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# **Placental Nitrosative Stress and Exposure to Ambient Air Pollution during Gestation: a Population Study**

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**Abbreviations:** 3-NTP, 3-nitrotyrosine; BMI, body mass index; CI, confidence interval; PM<sub>2.5</sub>, particulate matter with aerodynamic diameter  $\leq 2.5$   $\mu\text{m}$ ;

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**Running head:** Placental nitrosative stress and ambient air pollution

## ABSTRACT

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. We measured placental 3-NTp of 330 mother-newborn pairs, enrolled in the ENVIRONAGE birth cohort (2010 to 2013). Daily particulate matter  $\leq 2.5 \mu\text{m}$  (PM<sub>2.5</sub>), black carbon (BC), and nitrogen dioxide concentrations were interpolated for each mother's residence using a spatiotemporal interpolation method. Placental 3-NTp levels, adjusted for covariates, increased by 35.0% (95% confidence interval (CI): 13.9, 60.0%) for an interquartile range increment in entire pregnancy PM<sub>2.5</sub> exposure. The corresponding estimate for BC exposure was 13.9% (95% CI: -0.21, 29.9%). These results were driven by the first [PM<sub>2.5</sub>: 29.0% (95% CI: 4.9, 58.6%); BC: 23.6% (95% CI: 4.4, 46.4%)] and second gestational exposure window [PM<sub>2.5</sub>: 39.3% (95% CI: 12.3, 72.7)]. This link between placental 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 later in life through particle-mediated nitrosative stress during fetal life.

**Keywords:** 3-nitrotyrosine, ambient air pollution, nitrosative stress, placenta, fine particles, birth cohort, biomarker

## INTRODUCTION

Ambient air pollution has been linked to a variety of adverse health outcomes early in life on both the fetus and neonate such as infant mortality (1), birth weight (2, 3), birth length and head circumference (4), as well as to diseases later in life such as pulmonary and cardiovascular disorders, and even mortality (5, 6). Oxidative and nitrosative stress are two putative main mechanisms by which air pollutants may exert their toxic effects (7). Fine particles (particulate matter  $\leq 2.5 \mu\text{m}$ ; PM<sub>2.5</sub>), black carbon, ozone, nitrogen oxides, and transition metals are able to generate reactive oxygen species (8). 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 sensory receptors on the alveolar surface or particle-induced activation of inflammatory cells may release oxidative mediators (9) 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 (11). The peroxynitrite attack on tyrosine groups of proteins gives rise to the formation of 3-nitrotyrosine, This well-known marker for oxidative stress (12) and inflammation (13, 14) is a molecular fingerprint of peroxynitrite because of its positive association with the rate of protein degradation (15, 16). Increased 3-NTP levels have been observed in different illnesses and are regarded as a marker for Alzheimer's syndrome (17) and Parkinson's disease (18). During gestation, 3-nitrotyrosine formation in the placenta has been demonstrated in high-risk pregnancies including pre-eclampsia and pre-gestational diabetes (19, 20).

Evidence exists that 3-NTP, measured in blood plasma of bus drivers, is associated with traffic-related air pollution exposure (7), however the relationship between 3-nitrotyrosine in the placenta and exposure to ambient air pollutants during gestation has not yet been investigated. As maternal exposure to ambient air pollution in addition to pregnancy, a state of increased physiological oxidative stress, may exacerbate oxidative damage, we hypothesize that overproduction of free radicals may result in accumulation of 3-nitrotyrosine insults in placental tissue. Therefore, we studied the association between placental 3-nitrotyrosine and measures of ambient air pollutants including PM<sub>2.5</sub>, black carbon, and nitrogen dioxide exposure during various time windows of pregnancy.

## **METHODS**

### *Study population*

Our investigations were embedded in the ongoing ENVIRONAGE (ENVIRonmental influence on AGEing in early life) birth-cohort in Limburg (Belgium) (21, 22). We recruited mothers when they arrived for delivery in the weekend, as during this period no competition with other studies existed. Enrolment was spread equally over all seasons of the year and 61% of the eligible mothers participated in the birth cohort. The inclusion criteria were that mothers could fill out a Dutch language questionnaire and delivered a singleton. The study protocol was approved by the Ethical Committees of Hasselt University and East-Limburg Hospital (Genk, Belgium) and 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, pre-gestational body mass index (BMI), education, occupation, smoking status, alcohol consumption, use of

medication, parity, and newborn's ethnicity. Perinatal parameters such as birth date, gestational age, newborn's sex, birth weight and length, Apgar score, and ultrasonographic data were gathered immediately after delivery from the medical record. All neonates with an Apgar score ranging from 7 to 10, five minutes after birth were considered healthy. Maternal education was coded as "low" (no diploma or primary school), "medium" (high school) or "high" (college or university degree). We coded socioeconomic status (SES) according to the U.K. Office of National Statistics using the standard occupational classification hierarchy and condensed it into "low" (including occupation belonging to group 7-9), "middle" (group 4-6) and "high" (group 1-3) (23).

In the present study, 502 mother-newborn pairs were enrolled in the ENVIRONAGE birth cohort between February 2010 and May 2013. For practical and financial reasons, we had to select randomly 400 placentas which were used for multiple biomolecular assays. From these placentas were excluded, 46 biopsies with insufficient tissue, 7 with missing covariate data, 12 preterm newborns, and 5 mothers with gestational diabetes, resulting 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) (24) (Web Table 1).

### *Placental sampling*

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

### *3-Nitrotyrosine protein measurement*

After thawing, 10 mg placental tissue (w. w.) was homogenized in a mixture containing lysis buffer [10 mM tris-hydrochloric acid (pH 7.4), 150 mM sodium chloride, 1% Triton X-100, and Protease Inhibitor Cocktail, Complete, mini, (Roche, Basel, Switzerland)] by sonicating 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 supernatant was aliquoted and frozen at -20°C. Total protein concentration of the supernatant was determined with the Bio-rad protein assay (Biorad, Belgium) according to the manufacturer's instructions.

The amount of 3-nitrotyrosine in each sample was quantified with a competitive enzyme-linked immunosorbent assay (Oxiselect nitrotyrosine ELISA kit, Cell Biolabs, CA, USA). Briefly, 50 µL of the samples and nitrated bovine serum albumin standards were first added to a nitrated bovine serum albumin pre-coated enzyme immune-assay plate. After 10 min incubation, 50 µL of anti-3-nitrotyrosine antibody was added, followed by 1 hour incubation on an orbital shaker and three wash steps. Then, 100 µL of horseradish peroxidase conjugated secondary antibody was added, incubated for 1 hour and washed three times. Finally, 100 µL substrate solution was added and the 3-NTp content of unknown samples was determined by comparing with a curve of known nitrated bovine serum albumin standards. The 3-nitrotyrosine content (nM) was normalized to the placental protein content (mg) and expressed as nM/mg protein.

### *Exposure estimates*

Residential PM<sub>2.5</sub>, black carbon, and nitrogen dioxide exposure levels (µg/m<sup>3</sup>) were interpolated for each mother's residential address using a spatiotemporal interpolation method (25) that takes into account land cover data obtained from satellite images (CORINE land cover data set) and pollution data of fixed monitoring stations in combination with a dispersion model (26, 27). 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 leave-one-out cross-validation including 34 monitoring points for PM<sub>2.5</sub>, 14 for black carbon, and 44 for nitrogen dioxide. Validation statistics of the interpolation tool gave a spatiotemporal explained variance of more than 0.80 for PM<sub>2.5</sub> (27), 0.74 for black carbon (28), and 0.78 for nitrogen dioxide (27).

To explore potentially critical exposure windows during pregnancy, we averaged the daily interpolated exposure concentrations (µg/m<sup>3</sup>) for each pregnancy trimester (29), i.e., 1–13 weeks, 14–26 weeks, and 27 weeks to delivery. Date of conception was estimated based on ultrasound data. The exposure over the entire pregnancy time-window was calculated as the mean of all days between the estimated date of conception and date of delivery. When mothers moved during pregnancy, we recalculated exposure windows accounting for the residential changes during this period. Black carbon exposure data 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, NC, USA). Continuous data were presented as mean (standard deviation) and categorical data as frequencies and percentages. The 3-NTp level was log<sub>10</sub>-transformed to



improve normality of the distribution. Pearson correlation coefficients were calculated to evaluate the correlations between placental 3-NTP and gestational exposure to PM<sub>2.5</sub>, black carbon, or nitrogen dioxide. Multiple linear regressions were performed between the same variables to assess the independent associations while accounting for significant covariates and covariates based on literature evidence. These include newborn's sex, gestational age, maternal age, maternal education (low – middle – high), pre-gestational BMI, smoking status (never, past and current smoker), ethnicity (European – non-European origin) and seasonality (warm – 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 were presented as a percentage difference in 3-nitrotyrosine content for an interquartile range increment in PM<sub>2.5</sub>, black carbon or nitrogen dioxide exposure. In a sensitivity analysis, we performed linear regression analysis to examine the associations between 3-NTP and PM<sub>2.5</sub> exposure, excluding smokers, induced deliveries, caesarian births or non-European participants. To study the relative importance of the entire pregnancy PM<sub>2.5</sub> exposure, we mutually adjusted the main model for entire pregnancy black carbon or nitrogen dioxide exposure. In addition to maternal education, we also explored the significance of SES.

## **RESULTS**

### *Study population characteristics*

Demographic and lifestyle characteristics of the study population (n = 330) are presented in Table 1. Briefly, mothers had a mean (standard deviation) age of 29.4 (4.6) years and the pre-gestational BMI averaged 24.3 (4.6). Most of the mothers (55.2%, n = 182) obtained a higher education degree and 46 mothers (13.9%) reported to have smoked during pregnancy,

whereas 68.2% (n = 225) 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 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 3443 g (432) and 50.5 cm (2.0) respectively.

### *Exposure estimates*

Table 2 displays the distributions of estimated daily outdoor exposure levels of PM<sub>2.5</sub>, black carbon, and nitrogen dioxide for the different time windows of pregnancy. Mean (interquartile range) trimester-specific PM<sub>2.5</sub> exposure was 15.8 (8.8) µg/m<sup>3</sup> for the first trimester, 15.3 (7.4) µg/m<sup>3</sup> for the second trimester, 17.1 (9.4) µg/m<sup>3</sup> for the third trimester of pregnancy, and 16.1 (3.5) µg/m<sup>3</sup> for the entire pregnancy period. The corresponding exposure estimates for the various time windows were for black carbon exposure 0.90 (0.54), 0.95 (0.56), 1.05 (0.46), and 0.97 (0.36) µg/m<sup>3</sup>, and for nitrogen dioxide exposure 19.8 (9.1), 20.2 (8.8), 21.7 (8.4), and 20.5 (5.8) µg/m<sup>3</sup> respectively. For the entire pregnancy, the exposure to nitrogen dioxide and black carbon exposure correlated well with PM<sub>2.5</sub> exposure (r = 0.75 and r = 0.64, respectively).

### *Association between placental 3-nitrotyrosine and ambient air pollution during various time windows of pregnancy*

The geometric mean (range) of placental 3-nitrotyrosine was 3,735 (212 – 23,682) nM/mg protein. There was no association between placental 3-nitrotyrosine and covariates shown in Table 1, except for pre-gestational BMI [-3.2%, 95% confidence interval (CI): -5.2, -1.3; P = 0.006] and cold versus warm season [-21.8% (95% CI: -35.1, -5.8; P = 0.01)]. We observed a

positive and significant correlation between placental 3-nitrotyrosine and entire pregnancy exposure to PM<sub>2.5</sub> ( $r = 0.27$ ,  $P < 0.0001$ ), black carbon ( $r = 0.13$ ,  $P = 0.03$ ) or nitrogen dioxide ( $r = 0.15$ ,  $P = 0.007$ ) (Figure 1A-C). After adjustment for newborn's sex, gestational age, maternal age, maternal education, pre-gestational BMI, smoking status, ethnicity and seasonality (Table 3), we observed similar results as in the unadjusted model for the association between placental 3-nitrotyrosine and entire pregnancy PM<sub>2.5</sub> or black carbon exposure. For an interquartile range increment in PM<sub>2.5</sub> exposure over the entire pregnancy period, placental 3-nitrotyrosine increased with 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<sub>2.5</sub> 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,  $P = 0.07$ )].

### *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-nitrotyrosine and ambient air pollution. Excluding caesarian births

(n=16) from the analysis did not change our findings, except for entire pregnancy black carbon exposure ( $P$  value changed from 0.05 to 0.09). Models in which we included parity as covariate, did not alter the observed associations. Furthermore, mutual adjustment of the main entire pregnancy PM<sub>2.5</sub> exposure model for entire pregnancy nitrogen dioxide or black carbon exposure did not change our main findings. Maternal education was not associated with any of the air pollutants ( $P \geq 0.33$ ). In addition, we did not observe a significant association between SES and PM<sub>2.5</sub> ( $P = 0.52$ ) or black carbon ( $P = 0.14$ ) exposure. For nitrogen dioxide exposure, the lowest SES group showed higher average (standard deviation) residential nitrogen dioxide exposure 21.6 (4.6)  $\mu\text{g}/\text{m}^3$  vs 19.6 (4.6)  $\mu\text{g}/\text{m}^3$  ( $P = 0.02$ ). However, SES status was not associated with 3-nitrotyrosine and therefore did not fulfill the definition of a confounder. Nevertheless, our results did not alter if we changed maternal education by maternal SES in the model.

## DISCUSSION

The key finding of our study was that 3-nitrotyrosine levels in the placenta were positively associated with PM<sub>2.5</sub> during gestation and that the associations were driven by the exposures of the first and second gestational trimester. For black carbon, the association with 3-nitrotyrosine was mainly driven by the first trimester exposure window and no associations were found with nitrogen dioxide exposure. Furthermore, the results for PM<sub>2.5</sub> remained the same after additional adjustment for black carbon or nitrogen dioxide exposure. Hence, our findings highlight on the one hand the relevance of placental 3-nitrotyrosine as a biomarker of cumulative PM<sub>2.5</sub>-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 (30). The placenta also acts as a sensor of the maternal-fetal environment and adapts to both intrinsic and extrinsic factors (31). In mice as well as in humans, environmental insults such as fine particle air pollution may affect placental functional morphology and fetal growth (2, 32). These detrimental changes on fetal programming may predetermine the risk of disease later in life (33). 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 (34). In 50 bus drivers, an average PM<sub>2.5</sub> exposure of 32.1 µg/m<sup>3</sup> resulted in an increased oxidative and nitrosative stress as reflected by protein carbonyl and 3-nitrotyrosine levels in blood plasma in comparison to 50 matched controls (7). In our study, a much lower gestational PM<sub>2.5</sub> exposure (16.1 µg/m<sup>3</sup>) resulted in a wider range of 3-nitrotyrosine levels in placenta (212 – 23,682 nM/mg protein) compared to 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 exert pronounced perturbations on placental function, as has been found in placental vessels in pathologic pregnancies, e.g. pre-eclampsia and gestational diabetes (19, 20). The targets for protein nitration are also involved in trophoblast invasion and regulation of placental vascular reactivity, which subsequently may affect placental function (35). Therefore, nitrosative stress may be one of the underlying mechanisms that links altered placental function to changes in fetal programming (36).

Hence, we hypothesize that particulate matter-induced oxidative stress during pregnancy may alter the placental vascular function with the potential to affect fetal development and growth. An experimental study in diesel exhaust particles-exposed mice with adverse intrauterine conditions observed elevated 3-nitrotyrosine protein modification, predominantly in perivascular regions at the fetal side of the placenta, suggesting that diesel exhaust particles exposure *in utero* promotes vascular oxidative stress (37). In atherosclerotic mice, long-term exposure to cigarette smoke free from nicotine and tar particles (15 min/day, 6 days/week, for 16 weeks) accelerated the accumulation of total cholesterol levels in the aorta and increased 3-nitrotyrosine levels (38). However, in humans with stable coronary heart disease, acute exposure to ambient particles for two hours did not induce significant alterations in 3-NTP levels in exhaled breath condensate neither did it affect vascular function (39). In our study population, we did not find significant associations between placental 3-nitrotyrosine and birth weight ( $P = 0.26$ ) or birth length ( $P = 0.30$ ). This is in line with the finding in the entire ENVIRONAGE birth cohort showing the absence of a direct association between birth weight and gestational PM<sub>2.5</sub> exposure (40). This observation, however, does not necessarily imply that the stimulated reactive oxygen species formation associated with increased gestational PM<sub>2.5</sub> exposure would not influence more subtle biological events during gestational growth of the fetus, e.g. neurodevelopment (21). Furthermore, we found that gestational exposure to PM<sub>2.5</sub> and black carbon was associated with placental 3-nitrotyrosine, which was mainly driven by the exposure during the first and/or second trimesters. Recently, early and mid-pregnancy exposure windows were shown most critical for adverse newborn health outcomes such as preterm birth (41, 42). Moreover, Robledo *et al.* (43) 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-nitrotyrosine and nitrogen dioxide exposure either during or prior to pregnancy (Web Table 2). Nevertheless, the nitrosative stress-mediated health outcomes of ambient air pollution exposure during gestation are yet to be better characterized.

We cannot exclude 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 as well as in placental tissue and/or related to translocation of ultrafine particles directly from the lung to the blood stream from where they can pass the maternal-placental-fetal barrier (9, 30). Therefore, we can currently only speculate about the mechanism of action 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 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 (12). The generalizability of our findings may be limited with regard to the placenta as a whole as we used only one biopsy of the fetal side of the placenta. Nevertheless, we consider the fetal side of the placenta as the most representative for 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 a certain selection bias in our study population, as we can only recruit in the weekend and elective deliveries for medical reasons are scheduled during weekdays. Anyway, this selection procedure resulted in a healthy-pregnancy effect limiting confounding of complicated pregnancies on 3-NTP outcome. Notwithstanding observational studies only allow to

characterize associations, there is also experimental evidence supporting our findings (34, 37, 38).

To conclude, we observed a positive association between placental 3-NTP levels and exposure to PM<sub>2.5</sub> and possibly black carbon during pregnancy, which is in line with experimental evidence on cigarette smoke and diesel exhaust exposure. Further research is needed to elucidate the potential health consequences later in life through particle-mediated nitrosative stress during fetal life.



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**Table 1.** Characteristics of 330 Mother-Newborn Pairs from the ENVIRONAGE Birth Cohort (Limburg, Belgium, 2010-2013)

Characteristics	Mean (SD)	No., %
<b>Mother</b>		
Age, y	29.4 (4.6)	
Pre-gestational BMI <sup>a</sup>	24.3 (4.6)	
Mother's education		
Low		38, 11.5
Middle		110, 33.3
High		182, 55.2
Socioeconomic status		
Low		87, 26.3
Middle		187, 56.7
High		56, 17.0
Smoking		
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
<b>Newborn</b>		
Sex		
Boy		165, 50.0
Ethnicity		
European origin		286, 86.7
Gestational age, w	39.5 (1.1)	
Born at term (≥ 37 w)		330, 100
Caesarian birth		16, 4.9
Induced delivery		26, 7.9
Seasonality		
cold (Autumn - Winter)		190, 57.6
warm (Spring – Summer)		140, 42.4
Apgar score after 5 min		
7		7, 2.1
8		18, 5.4
9		87, 26.4
10		218, 66.1
Birth weight, g	3443 (432)	
Birth length, cm	50.5 (2.0)	

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

<sup>a</sup>BMI expressed as kg/m<sup>2</sup>

**Table 2.** Distribution of Interpolated Air Pollution Exposure during Pregnancy from a Spatiotemporal and Dispersion Model among 330 Mother-Newborn Pairs of the ENVIRONAGE Birth Cohort (Limburg, Belgium, 2010-2013)

<b>Time window exposure, <math>\mu\text{g}/\text{m}^3</math></b>	<b>Mean (SD)</b>	<b>5<sup>th</sup> percentile</b>	<b>25<sup>th</sup> percentile</b>	<b>Median</b>	<b>75<sup>th</sup> percentile</b>	<b>95<sup>th</sup> percentile</b>	<b>IQR</b>
<b>PM<sub>2.5</sub></b>							
Entire pregnancy	16.1 (2.4)	12.4	14.2	16.1	17.6	20.3	3.5
Trimester 1	15.8 (5.6)	9.1	11.2	13.7	20.1	26.0	8.8
Trimester 2	15.3 (4.8)	9.6	11.3	14.6	18.8	24.4	7.4
Trimester 3	17.1 (5.7)	9.3	12.0	16.8	21.5	27.0	9.4
<b>BC<sup>a</sup></b>							
Entire pregnancy	0.97 (0.28)	0.56	0.77	0.92	1.12	1.53	0.36
Trimester 1	0.90 (0.39)	0.40	0.59	0.85	1.13	1.61	0.54
Trimester 2	0.95 (0.41)	0.38	0.63	0.91	1.18	1.74	0.56
Trimester 3	1.05 (0.37)	0.52	0.78	1.03	1.24	1.72	0.46
<b>NO<sub>2</sub></b>							
Entire pregnancy	20.5 (4.5)	14.0	17.3	20.2	23.2	29.1	5.8
Trimester 1	19.8 (6.3)	10.5	14.9	19.4	24.0	31.7	9.1
Trimester 2	20.2 (6.3)	11.3	15.6	19.8	24.4	30.8	8.8
Trimester 3	21.7 (6.1)	12.0	17.3	21.6	25.7	32.0	8.4

Abbreviations: BC, black carbon; IQR, interquartile range; NO<sub>2</sub>, nitrogen dioxide; PM<sub>2.5</sub>, particulate matter with aerodynamic diameter  $\leq 2.5$   $\mu\text{m}$ ; SD, standard deviation.

<sup>a</sup>Data available for 271 mother-newborn pairs.

**Table 3** Estimated Increase (%) in Placental 3-Nitrotyrosine Content Associated with an IQR Increment of PM<sub>2.5</sub>, BC, and NO<sub>2</sub> Air Pollution during Entire Pregnancy and the Three Gestational Trimesters among 330 Mother-Newborn Pairs from the ENVIRONAGE Birth Cohort (Limburg, Belgium, 2010-2013)

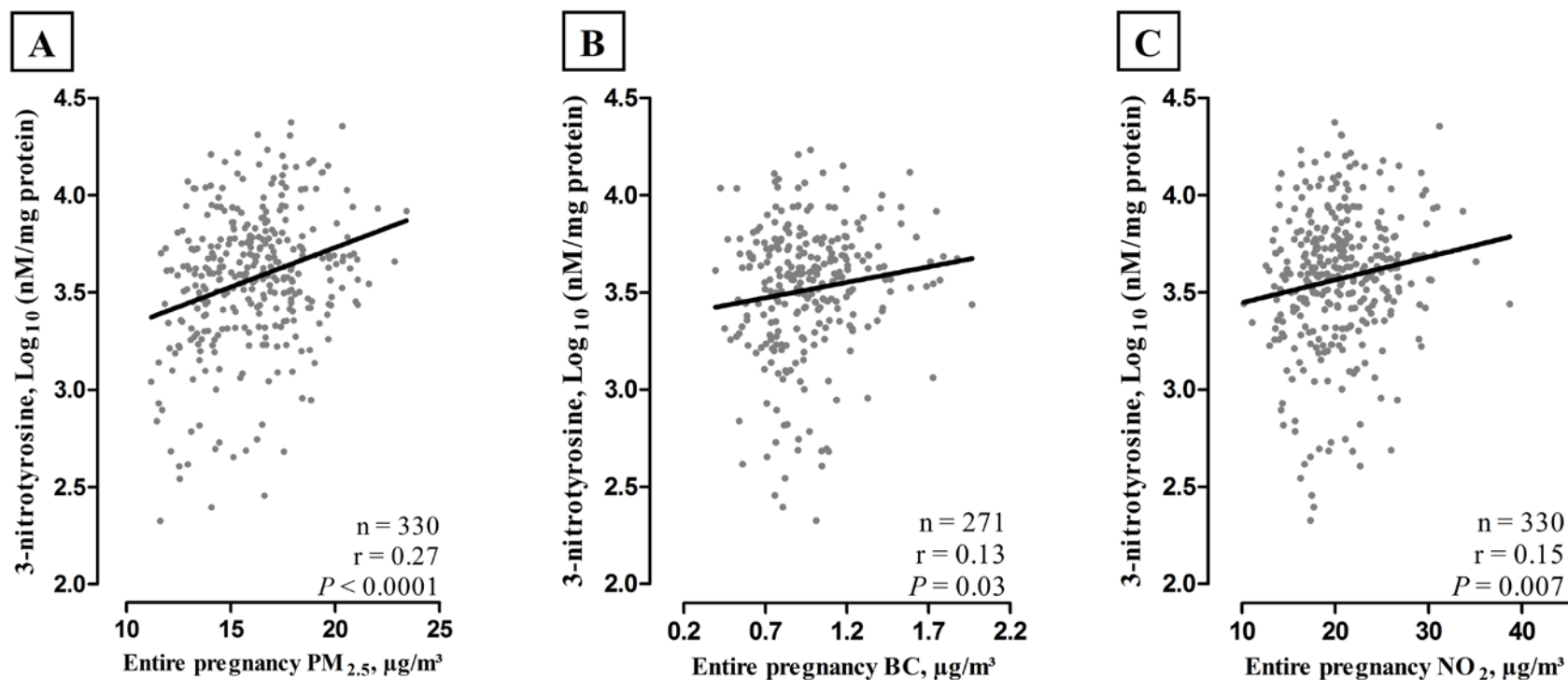
<b>Exposure time window, µg/m<sup>3</sup></b>	<b>Adjusted regression coefficients<sup>a</sup>, %</b>	<b>95% CI</b>	<b>P-value</b>
<b>PM<sub>2.5</sub></b>			
Entire pregnancy	35.0	13.9, 60.0	0.0006
Trimester 1	29.0	4.9, 58.6	0.02
Trimester 2	39.3	12.3, 72.7	0.003
Trimester 3	13.2	-9.4, 41.3	0.27
<b>BC<sup>b</sup></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
<b>NO<sub>2</sub></b>			
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

Abbreviations: BC, black carbon; CI, confidence interval; IQR, interquartile range; NO<sub>2</sub>, nitrogen dioxide; PM<sub>2.5</sub>, particulate matter with aerodynamic diameter ≤ 2.5 µm;

<sup>a</sup> Estimates (95% CI) are adjusted for newborn's sex, gestational age, maternal age, maternal education, pre-gestational BMI, smoking status, ethnicity and seasonality.

<sup>b</sup> Models carried out for 271 mother-newborn pairs.

## FIGURES



**Figure 1** Pearson Correlation between Placental 3-Nitrotyrosine and Entire Pregnancy Exposure to A) particulate matter  $\leq 2.5 \mu\text{m}$  (n = 330, r = 0.27,  $P < 0.0001$ ), B) black carbon (n = 271, r = 0.13,  $P = 0.03$ ), and C) nitrogen dioxide (n = 330, r = 0.15,  $P = 0.007$ ) among Mother-Newborn Pairs from the ENVIRONAGE Birth Cohort (Limburg, Belgium, 2010-2013). Abbreviations: BC, black carbon; NO<sub>2</sub>, nitrogen dioxide; PM<sub>2.5</sub>, particulate matter with aerodynamic diameter  $\leq 2.5 \mu\text{m}$ .