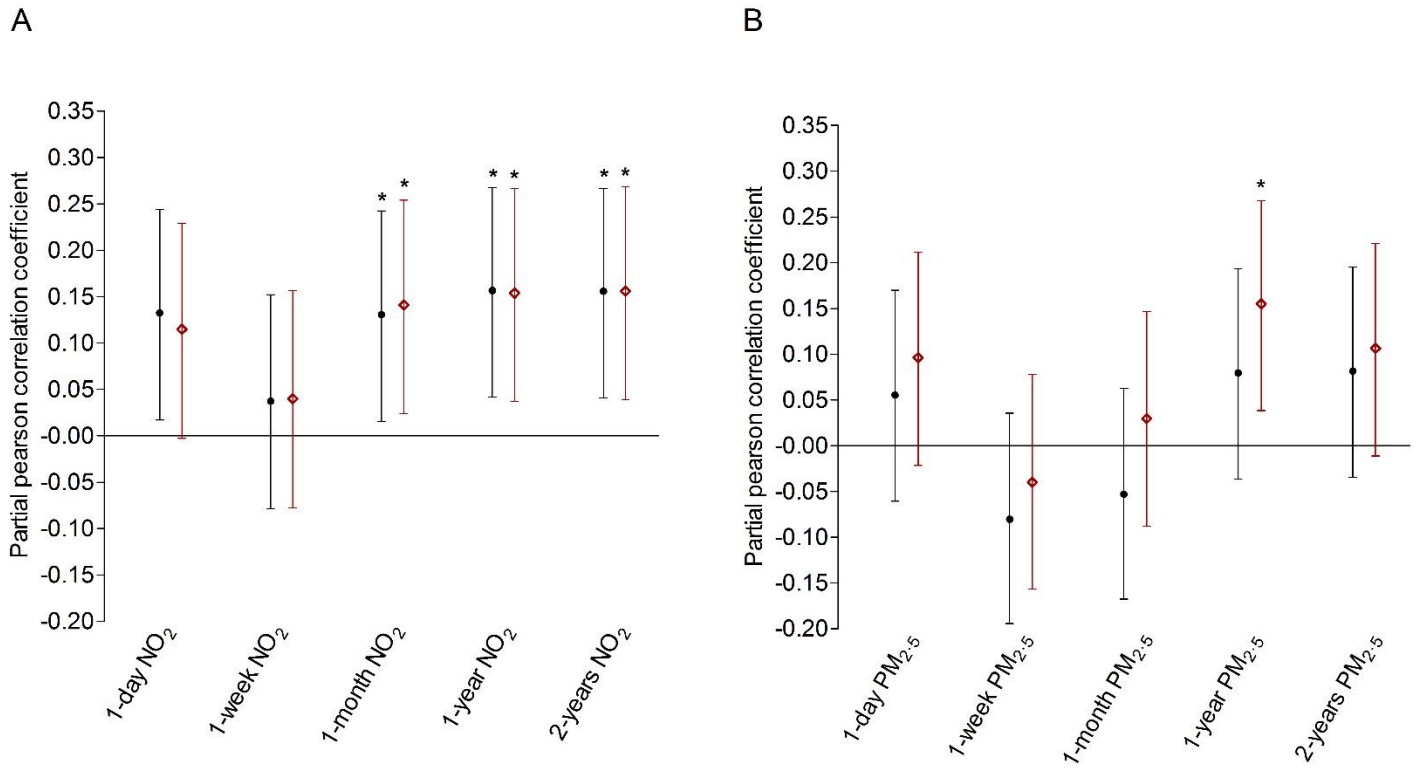

Figure S3. Unadjusted and adjusted (partial) Pearson correlation coefficient (95% Confidence Intervals) between urinary black carbon load and residential recent (1-day and 1-week before urine sampling), medium-term (1-month before urine sampling), or chronic (1-year and 2-years before urine sampling) NO_2 (A) and $PM_{2.5}$ (B) exposure (*n*=289). Partial correlation coefficients were adjusted for *a priori* chosen covariates including sex, age, BMI, mother's education, month of examination, and urinary osmolality. * $P < 0.05$.

Table S1. Recent, medium-term and chronic NO_2 and $PM_{2.5}$ exposure characteristics (n=289)

	Median	**25th percentile**	**75th percentile**
Recent			
NO_2, µg/m³			
1-day	26.0	19.5	31.7
1-week	24.2	20.4	28.7
$PM_{2.5}$, µg/m³			
1-day	11.4	8.7	21.8
1-week	13.1	9.4	17.3
Medium-term			
NO_2, µg/m³			
1-month	22.4	20.2	24.1
$PM_{2.5}$, µg/m³			
1-month	11.9	10.7	14.0
Chronic			
NO_2, µg/m³			
1-year	21.4	19.9	22.7
2-years	22.2	20.4	23.5
$PM_{2.5}$, µg/m³			
1-year	14.3	13.8	15.2
2-years	15.2	14.3	15.5

Table S2. Sensitivity analysis on the association between urinary carbon load and annual residential black carbon exposure.

	Urinary carbon load, x 10^5 particles/mL	95% CI, x 10^5 particles/mL			*P*-value
Unadjusted model	4.26	0.71	to	7.80	0.019
+ osmolality	3.96	0.41	to	7.50	0.029
+ creatinine	3.63	0.02	to	7.23	0.049
+ osmolality and creatinine	3.58	-0.02	to	7.18	0.051
Main model	5.34	1.56	to	9.11	0.006
+ creatinine	5.03	1.21	to	8.84	0.010
+ physical activity	5.15	1.37	to	8.94	0.008
+ education father	4.87	1.08	to	8.66	0.012
+ highest occupation either parents	4.80	0.93	to	8.66	0.015
+ passive smoking exposure	5.24	1.61	to	9.18	0.005
+ vegetables or fruit from own garden	5.44	1.66	to	9.22	0.005
+ recent black carbon exposure (1-week)	5.49	1.62	to	9.63	0.006

The unadjusted model describes the association between urinary carbon load and 1-year residential black carbon exposure. The main model describes the same association, adjusted for gender, age, BMI, mother's education, month of examination, and urinary osmolality. Sensitivity analysis shows the estimates presented as a change in urinary carbon load (particles/mL) for an IQR increment (0.21 μg/m³) in annual residential black carbon exposure, with additional adjustment for osmolality and/or creatinine in the unadjusted model, and creatinine, physical activity, education of the father, passive smoking exposure, vegetable or fruit intake from own garden, or short-term (1-week) residential black carbon exposure in the main model.

GENERAL DISCUSSION

Cognitive impairment and behavioral problems are considered one of the most important factors that contribute to reduced quality of life, placing an enormous burden on the familial and societal education and healthcare systems.[260] The prevention of problems during growth and maturation of the brain in early childhood, the understanding of maladaptive trajectories that operate in early life, and the identification of hazardous environmental influences represent research priorities of great importance for preserving a healthy population.

Growing scientific evidence is documenting ambient air pollution as a developmental neurotoxicant.[123, 261] Experimental and epidemiological studies suggest that the effects of air pollution on neurodevelopment likely begin *in utero*.[261, 262] In this doctoral dissertation, we assessed the extent and implications of air pollution on (neuro)development for two periods of life, namely the prenatal period and childhood. First, we used the placenta as a surrogate tissue for studying fetal (neuro)development by examining the associations between molecular signatures of (neuro)development and prenatal air pollution exposure. Second, in children aged 9-12 years, we studied the link between recent *versus* chronic air pollution exposure and cognitive function, and explored a new internal biomarker reflecting ambient air pollution. A schematic overview of this doctoral dissertation is presented in **Figure 1**.

Novelties of this dissertation include:

- **Novel biomarkers reflecting ambient air pollution exposure**
  - Placental nitrosative stress
  - Urinary carbon load
- **Alterations in placental molecular signatures of (neuro)development associated with prenatal exposure to air pollution**
  - Transcriptional changes in the Brain-derived neurotrophic factor (*BDNF*) signalling pathway
  - Methylation differences of Leptin (*LEP*)
- **Differential cognitive processes affected by recent or chronic exposure to ambient air pollution**

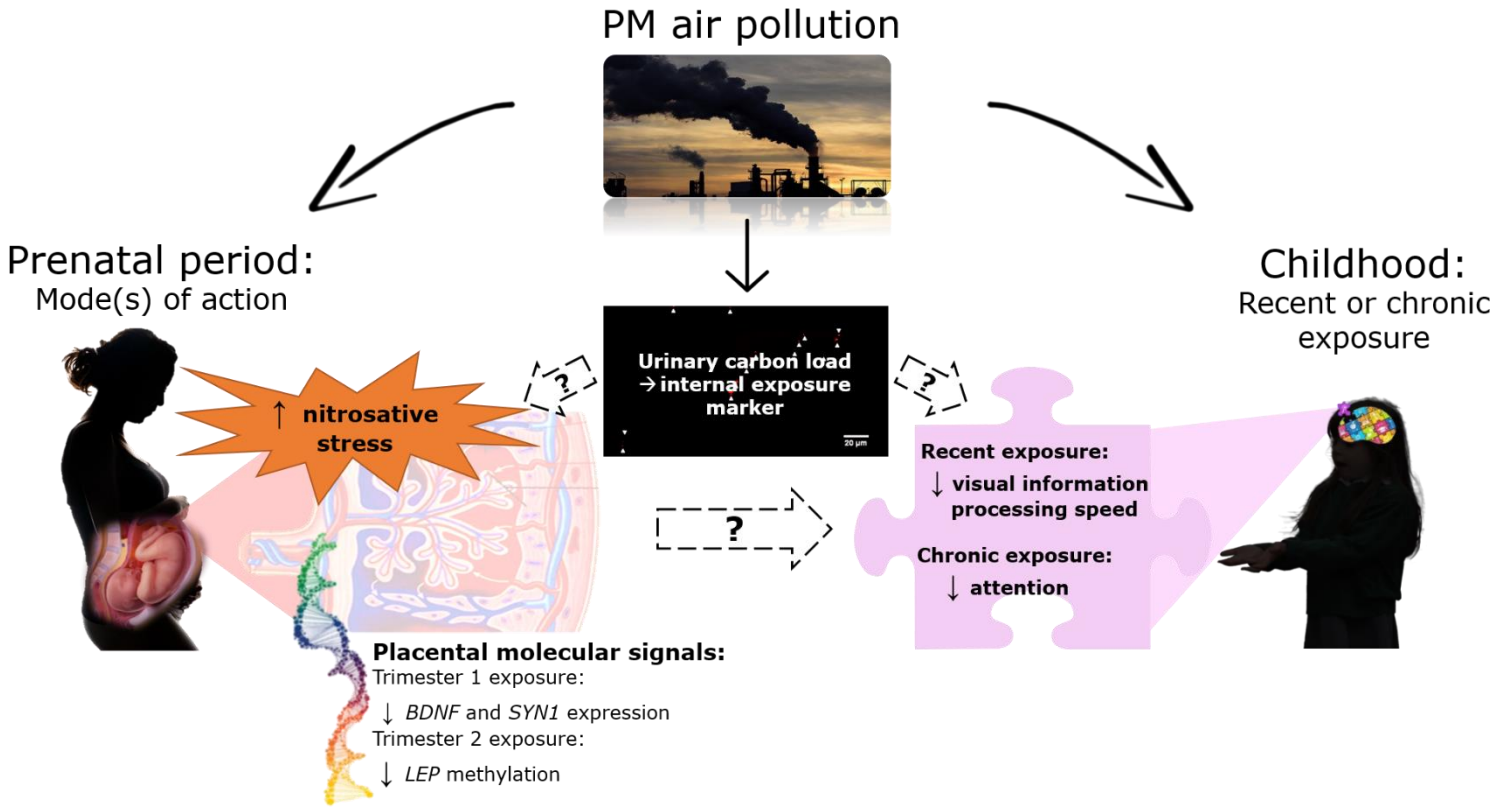

Figure 1: Schematic overview of this doctoral dissertation

NOVEL BIOMARKERS REFLECTING AMBIENT AIR POLLUTION EXPOSURE

Quantifying human exposure to air pollutants is a challenging task. Ambient concentrations of air pollutants at potentially harmful levels are ubiquitous in urban areas and subject to high spatial and temporal variability. At the same time, every individual has unique activity-patterns.[263] Traditionally, exposure to ambient air pollution, including $PM_{2.5}$ and BC, are estimated based on spatial temporal interpolation models and thus not measured at the individual level. Personal exposure assessment would be preferred despite being laborious and resource intensive as it needs specialized personnel and expensive equipment.

Epidemiological studies based on spatial temporal interpolation models or personal monitoring of air pollution are extremely valuable, nevertheless their scientific contribution can be enhanced by and integrated with the use of biomarkers. Biomarkers have been introduced under the assumption that they could improve the investigation of health effects of air pollution and other exposures, by (i) improving the personal exposure assessment because of the close biological link with the individual, (ii) increasing the understanding of mechanisms of action, e.g. by measuring intermediate biomarkers, and (iii) allowing the investigation of individual susceptibility.[264]

Placental nitrosative stress and prenatal air pollution exposure

Recent efforts have turned toward exploiting biologically active chemical components generated by particulate matter (PM), such as reactive oxygen species (ROS), as better predictors of health effects associated with PM in comparison to the mass metric of PM (μg/m³). Reactive oxygen species that are formed through PM include molecules such as hydrogen peroxide, ions such as hypochlorite and peroxynitrite, and radicals such as hydroxyl.[17] In **Chapter 1**, we assessed in placental tissue whether 3-nitrotyrosine (3-NTp), a biomarker of oxidative/nitrosative stress, was associated with prenatal ambient air pollution exposure. We investigated 3-NTp as it is the stable product of protein nitration involving the reaction of tyrosine hydroxyl groups with the reactive intermediate

peroxynitrite (**Figure 2**). Therefore, we hypothesized that the concentration of 3-NTp may be a biomarker reflecting the level of PM air pollution exposure.

The key finding of our study was that placental 3-NTp levels increased with 35.0% (95% CI: 13.9 to 60.0%) for an interquartile range increment (3.5 μg/m³) of exposure to $PM_{2.5}$ during the entire pregnancy. This observation of an elevated nitrosative stress response highlights the relevance of placental 3-nitrotyrosine as a biomarker for cumulative prenatal $PM_{2.5}$ exposure. Our findings are supported by several other experimental or occupational studies which have shown that 3-NTp is positively associated with traffic-related air pollution exposure.[87, 112, 115] Furthermore, evidence supports the *in vivo* formation of 3-NTp in diverse pathologic conditions, such as multiple sclerosis,[265] autoimmune diseases,[266] cardiovascular disease,[267] and pre-eclampsia[98]. Whether a PM-induced placental nitrosative stress response is a better predictor of health effects associated with PM exposure warrants further research.

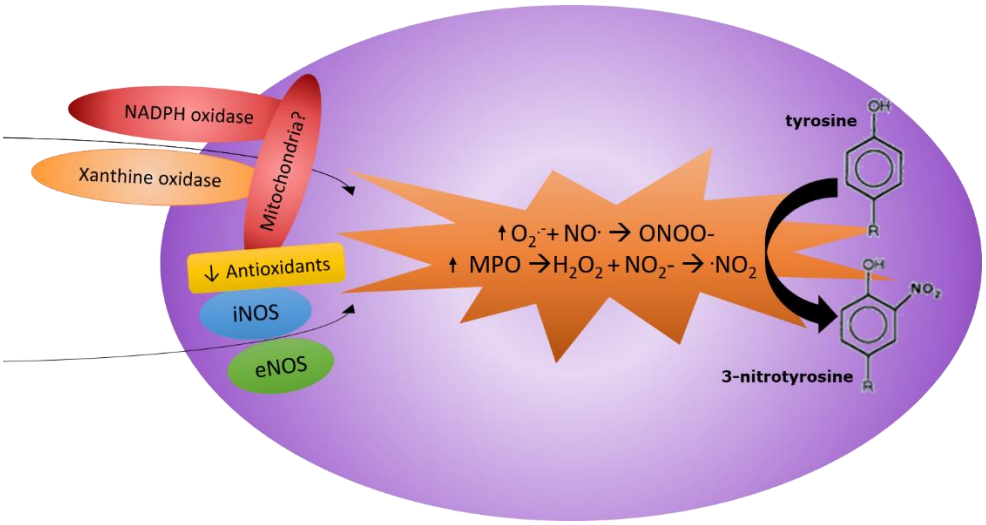

Figure 2. Simplified schematic illustration of common forms of reactive oxygen and nitrogen species. Protein tyrosine nitration is mainly mediated by different biochemical processes: i) superoxide radicals ($O2^{\cdot-}$) derived from different sources such as Xanthine oxidase, Nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase, mitochondria, and can react with the nitrogen oxide radical (NO·) derived from uncoupled endothelial NO synthase (eNOS) to generate the reactive nitrogen species peroxynitrite ($ONOO^-$). ii) (myelo)peroxidase (MPO)-catalyzed nitrogen dioxide radical (·NO_2) can be formed from hydrogen peroxide (H_2O_2) and nitrite (NO_2-). ONOO- or ·NO_2 react with protein tyrosine residues to generate 3-nitrotyrosine.

The associations observed between placental 3-NTp and prenatal $PM_{2.5}$ exposure were mainly driven by the exposure incurred during the time windows of the first and second trimesters of gestation. These findings emphasize the importance of investigating critical exposure windows. Susceptibility to PM exposure may fluctuate as certain periods during pregnancy are more vulnerable to environmental stressors. It has been shown that the early and midpregnancy exposure windows are more critical for adverse health outcomes such as preterm birth.[119, 268]

To evaluate whether placental 3-NTp could be used as *a* pseudo-biomarker of prenatal $PM_{2.5}$ exposure, we compared in **Chapter 3**, the associations between prenatal $PM_{2.5}$ exposure and 3-NTp with the placental *LEP* methylation status. This study showed that both $PM_{2.5}$ exposure during the second trimester of pregnancy and placental 3-NTp were inversely associated with placental *LEP* methylation. Furthermore, the sensitivity analysis showed suggestive involvement of placental 3-NTp in the association between placental *LEP* methylation and prenatal $PM_{2.5}$ exposure. These findings may be indicative of an underlying role of nitrosative stress in $PM_{2.5}$-associated placental epigenetic events. Nitrosative stress involves nitric oxide (NO), one of the reactive intermediates in the formation of 3-NTp, which has been shown to directly and indirectly affect numerous epigenetic mechanisms. Recent literature demonstrates the ability of NO to dramatically alter global histone post-translational modifications and DNA adducts by directly inhibiting epigenetic enzymes or indirectly by altering enzyme expression levels.[269] With respect to DNA methylation, several *in vitro* studies have linked NO production or nitric oxide synthase 2 (NOS2) expression with changes in DNA methylation.[270-272] However, direct mechanisms by which NO alters DNA methylation remain to be elucidated. Our hypothesis of a nitrosative stress-induced epigenetic alteration in the placenta warrants further research because on the basis of the current findings, we can only speculate about the precise mechanism underlying the observed relationships.

Urinary environmental carbon load in children

From the year 2000 onwards, many reports examined the possibilities of extrapulmonary particle translocation to the systemic circulation, organs such as liver and kidney, and even the brain.[14] In general, most studies used artificial particles that were inhaled or intratracheally administered into the lung to investigate the fate of particles ≤100 nm. For this purpose, rat models were frequently used. These experimental studies have demonstrated that ultrafine particles can translocate from the lung into the systemic circulation. In humans, only a few reports exist, using inhalation of Technegas (*i.e.*, a cluster of nanosized ~35 nm pure carbon platelets of hexagonal shape fully encapsulating Technetium metal crystals), that reported no translocation to the systemic circulation or remote organs.[273] These studies are limited as they are mainly based on labelling techniques and short-term exposure conditions.

Considering the likelihood that particles may translocate to the systemic circulation and are cleared via the kidneys into the urine, we investigated in **Chapter 5** whether urinary environmental carbon load could be used as a novel internal biomarker reflecting exposure to ambient black carbon particles. The innovative information and originality from our work is that we employed a novel label-free and biocompatible imaging technique using white-light generation of carbonaceous particles under femtosecond illumination.[240] This new method is very specific and suitable for the detection of carbonaceous particles in urine as other types of particles do not bear the heterogeneous and absorptive characteristics to give rise to the white-light phenomenon. Furthermore, the technique does not require additional labelling or preparation procedures, which is different from most studies investigating particle translocation.

A second novelty is that urinary carbon load mirrors medium-term and chronic ambient black carbon exposure. This innovative biomarker of exposure differs from other biomarkers used in the past in such a way that they do not specifically relate to combustion-associated air pollution (*i.e.*, traffic) and mostly reflect transient or short-term exposure, such as *trans,trans*-muconic acid, a metabolite of traffic-linked benzene measurable in urine.[251, 274] Moreover, our novel detection method of black carbon in biological media is noninvasive, which is at variance with recent efforts to assess carbon load in alveolar macrophages

using light microscopy,[253, 254] a technique requiring semi-invasive collection of induced sputum from the central airways.[253]

Although urinary carbon load can be used as an internal biomarker of ambient black carbon exposure, *e.g.* from traffic, additional research is required for the complete understanding of the physical features of this detection technique. It is established that the carbonaceous particles generate white-light under femtosecond laser illumination, however the physical mechanism behind this phenomenon is still unknown. Furthermore, our technique cannot distinguish between individual particles or aggregates, because it fails to recognize particle size. In addition, it would be of interest to determine whether all particles show up under femtosecond laser illumination or only a subset.

Whilst our results reflect the renal clearance of black carbon particles from the circulation into the urine, we cannot extrapolate to other organs or bodily fluids (blood, lymphatic system), such as the translocation from lungs to the circulation. For future purposes, it would be of interest to optimize this new detection method for other body fluids and human tissues to investigate more in depth the toxicokinetics of the translocation of black carbon particles throughout the human body. In addition, the utility of this biomarker to unravel particulate matter-related toxicodynamic health effects warrants further exploration.

ALTERATIONS IN PLACENTAL MOLECULAR SIGNATURES OF (NEURO)DEVELOPMENT ASSOCIATED WITH PRENATAL EXPOSURE TO AIR POLLUTION

Brain and CNS development begin in the womb, with most of the structural features of the brain formed in the first trimester of pregnancy. The brain forms a network of interconnected cells (*i.e.* neurons) that spreads across different anatomic regions and connects to peripheral tissues.[275, 276] These structures continue to grow and develop throughout pregnancy and after birth until the age of adolescence. During this period, the various structural components of this network are most sensitive to stimulation from the environment eliciting adaptive changes. These subtle, individual differences *in utero* may affect developmental processes later in life by changing normal trajectories.[277, 278]

A wide variety of biologically unfavourable environmental factors are believed to play some role in the etiology of these differences by inducing inflammatory, oxidative and nitrosative stress responses, mitochondrial dysfunction, apoptosis, and epigenetic dysregulation. Moreover, there is growing awareness that prenatal epigenetic dysregulation due to adverse *in utero* environments not only affects fetal brain development, but also predisposes an individual to neurodevelopmental, behavioral and cognitive deficits later in life.[279, 280] In **Chapters 2 and 3**, we investigated whether prenatal $PM_{2.5}$ exposure was associated with placental differences in DNA methylation or gene expression of factors involved with (neuro)development.

In animals, studies evidenced that subtle variations in the intrauterine environment can cause recognizable differences in brain structure.[281] For humans however, ethical constraints make it problematic to investigate the developing fetus or brain in a direct way. Therefore, other biological matrices are used as a surrogate such as cord blood and placenta. At delivery, the placenta represents a precious source of the morphological, functional, and molecular information accumulated during the gestational period. These biological signatures enshrined in the placenta as a result of various external exposures make the placenta a suitable, non-invasive matrix for research pertaining to the pregnancy period.

In **Chapter 2**, we focused on the Brain-derived neurotrophic factor (*BDNF*) signalling pathway, which during pregnancy is involved in placental development, fetal growth, glucose metabolism, and energy homeostasis. We observed for a 5 $\mu g/m^3$ increment in the first trimester $PM_{2.5}$ exposure window, a 15.9% decrease (95% CI: -28.7, -3.2%, p=0.015) in relative gene expression of placental *BDNF* at birth. Correspondingly, a 24.3% decrease (95% CI: -42.8, -5.8%, p = 0.011) in Synapsin 1 (*SYN1*), a more downstream target of the *BDNF* signalling pathway, was observed. Multi-gene models showed that the Son of sevenless (*SOS*) cascade and Phospholipase gamma (*PLCG*) cascade were negatively associated with trimester 1 $PM_{2.5}$ exposure. These findings, however, need to be interpreted with caution as the multi-gene models can be driven by specific genes (i.e. *BDNF* and *SYN1*). These results provide suggestive evidence that $PM_{2.5}$ exposure early in pregnancy may affect specific developmental targets such as *BDNF* and *SYN1*, both involved in normal neurodevelopmental trajectories, but potentially also their downstream signalling. It remains challenging to find out whether these placental

changes in gene expression have any consequence as to cognitive function in childhood.

In **Chapter 3,** placental DNA methylation of the Leptin (*LEP*) promoter was investigated in association with prenatal $PM_{2.5}$ exposure. During pregnancy, placental LEP plays a functional role in embryo implantation, intrauterine development, and fetal growth.[168] The key finding of this study was a significant inverse association between second-trimester $PM_{2.5}$ exposure and placental *LEP* methylation at birth. The effect estimate was a 1.4% lower methylation (95% CI: -2.7, -0.19%, p = 0.02) for an interquartile range increment in $PM_{2.5}$ exposure (7.5 μg/m³). Furthermore, we also examined whether placental *LEP* methylation was associated with birth weight, but we did not observe a significant association (p=0.52). An unaltered birth weight does not necessarily mean that the $PM_{2.5}$-induced placental *LEP* methylation changes may not have long-term consequences. For instance, the Dutch Hunger Winter Study showed that normal birth weight children born to mothers exposed to undernutrition during early gestation nevertheless experience increased rates of obesity later in life.[282] Therefore, future studies are needed to establish potential health consequences of the current *LEP* methylation findings.

Additionally, it is plausible that other aspects of the physiological importance of placental LEP for gestational growth are to be sought elsewhere. Based on literature evidence, it has been shown that placental LEP may be involved in immunomodulation[190] and vascularization.[170, 186, 187] Therefore, it is not unreasonable to hypothesize that gestational $PM_{2.5}$ exposure results in hypomethylation of the placental *LEP* status with possible involvement in placental immunomodulation and vascularization. Furthermore, in our study, we also showed a negative association of placental *LEP* methylation with nitrosative stress which may be indicative for a vascular response. The potential impact of the placental *LEP* methylation status induced by $PM_{2.5}$ exposure on vascularization should be addressed in future experimental and epidemiological studies.

To summarize, we have shown adverse molecular signatures at birth in association with different critical $PM_{2.5}$ exposure windows during pregnancy. In the past, several authors considered the importance of the course of exposure and how this affects the observed outcomes. In 1973, Moore and Persaud presented a timeline

for human development identifying highly sensitive windows for morphological abnormalities and compared them to less sensitive periods that were more likely to be associated with physiological defects and minor morphological abnormalities.[283] Later, an adaptation of these efforts was made by Selevan and Lemasters to commonly studied adverse outcomes for humans such as transplacental carcinogenesis and lowered birth weight.[284] Indeed, in the prenatal period, exposures often vary throughout pregnancy due to changes in internal dose, resulting from alterations in absorption, distribution, metabolism, and excretion.[285] Therefore, fluctuations may be observed in the molecular signatures at birth depending on the exposure window during pregnancy. Our findings, however, are limited as we cannot extrapolate the changes in molecular targets to fluctuations during pregnancy. Nevertheless, our studies contribute to the understanding of the mode(s) of action through which air pollution may affect (neuro)developmental processes.

DIFFERENTIAL COGNITIVE PROCESSES AFFECTED BY RECENT OR CHRONIC EXPOSURE TO AMBIENT AIR POLLUTION

Over the last decade, evidence of cognitive deficits in response to components of air pollution in human studies has become more compelling. Differences in a range of cognitive outcomes have been shown among several studies between all ages living in more and less polluted environments.[55, 286] In general, adults show accelerated cognitive aging in those exposed to higher levels of air pollution, based on tests of visuo-motor abilities, memory and learning. Children exposed to higher levels of air pollution performed poorer on neurodevelopmental, intelligence, and memory measures. However, the consistency of the findings is their main limitation.

To support the hypothesis that air pollution affects cognitive functioning, we investigated in **Chapter 4**, the association between different cognitive processes and recent (a few days) or chronic (annual) exposure to ambient air pollution in primary schoolchildren. The part of our study on chronic PM exposure at residence showed negative associations with sustained and selective attention. These results are consistent with previous reports.[287] To examine the recent exposure effects of

air pollution, we used a repeated measurement study design without the risk of confounding by person-related characteristics. The main effects of the recent PM exposure conditions was a decrease in selective attention and visual information processing speed. At the same time of our investigation, a longitudinal observational study showed in 2,618 primary schoolchildren that traffic-related particles were associated with a reduction in working memory.[288] We did not find an association with short-term memory. One recent study reported that attention processes were negatively associated with both short-term exposure to NO_2 and elemental carbon,[289] which supports our results of a selective attention deficit with recent exposure to PM.

To the best of our knowledge, we were the first to investigate the cognitive effects of day-to-day variations in levels of air pollution exposure. Our findings corroborate current evidence on air pollution-induced neurotoxicity and highlight the importance of air pollution reduction in the vicinity of primary schools which would result in beneficial effects on cognition. It is also clear that further research is needed to better characterize the health hazard of recent exposure conditions as the robustness and consistency of cognitive responses might be less outspoken than in the case of chronic exposure.

SCIENTIFIC RELEVANCE AND PERSPECTIVES

The scientific relevance of this doctoral dissertation is not only based on its strengths but also builds on some limitations which convey suggestions for future research perspectives.

The current understanding of the impact of air pollution on cognition is still in its infancy. To contribute to this field of knowledge, we have focussed our investigations on two vulnerable stages of life, the prenatal period and childhood.

First, we identified two novel biomarkers reflecting ambient air pollution exposure, one in placental tissue (prenatal period) and one in urine (childhood). Placental 3-NTp is a reflection of PM-induced oxidative stress, which helps to understand putative toxic mechanisms and may be a better predictor of health consequences. The urinary carbon load will improve exposure assessment as it may reflect the internal dose of black carbon exposure on an individual basis. Nevertheless, this newly developed technique needs further toxicokinetic studies

to clarify the fate of inhaled carbonaceous particles and to substantiate the translocation pathway of those particles across different compartments of the human body. Although the biomarkers placental 3-NTp level and urinary carbon load are evaluated in specific biological matrices, it does not mean they are limited to these specimens. Future studies should examine the potential of these markers in other bodily fluids or tissues.

Second, in the prenatal period we looked at critical exposure windows for fetal susceptibility to air pollution. We evidenced the existence of more critical developmental exposure windows for susceptibility across the different stages of *in utero* life, which will be helpful to improve health risk assessment with the focus on the most sensitive exposure window(s) during pregnancy. Furthermore, examination of potential underlying mechanisms by which air pollution may act on cognitive functioning will increase our knowledge of the mode(s) of action. We provide suggestive evidence for contributory mechanisms such as altered methylation and expression levels of genes linked to (neuro)developmental and cognitive processes later in life.

Third, in primary schoolchildren we observed differential adverse effects on cognitive processes associated with recent or chronic air pollution exposure. These findings highlight the relevance of not only investigating the impact of different time periods of exposure, but also more precise characterization of the specific behavioural domains of cognitive function that are being affected. Furthermore, these results support the current evidence that air pollution affects cognitive function, even at exposures well below the European Union exposure limits.

The main limitation of this doctoral dissertation is that in the timeframe of my doctoral project, I could not investigate the triple association paradigm of the link between PM air pollution exposure, molecular signatures identified in the placenta, and health outcomes *in casu* the cognitive performance in childhood. Currently, a follow-up study of the ENVIR*ON*AGE birth is ongoing in which cognitive performance tests are administered to children of 4-6 years old. Investigating the association between the placental molecular signatures identified at birth and the 4-6 year cognitive performance would be of interest to observe whether early life alterations may have consequencens in early childhood.

In Belgium, ambient air pollution is still an intermittent problem as to meeting the European Union limit values. In the past years, the degradation of psycho-emotional health and prevalence of neurological disorders has strongly increased. They produce a range of symptoms and functional limitations that pose daily challenges to individuals and their families. In addition, neurological disorders pose an economic burden to society as costs of services such as hospital admission, community resources and carers, rise considerably. This burden may magnify as Belgium's population ages which forces policy-makers to tackle these issues.

Prevention of disease and disability by risk reduction remains an important strategy, which may already start at the earliest stages of life. Within this context, this thesis contributes to the understanding of how air pollution affects neurodevelopmental processes within the field of environmental and molecular epidemiology. Our findings provide new ideas for future efforts to investigate potential underlying action mechanisms of early-life air pollution exposures and cognitive function. Furthermore, as all our results (Flanders, Belgium) were observed at average air pollution levels below the current European Union guidelines, it is fair to persist increasing the pressure on the Belgian government and their citizens to continue to reduce air pollution levels.

"Almost all aspects of life are engineered at the molecular level, and without understanding, we can only have a very sketchy understanding of life itself"

Francis Crick (*What Mad Pursuit*, 1988)

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DANKWOORD

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Resultaten

Resultaten, dat boek je niet enkel alleen! Daar heb je een hele reeks aan faciliteiten voor nodig 😊:

1. Collega's.. 'Alone we can do so little, together we can do so much'

Prof. em. dr. Harry Roels, een speciale dank naar jou om mijn artikels tot een hoger niveau te brengen door kritisch mijn werk na te lezen! Karen, Michelle, en Janneke, bedankt voor jullie begeleiding in mijn toch wel diverse onderwerpen. De 'oldies' - Nicky, Michal, Bianca, Bram, Esmée en Eline – wat hebben we toch mooie tijden met elkaar beleefd! Een flesje rood of wit, dat maakte niet uit, het waren er alleen maar veel 😊. Wat salsa in de heupen om alles van ons af te schudden! En af en toe werd er ook wel gewerkt.. niet alleen veldwerk, labowerk, of analysewerk maar ook avondwerk zoals netwerken zonder te overwerken (recepties,..😉). Gelukkig was er cafeïne om dit allemaal te bolwerken. Vervolgens de 'C107b' – Annette, Maria, Narjes, Ellen, Dries en Diana – Door jullie is veel exponentieel gestegen.. niet alleen het aantal doctoraatstudenten, maar ook het aantal placenta's, het aantal teambuildings en het gemiddelde alcoholverbruik, … doch één significante daling, namelijk het aantal sterren van onze verblijfplaatsen op congressen 😊. Hoewel de luxe afnam, werden er veel memorabele momenten beleefd.. 'de intoxicatie van non-alcoholische dranken' gevolgd door 'romantiek

met rode rozen' om daarna 'de weg terug te vinden met een ebola-kaart of desnoods met de hulp van een smeerpoes'. Dan wil ik hier ook de 'newbies' aan bod laten komen – Leen, Kristof, Yinthe, Katrien, Charlotte, Rossella, en Hanne – Wat hebben jullie nieuw leven gebracht in de brouwerij van de groep Epidemiologie! Jullie enthousiasme en energie zijn zeer aantrekkelijk! Bij sommigen is de inzet zo groot dat 'slapen' tijdens housewarmings onvermijdelijk is ☺. Ook nog een extra speciaal bedankje voor Martien en Marijke, jullie luisterend oor en advies gaven me altijd weer een nieuwe 'boost' om ervoor te gaan! Aan alle andere collega's van het CMK, bedankt voor de toffe middagpauzes!

2. Vrienden.. 'One of the most beautiful things in life is true friendship'

Leentje en Inse, ik kan eigenlijk niet in woorden beschrijven wat jullie voor mij betekenen, maar hier een ludieke poging.. vanaf dat ik begonnen ben op de universiteit gaan jullie al mee op reis met 'SNelly-travel'. We hebben heel wat avonturen beleefd, en er zullen er ook nog velen komen. Maar vooral wil ik jullie bedanken om mee deze vlucht te nemen! Want soms was er wel eens wat turbulentie, maar dankzij jullie als gezelschap waren de eindbestemmingen steeds de moeite waard! Lobke, jij bent er in gedachte nog altijd bij! Vervolgens om zeker niemand te vergeten, spreek ik jullie allen tegelijk aan: 'Dear best friends, I just wanna say thank you for being awesome, for always being there and for always making me feel happy ☺'

3. Familie.. 'Where life begins and love never ends'

Mijn ouders wil ik graag bedanken om mij de mogelijkheid te bieden om een universitaire opleiding te voltooien, maar zeker ook voor hun wijze raad. In het bijzonder, mijn bomma'tje en tante Zan.. zonder jullie zou mijn wereldje af en toe in elkaar gestort zijn, maar jullie zorgden altijd voor de juiste ondersteuning om mij recht te houden. En wat een geluk heb ik om een broer te hebben, want 'een broer' hebben maakt je blijkbaar succesvoller in het leven ☺. Als laatste wil ik ook de rest van mijn familie bedanken voor de vele interesse in mijn doctoraatsonderzoek!

Conclusie

Ik heb de beste collega's, vrienden en familie die me door dik en dun steunen!

CURRICULUM VITAE

Nelly Saenen was born in Hasselt (Belgium) on October 10th 1988. After she graduated from secondary school at Virga Jesse College Hasselt in 2006, she studied Bachelor Biology and Master Environmental Health Sciences at Hasselt University. During her masterthesis, she investigated gene expression patterns to monitor stress experienced by humans in spaceflight analogues, which gave her the opportunity to specialize in molecular biology. She graduated in 2011 *cum laude*. In the same year, she started her PhD in molecular epidemiology at the Centre for Environmental Sciences at Hasselt University, under supervision of Prof. dr. Tim Nawrot. The aim of her research was to establish epidemiological evidence for indicators of air pollution exposure on neurological development at a molecular and cognitive performance level. Besides teaching activities, she followed in 2012 an international course "Molecular Epidemiology of Chronic Diseases" in Maastricht, The Netherlands. She presented her results at several conferences including ISEE in Basel and Rome, ISEE-Young in Barcelona and Utrecht, and Healthy Living in Maastricht. In 2015, she received at the ISEE-Young in Utrecht the 'Occupational and Environmental Medicine Award' for her poster presentation on placental nitrosative stress and air pollution exposure.

LIST OF PUBLICATIONS

INTERNATIONAL PEER-REVIEWED PUBLICATIONS

1. Bijnens E, Pieters N, Dewitte H, Cox B, Janssen BG, **Saenen N**, Dons E, Zeegers MP, Int Panis L, Nawrot TS. Host and Environmental predictors of exhaled breath temperature in the elderly*. BMC Public Health*. 2013; 13: 1226.

2. **Saenen ND**, Plusquin M, Bijnens E, Janssen BG, Gyselaers W, Cox B, Fierens F, Molenberghs G, Penders J, Vrijens K, De Boever P, Nawrot TS. In utero fine particle air pollution and placental expression of genes in the Brain-derived neurotrophic factor signaling pathway: an ENVIR*ON*AGE birth cohort study. *Environ Health Perspect*. 2015; 123(8):834-840.

3. Vriens A, Nawrot TS, **Saenen ND**, Provost EB, Kicinski M, Lefebvre W, Vanpoucke C, Van Deun J, De Wever O, Vrijens K, De Boever P, Plusquin M. Recent exposure to ultrafine particles in school children alters miR-222 expression in the extracellular fraction of saliva. *Environ Health*. 2016; 15(1): 80.

4. **Saenen ND**, Provost EB, Viaene MK, Vanpoucke C, Lefebvre W, Vrijens K, Roels HA, Nawrot TS. Recent versus chronic exposure to particulate matter air pollution in association with neurobehavioral performance in a panel study of primary schoolchildren. *Environ Int*. 2016; 95: 112-119.

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7. Janssen BG*, **Saenen ND***, Roels HA, Madhloum N, Gyselaers W, Lefebvre W, Penders J, Vanpoucke C, Vrijens K, Nawrot TS. Fetal thyroid function, birth weight, and *in utero* exposure to fine particle air pollution: a birth cohort study. *Environ Health Perspect*. 2017; 125(4): 699-705
**Authors equally contributed.*

8. Kicinski M*, **Saenen ND*,** Viaene MK, Den Hond E, Schoeters G, Plusquin M, Nelen V, Bruckers L, Sioen I, Loots I, Baeyens W, Roels HA, Nawrot TS. Urinary t,t-muconic acid as a proxy-biomarker of car exhaust and neurobehavioral performance in 15-year olds. *Environ Res*. 2016; 151: 521-527.
** Authors equally contributed.*

9. Madhloum N, Janssen BG, Martens DS, **Saenen ND**, Bijnens E, Gyselaers W, Penders J, Vanpoucke C, Lefebvre W, Plusquin M, Nawrot TS. Cord plasma insulin and in utero exposure to ambient air pollution. *Environ Int.* 2017; 105: 126-132

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11. **Saenen ND***, Bové H*, Steuwe C, Roeffaers MBJ, Provost EB, Lefebvre W, Vanpoucke C, Ameloot M, Nawrot TS. Children's urinary environmental carbon load: a novel marker reflecting residential ambient air pollution exposure?. *Am J Respir Crit Care Med.* 2017; 196(7):873-881
** Authors equally contributed.*

12. Provost EB, Int Panis L, **Saenen ND**, Kicinski M, Louwies T, Vrijens K, De Boever P, Nawrot TS. Recent versus chronic fine particulate air pollution exposure as determinant of the retinal microvasculature in school children. *Environ Res*. 2017; 159: 103-110

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15. **Saenen ND***, Provost EB*, Cuypers A, Kicinski M, Pieters N, Plusquin M, Vrijens K, De Boever P, Nawrot TS. Child's mitochondrial DNA content modifies the association between heart rate variability and air pollution exposure at school. *Submitted.*
** Authors equally contributed.*

CONFERENCE MATERIAL

1. **Saenen ND**, Plusquin M, Bijnens E, Janssen BG, Gyselaers W, Cox B, Fierens F, Molenberghs G, Penders J, Vrijens K, De Boever P, Nawrot TS. In utero fine particle air pollution and placental expression of genes in the Brain-derived neurotrophic factor signaling pathway: an ENVIR*ON*AGE birth cohort study. *International Society Environmental Epidemiology (ISEE): 'Environment and Health – Bridging South, North, East and West'*, Basel, Switzerland, 19-23 Aug 2013 (**poster presentation**).

2. **Saenen ND**, Plusquin M, Bijnens E, Janssen BG, Gyselaers W, Cox B, Fierens F, Molenberghs G, Penders J, Vrijens K, De Boever P, Nawrot TS. In utero fine particle air pollution and placental expression of genes in the Brain-derived neurotrophic factor signaling pathway: an ENVIR*ON*AGE birth cohort study. *ISEE Young Researchers conference on Envrionmental Epidemiology*, CREAL, Barcelona, Spain, 20-21 Oct 2014 (**oral presentation**).

3. **Saenen ND**, Janssen BG, Roels HA, Madhloum N, Gyselaers W, Lefebvre W, Penders J, Vanpoucke C, Vrijens K, Nawrot TS. Fetal thyroid function, birth weight, and *in utero* exposure to fine particle air pollution: a birth cohort study. Healthy Living Conference, Maastricht, The Netherlands, 25 -27 Jun 2015 (**oral presentation**).

4. **Saenen ND**, Vrijens K, Janssen BG, Madhloum N, Peusens M, Gyselaers W, Vanpoucke C, Lefebvre W, Roels HA, Nawrot TS. Placental nitrosative stress and exposure to ambient air pollution during gestation: a population study. *ISEE young Researchers conference on Environmental Epidemiology*, Utrecht, the Netherlands, 2-3 Nov 2015 *(***poster presentation***).*

5. **Saenen ND**, Vrijens K, Janssen BG, Roels HA, Vanden Berghe W, Gyselaers W, Vanpoucke C, De Boever P, Nawrot TS. Sex-specific placental epigenetic changes of early neurodevelopment genes and particulate matter air pollution exposure. *ISEE young Researchers conference on Environmental Epidemiology*, Utrecht, the Netherlands, 2-3 Nov 2015 *(***oral presentation***).*

6. **Saenen ND,** Kicinski M, Viaene MK, Den Hond E, Schoeters G, Plusquin M, Nelen V, Bruckers L, Sioen I, Loots I, Baeyens W, Roels HA, Nawrot TS. Urinary t,t-muconic acid as a proxy-biomarker of car exhaust and neurobehavioral performance in 15-year olds. *International Society Environmental Epidemiology (ISEE): 'Old and new risks: challenges for environmental epidemiology'*, Rome, Italy, 01-04 Sep 2016 (**poster presentation**).

7. **Saenen ND**, Vrijens K, Janssen BG, Roels HA, Neven KY, Vanden Berghe W, Gyselaers W, Vanpoucke C, Lefebvre W, De Boever P, Nawrot TS. Lower placental Leptin promoter methylation in association with fine particulate matter air pollution during pregnancy and placental nitrosative stress at birth in the ENVIR*ON*AGE cohort. *International Society Environmental Epidemiology (ISEE): 'Old and new risks: challenges for environmental epidemiology'*, Rome, Italy, 01-04 Sep 2016 (**oral presentation**).

AWARDS

1. Occupational and Environmental Medicine Award,
   bestowed in November 2015 at ISEE-Young Researchers conference on Environmental Epidemiology in Utrecht, The Netherlands