Prenatal ambient air pollution exposure, infant growth and placental mitochondrial DNA content in the INMA birth cohort

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Abstract

BACKGROUND: The association between prenatal air pollution exposure and postnatal growth has hardly been explored. Mitochondrial DNA (mtDNA), as a marker of oxidative stress, and growth at birth can play an intermediate role in this association.

OBJECTIVE: In a subset of the Spanish birth cohort INMA we assessed first whether prenatal nitrogen dioxide (NO_2) exposure is associated with infant growth. Secondly, we evaluated whether growth at birth (length and weight) could play a mediating role in this association. Finally, the mediation role of placental mitochondrial DNA content in this association was assessed.

METHODS: In 336 INMA children, relative placental mtDNA content was measured. Land-use regression models were used to estimate prenatal NO_2 exposure. Infant growth (height and weight) was assessed at birth, at 6 months of age, and at 1 year of age. We used multiple linear regression models and performed mediation analyses. The proportion of mediation was calculated as the ratio of indirect effect to total effect.

RESULTS: Prenatal NO_2 exposure was inversely associated with all infant growth parameters. A 10 $\mu g/m^3$ increment in prenatal NO_2 exposure during trimester 1 of pregnancy was significantly inversely associated with height at 6 months of age (-6.6%; 95%CI: -11.4, -1.9) and weight at 1 year of age (-4.2%; 95%CI: -8.3, -0.1). These associations were mediated by birth length (31.7%; 95%CI: 34.5, 14.3) and weight (53.7%; 95%CI: 65.3, -0.3), respectively. Furthermore, 5.5% (95%CI: 10.0, -0.2) of the association between trimester 1 NO_2 exposure and length at 6 months of age could be mediated by placental mtDNA content.

CONCLUSIONS: Our results suggest that impaired fetal growth caused by prenatal air pollution exposure can lead to impaired infant growth during the first year of life. Furthermore, molecular adaptations in placental mtDNA are associated with postnatal consequences of air pollution induced alterations in growth.

Keywords: Prenatal air pollution; Nitrogen dioxide; Infant growth; Mitochondrial DNA content; Mediation

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1. Introduction

In the last decade, numerous studies have reported an association between prenatal ambient air pollution exposure and adverse birth outcomes. Prenatal nitrogen dioxide (NO_2) has been associated with low birth weight, intra-uterine growth retardation, and preterm birth, even at low levels of air pollution (Pedersen et al. 2013; Stieb et al. 2012). The fetus may be particularly susceptible to air pollution exposure, because of its physiologic immaturity and its higher rates of cell proliferation (Grandjean et al. 2008). The impact of NO_2 exposure on the fetus is important for public health because fetal growth, and birth size and weight of newborns are important predictors of the future health status during childhood and adulthood (Barker 1995, 2004). Infant growth is believed to be a continuation of *in utero* growth and is influenced predominantly by factors determining intra-uterine growth and nutrition (Hindmarsh et al. 2008); consequently, exposure to NO_2 during pregnancy could also affect infant growth. Nonetheless, little is known about how these intra-uterine effects may translate into variations in growth patterns of children after birth. Additionally, infant growth can be influenced by both genetic and environmental factors (Victora et al. 2008). Adverse infant growth may be an important determinant of obesity and related health problems later in life (Godfrey and Barker 2000; Olsen et al. 2001).

The placenta is a metabolically active organ that connects and separates two genetically distinct individuals: the mother and the fetus. It plays an essential role in nutrient transfer, growth and organ development. The placenta is a unique vascular organ that requires a constant source of energy. This energy provision is regulated by mitochondrial function of placental cells (Myllynen et al. 2005). Mitochondria, the energy producers of the cells, are the major intracellular sources of reactive oxygen species (ROS), which are generated under normal conditions as by-product of oxidative phosphorylation. Mitochondria are the primary targets of oxidative stress because mitochondrial DNA (mtDNA) lacks protective strategies associated with nuclear DNA. Consequently, mitochondria are uniquely sensitive to environmental toxicants (Lee and Wei 2000). Furthermore, mtDNA content is correlated with the size and number of mitochondria, which have been shown to change under different energy demands, as well as different environmental conditions (Clay Montier 2009). NO_2 has a strong oxidation capacity by generating ROS and reactive nitrogen species (RNS). Some studies have implicated that mitochondrial function can be negatively affected by environmental toxicants stimulus, such as NO_2, $PM_{2.5}$, and black carbon (Colicino et al. 2014; Li et al. 2003; Meyer et al. 2013; Yan et al. 2015). Fetus adapt their mitochondrial structure and metabolism when the supply of nutrients is limited (Gemma et al. 2006). Changes in placental mtDNA content may represent a biological effect along the path linking air pollution to effects on the infant.

Recently, it was shown that placental mtDNA content was influenced by prenatal particulate matter <10μm (PM_{10}) and nitrogen dioxide (NO_2) exposure (Clemente et al. 2016; Janssen et al. 2012). Furthermore, in our previous study we showed that placental mtDNA content was significantly associated with birth weight and that it could be one of the mediators of the inverse association between prenatal NO_2 exposure and birth weight (Clemente et al. 2016). These findings raise the question of whether prenatal air pollution exposure may also result in subsequent changes in infant growth and whether placental mtDNA alterations can be linked to these outcomes in later life.

In the current study we therefore evaluated firstly whether prenatal NO_2 exposure is associated with infant growth (height and weight) at 6 months and 1 year of age. Secondly, we evaluated whether growth deficits at birth (length and weight) play a mediating role in this association. Finally, the mediating role of placental mtDNA content in the association between prenatal air pollution exposure and infant growth was assessed.

2. Methods

2.1. Study design and population

INMA (INfancia y Medio Ambiente; Environment and Childhood) is a birth cohort study that recruited pregnant women in seven regions, following a common protocol (Guxens et al. 2012). In this study we used participants with singleton live-born infants from three INMA regions (Valencia, Sabadell and Gipuzkoa). Pregnant women were enrolled between 2004 and 2008 during the first trimester of pregnancy at primary health care centers or public hospitals if they fulfilled the inclusion criteria: singleton pregnancy, intention to deliver at the reference hospital, ≥ 16 years of age, no problems of communication, and no assisted conception. Of all eligible women, 57% agreed to participate. Study approval was obtained from the ethics committees of each participating center and informed consents were obtained from the mothers.

In INMA, placentas were randomly collected in approximately one of four deliveries (n = 502). The present analysis included 336 randomly selected mother-newborn pairs from whom placentas were collected and placental mtDNA content measured. The main characteristics of our study population including maternal age, smoking during pregnancy, maternal education, parity, gestational age, ethnicity, and prenatal NO_2 exposure are in line with the INMA participants that provided placental samples, but were not included in this study (Supplemental Material, Table S1 and Table S2). Therefore our population of mother-newborn pairs is representative for those who were not included in the analysis.

2.2. Ambient air pollution assessment

Ambient concentrations of nitrogen dioxide (NO_2) were measured with the aid of passive samplers (Radiello, Fundazione Salvatore Maugeri, Padua, Italy) installed in several sampling campaigns each lasting seven days and distributed across the study areas according to geographic criteria, taking into account the expected pollution gradients and the distribution of the residences of the participating women.

The methodology has been described in detail elsewhere (Aguilera et al. 2008; Iniguez et al. 2009). Briefly, area-specific land use regression (LUR) models were used to predict NO_2 levels at women's residential addresses, using the average of the NO_2 levels registered across campaigns to represent an annual mean level, together with land use (agricultural, industrial or urban), traffic-related variables, and altitude. Residential NO_2 estimations from LUR were then adjusted to time of pregnancy for each woman, using daily records from the monitoring network stations covering the study area. This model also took into account residential changes if women lived at least 2 months of pregnancy in the new residence. The validation statistics gave a spatial explained variance (R^2) for annual mean NO_2 from 0.51 to 0.75 in the three INMA regions (Aguilera et al. 2008).

In order to explore potentially critical exposures during pregnancy, individual NO_2 concentrations were calculated for different periods of pregnancy: trimester 1 (1-13 weeks), trimester 2 (14-28 weeks), trimester 3 (29 weeks to delivery), and for the entire pregnancy.

2.3. Placental mtDNA content

As previously described (Clemente et al. 2016), placentas were entirely frozen after delivery at -20°C and afterwards at -86°C. Placentas were thawed minimally to obtain tissue biopsies for DNA extractions. To minimize the impact of within-placental variability, biopsies were all taken 1-1.5 cm below the chorio amniotic membrane at a fixed location and preserved at -80°C (Janssen et al. 2012). Briefly, DNA was extracted from placental tissue cells and quantified. MtDNA content was measured in placental tissue cells by determining the ratio of two mitochondrial gene copy numbers (mitochondrial encoded NADH dehydrogenase subunit 1 (*MT-ND1*) and mitochondrial forward primer for nucleotide 3212 and reverse primer from nucleotide 3319 (*MTF3212/R3319*)) to two single-copy nuclear control genes (acidic ribosomal phosphoprotein P0 (*RPLP0*), and beta-actin (*ACTB*)) using the 7900HT Fast Real-Time PCR System (Life Technologies, Foster City, CA, United States) (Janssen et al. 2012). Samples were run randomly in triplicate and each plate included six inter-run calibrators to account for inter-run variability. qBase software (Biogazelle, Zwijnaarde, Belgium) automatically averaged triplicate measurements that pass quality control and normalizes the data to nuclear reference genes while correcting for run-to-run differences (Hellemans et al. 2007).

2.4. Infant growth

Birth weight was recorded by trainee midwifes at delivery whereas birth length was measured by a nurse when the neonate arrived at the hospital ward within the first 12 hours of life.

Repeated height and weight measures from birth to 6 months of age were extracted from medical records. For infants without weight measures available within ± 14 days of their exact 6-month anniversary (n=17, 5.1% of the main analysis sample), we used the 2^{nd}-order Reed sex-specific early infancy growth models to predict the weight of children as described previously (Valvi et al. 2013). Child height and weight were measured at 1 year of age using standard protocols, with light clothing and without shoes. Age- and sex-specific z-scores for height and weight at birth, at 6 months and 1 year of age were calculated using the World Health Organization (WHO) referent (de Onis et al. 2009). The change in length and height z-score was calculated as the length/height z-score at follow up (6 months and 1 year of age) minus the length/weight z-score at birth divided by the timespan between birth and follow-up.

2.5. Covariates

Information on maternal age, ethnicity, education, smoking status, place of residence, pre-pregnancy BMI, and parity was obtained by self-reported questionnaires administrated by trained interviewers at 1^{st} and 3^{rd} trimester of pregnancy. Child sex and date of birth was obtained from clinical records.

2.6. Statistical analysis

Continuous data were checked for normality using the Shapiro-Wilk test statistic. Continuous data were presented as mean ± SD and categorical data as frequencies and percentages. Average placental mtDNA was log10-transformed to improve the normality of the distributions and described by geometric mean and 25th-75th percentile. Collinearity was assessed among the different exposure windows by calculating variance inflation factors (VIF). Multiple linear regression models were used to assess the association between (i) prenatal NO_2 exposure and infant growth (height and weight at 6 months and at 1 year of age) and between (ii) placental mtDNA content and infant growth.

Covariates used in the models were chosen *a priori*, including newborn's sex (male, female), gestational age (linear and quadratic term), maternal age (years), maternal pre-pregnancy BMI (kg/m^2), ethnicity (European, non-European), maternal education (primary, secondary, university), smoking during pregnancy (never, quit smoking before week 12, during entire pregnancy), parity (nulliparous, multiparous), season of birth (January-March, April-June, July-September, October-December), and region (Sabadell, Valencia, Gipuzkoa). We presented adjusted models because they yielded similar results than the unadjusted ones.

Prior to the mediation analysis we explored if placental mtDNA content, birth weight and birth length were effect modifiers of the association between prenatal NO_2 exposure and height/weight at 6 months and 1 year of age. This was done by adding an interactionterm combining the NO_2 exposure with placental mtDNA content or interactionterms combining the NO_2 exposure with birth weight/length. Several mediation analyses were performed. Firstly, we investigated if birth length mediated the association between prenatal NO_2 exposure and length in the infants at 6 months and 1 year of age. Secondly, we assessed whether birth weight mediated the association between prenatal NO_2 exposure and weight in the infants at 6 months and 1 year of age. Thirdly, we investigated whether placental mtDNA content was a mediator of the association between prenatal NO_2 exposure and the different infant growth characteristics (height and weight at 6 months and 1 year of age). We only performed the mediation analysis when there was a significant association between the outcome and the exposure, a significant association between the exposure and the mediator, and a significant association between the outcome and the mediator. To perform these mediation analyses we used the SAS macro developed by Valeri and VanderWeele (Valeri and VanderWeele 2013). In this macro, the direct effect (DE), indirect effect (IE) and total effect (TE) were determined. The DE represents the effect of exposure on the outcome after controlling for the mediator whereas the IE is the effect of exposure operating through the mediator. The proportion of mediation was calculated as the ratio of IE to TE. For example, if the proportion of mediation is 0.32 this means that 32% of the total effect can be explained by the mediating variable in question.

3. Results

3.1. Characteristics and exposure levels of the study population

Table 1 summarizes the characteristics of the 336 mother-newborn pairs. Briefly, mean maternal age was 32.2 years. Pre-gestational BMI of the participating mothers averaged 23.5 kg/m² and 44.6% of the mothers never smoked cigarettes. The newborns, 168 of which were girls (50.0%), had a mean gestational age of 39.9 weeks. More than 90% of the newborns were European.

Table 1. Study population characteristics (n=336)

Characteristics	Mean ± SD range or number (%)
Maternal	
Age, years	32.2 ± 3.9
Smoking	
Never	150 (44.6)
Quit smoking before week 12	128 (38.1)
During entire pregnancy	58 (17.3)
Education	
Primary school or none	68 (20.2)

Secondary school	154 (45.8)
University	114 (33.9)
Parity	
1	184 (54.8)
2	128 (38.1)
≥3	24 (7.1)
Pre-pregnancy BMI, kg/m^2	23.5 ± 4.3
Region	
Gipuzkoa	154 (45.8)
Sabadell	121 (36.0)
Valencia	61 (18.2)
Newborn	
Gestational age, weeks	39.9 ± 1.4
Sex	
Male	168 (50.0)
Female	168 (50.0)
Ethnicity	
European	306 (91.1)
Non-European	30 (8.9)
Season at birth	
January-March	76 (22.6)
April-June	89 (26.5)
July-September	88 (26.2)
October-December	83 (24.7)
Birth weight, g	3,284 ± 429
Weight at age 6 months, kg	7.7 ± 0.8
Weight at age 1 year, kg	9.8 ± 1.1
Birth length, cm	49.4 ± 2.1
Height at age 6 months, cm	67.2 ± 2.4
Height at age 1 year, cm	75.4 ± 2.8
Placental mtDNA content	1.5 (1.2-1.8)

Continuous covariates expressed by mean and standard deviation (SD) (normally distributed) or geometric mean and 25– 75th percentile (not normally distributed); categorical covariates described by numbers and frequencies (%).

Table 2 displays the daily outdoor NO_2 exposure levels averaged for the different exposure periods. Average (25th-75th percentile) period-specific NO_2 exposure was 27.0 (16.8-34.7) μg/m³ for trimester 1, 26.0 (16.7-32.6) μg/m³ for trimester 2, 26.4 (17.0-33.4) μg/m³ for trimester 3, and 26.2 (17.4-33.3) μg/m³ for the entire pregnancy.

Table 2. Descriptive statistics of prenatal NO2 exposure (μg/m³) in the INMA study (N=336)

							Correlation[a]			
NO_2 exposure (μg/m^3)	**Mean ± SD**	**P5**	**P25**	**P50**	**P75**	**P95**	**Trimester 1**	**Trimester 2**	**Trimester 3**	**Entire pregnancy**
Trimester 1	27.0 ± 13.0	5.6	16.8	24.8	34.7	74.2	1			
Trimester 2	26.0 ± 11.9	5.7	16.7	24.7	32.6	74.7	0.85*	1		
Trimester 3	26.4 ± 12.5	5.7	17.0	24.0	33.4	74.4	0.78*	0.85*	1	
Entire pregnancy	26.2 ± 11.6	5.7	17.4	24.6	33.3	66.7	0.91*	0.92*	0.94*	1

[a]Spearman correlation coefficients between different exposure periods

*P-value <0.0001

3.2. Association between prenatal NO_2 exposure and infant growth

Table 3 displays the percent change in z-scores for height and weight at 6 months and at 1 year of age for every 10 μg/m³ increment in NO_2 exposure during the different exposure windows of pregnancy. A 10 μg/m³ increment in prenatal NO_2 exposure was inversely and significantly associated with height at 6 months, especially during trimester 1 (-6.64%; 95%CI: -11.38, -1.90) and trimester 2 (-5.56%; 95%CI: -10.86, -0.26). Furthermore, each 10μg/m³ increment in NO_2 levels during trimester 1 of pregnancy was inversely and significantly associated with weight at 1 year (-4.21%; 95%CI: -8.34, -0.09). Prenatal NO_2 exposure was negatively but not significantly associated with weight at 6 months, and height at 1 year of age. VIF values in our collinearity diagnostics ranged from 4.74 to 7.39 for NO_2, indicating elevated levels of collinearity in the multi trimester models. These models show results in the same direction, but the confidence intervals are much wider (see Supplemental Materials, Table S3). The multi collinearity between the different exposure windows probably contribute to these wider confidence intervals that we observe.

Furthermore, prenatal NO_2 exposure was not associated with a change in weight z-scores between birth and 6 months/1 year of age and between 6 months and 1 year of age (see Supplemental Materials, Table S4). Only first trimester exposure to NO_2 was significantly positively associated with a change in length z-scores between 6 months and 1 year of age (0.8%; 95%CI: 0.17, 1.43). Prenatal NO_2 exposure was not significantly associated with length z-scores between birth and 6/12 months of age (see Supplemental Materials, Table S4).

Table 3. Association between maternal NO_2 exposure in different exposure periods of pregnancy and infant growth in the INMA study

	N	Change (%)	95% CI	P-value
zHeight at 6 months				
NO_2 Trimester 1	286	-6.64	-11.38, -1.90	<0.01
NO_2 Trimester 2	286	-5.56	-10.86, -0.26	0.04
NO_2 Trimester 3	286	-2.22	-7.13, 2.69	0.37
NO_2 Entire pregnancy	286	-5.20	-10.8, 0.41	0.07
zWeight at 6 months				
NO_2 Trimester 1	289	-3.32	-7.32, 0.67	0.10
NO_2 Trimester 2	289	-3.26	-7.6, 1.07	0.14
NO_2 Trimester 3	289	-2.16	-6.23, 1.92	0.30
NO_2 Entire pregnancy	289	-2.94	-7.62, 1.75	0.22
zHeight at 1 year				
NO_2 Trimester 1	286	-3.70	-9.58, 2.19	0.22
NO_2 Trimester 2	286	-3.53	-9.86, 2.54	0.24
NO_2 Trimester 3	286	-3.71	-9.96, 2.54	0.24
NO_2 Entire pregnancy	286	-4.51	-11.42, 5.97	0.20
zWeight at 1 year				
NO_2 Trimester 1	289	-4.21	-8.34, -0.09	0.04
NO_2 Trimester 2	289	-3.45	-7.99, 1.08	0.13
NO_2 Trimester 3	289	-2.47	-6.71, 1.77	0.25
NO_2 Entire pregnancy	289	-3.97	-8.84, 0.89	0.11

Effect size was estimated for each 10 μg/m^3 increment in exposure to NO_2 at each mother's residence during the corresponding period;

Models were adjusted for newborn's sex, maternal age, smoking status, gestational age, gestational age^2, maternal pre-pregnancy BMI, parity, ethnicity, season, and education;

3.3. Association between placental mtDNA content and infant growth

Table 4 shows that placental mtDNA content was significantly and positively associated with length at birth (0.29g; 95%CI: 0.04, 0.55). Furthermore, placental mtDNA content was significantly and positively associated with height at 6 months (5.90%; 95%CI: 0.60, 13.24) (Table 4). Placental mtDNA content was positively but not significantly associated with weight at 6 months, and with weight and height at 1 year of age. Furthermore, placental mtDNA content was not associated with a change in weight/height z-scores between birth and 6 months/1 year of age and between 6 months and 1 year of age (Data not shown).

Table 4. Association between placental mtDNA content and infant growth outcomes in INMA

	N	Change	95% CI	P-value
Length at birth, cm	336	0.29	0.04, 0.55	0.02
Weight at birth, g[a]	336	73.8	18.60, 127.21	<0.01
zHeight at 6 months, %	286	5.90	0.60, 13.24	0.03
zWeight at 6 months, %	289	3.62	-1.34, 6.95	0.15
zHeight at 1 year, %	286	4.60	-1.50, 9.19	0.19
zWeight at 1 year, %	289	3.10	-1.25, 7.39	0.13

Effect size was estimated for each IQR (0.18) increase in placental mtDNA content;
Models were adjusted for newborn's sex, maternal age, smoking status, gestational age, gestational age^2, maternal pre-pregnancy BMI, parity, ethnicity, season, and education;
[a]These results were presented in a previous paper based on the same cohorts (Clemente et al. 2016)

3.4. Mediation analyses

The added interactionterm combining the NO_2 exposure with placental mtDNA content and the interactionterms combining the NO_2 exposure with birth weight/length in the association between prenatal NO_2 exposure and the height/weight at 6 months and 1 year of age was not significant (data not shown). Meaning that neither placental mtDNA content nor birth weight/length were effect modifiers of the association between prenatal NO_2 exposure and infant growth.

We tested whether birth outcomes (birth length and weight) and mtDNA content could be mediators of the association between prenatal NO_2 exposure and infant growth. Prenatal NO_2 exposure during all the three trimesters of pregnancy and during the entire pregnancy was associated with birth weight, birth length, and mtDNA content (previous study (Clemente et al. 2016) except for birth length and Supplemental Material, Table S5). Mediation analysis showed that birth length mediated 31.7% (DE = -4.78%, p = 0.02; IE = -2.39, p = 0.02) and placental mtDNA content 5.5% (DE = 5.6%, p = 0.02; IE = -0.32, p = 0.04) of the inverse association between prenatal NO_2 exposure during trimester 1 and infant height at age 6 months (Figure 1A).

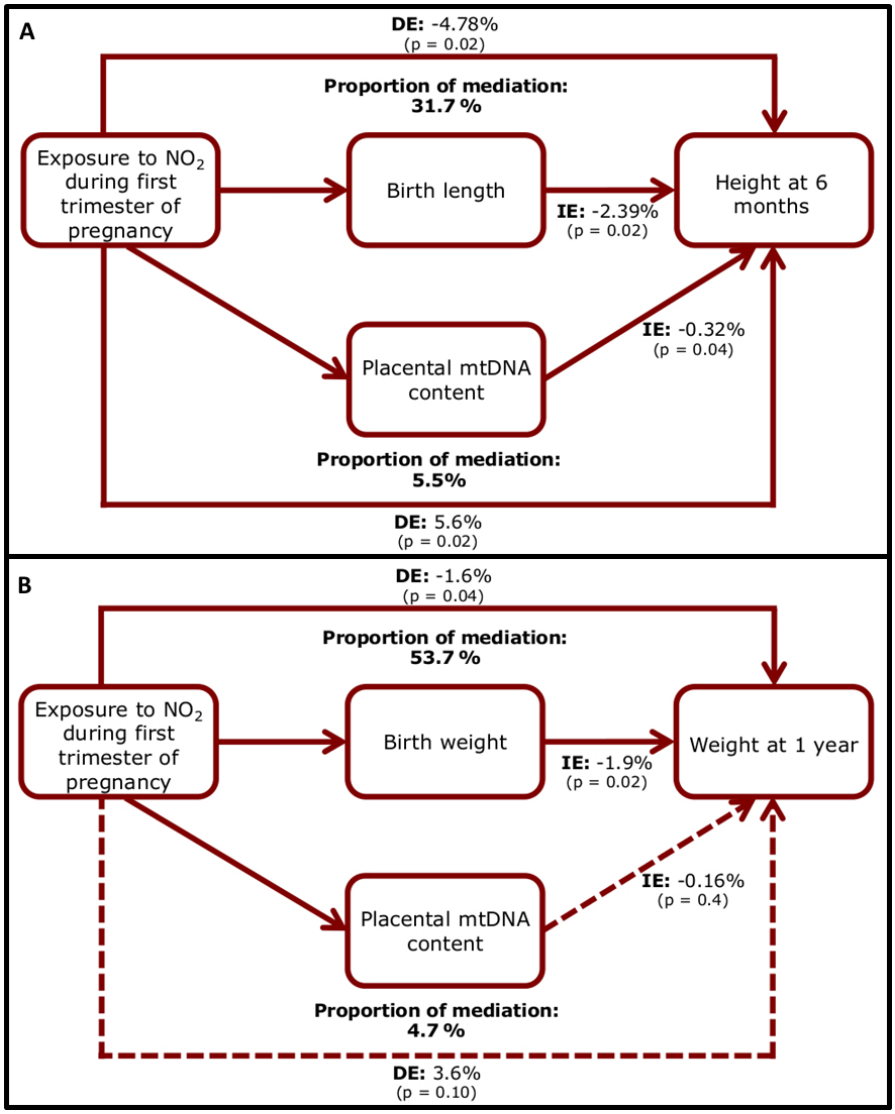

Figure 1. Mediation analyses. This figure shows the estimated proportion of the association between a 10 µg/m³ increment in NO_2 exposure during the first trimester of pregnancy and height at 6 months of age (A), and weight at 1 year of age, mediated through placental mtDNA content. Furthermore, it also includes mediation analysis showing the estimated proportion of the association between NO_2 exposure during the first trimester of pregnancy and both height at 6 months, mediated through birth length (A), and weight at 1 year of age, mediated through birth weight (B). The figure displays the estimates of the indirect effects (IE), the estimates of the direct effect (DE), and the proportion of mediation (IE/DE+IE).

4. Discussion

The present study indicates that prenatal air pollution exposure during early pregnancy results in significant growth deficits in newborns, and shows that these deficits continue to be seen at 6 months and 1 year of age. Furthermore, this study shows that growth at birth could mediate the

effect of prenatal air pollution exposure on postnatal growth. Additionally, we showed that the association between prenatal NO_2 exposure and length at 6 months of age could be mediated by placental mtDNA content.

The fetus and the infant are especially vulnerable to ambient air pollutants due to their differences in exposure, physiological immaturity, and long life expectancy after exposure, compared to adults (Lacasana et al. 2005). The analysis of birth outcomes in our study documented significant inverse associations between prenatal exposure to NO_2 and length of the newborn. Additionally, in a previous study we showed that birth weight was significantly inversely associated with prenatal exposure to NO_2 (Clemente et al. 2016). Several studies have estimated the impact of air pollution on anthropometric parameters at birth such as length, and weight (Glinianaia et al. 2004; Lacasana et al. 2005; Maisonet et al. 2004; Proietti et al. 2013; Sram et al. 2005). An earlier observation in INMA on the participants from the region of Valencia reported a significant decrease in birth length of 0.27 cm (95% CI: -0.51, -0.03) (Iniguez et al. 2012). Our birth outcome results are also consistent with the previous reported findings based on the same regions (Estarlich et al. 2011).

Exposure to air pollution during pregnancy may have long-term implications: Impaired fetal growth is believed to negatively influence infant growth and is also a risk factor for a number of adult chronic diseases such as cardiovascular diseases, and diabetes (Barker 2004). The results of this study show that air pollution exposure during the beginning of pregnancy is significantly negatively associated with height at 6 months of age and weight at 1 year of age. Our observation that prenatal NO_2 exposure during pregnancy appears to affect early postnatal growth in a negative manner is also consistent with two studies on the effect of smoking during pregnancy. A study in Brazil demonstrated that children exposed to maternal smoking during pregnancy showed persistent lower height-for-age from birth to adolescence compared to non-exposed (Muraro et al. 2014). Another study in Turkey showed that infants of mothers that smoked during pregnancy had significant weight and length deficits at birth compared with nonsmokers' infants. Moreover, those infants continued to show significant deficits in height and weight at 6 months of age (Fenercioglu et al. 2009). So far, only one study has focused on the association between prenatal ambient air pollution and infant growth. This study in South Korea reported that prenatal PM_{10} exposure significantly lowered children's weight at 1 year of age (Kim et al. 2016). The results of this study are consistent with our results that showed that prenatal NO_2 exposure is significantly negatively associated with infant weight at 1 year of age. As already mentioned, infant growth is believed to be a continuation of *in utero* growth (Hindmarsh et al. 2008); this present study showed a mediation effect of fetal growth on the association between prenatal NO_2 and infant growth. This indicates that infant growth can be influenced by factors determining intra-uterine growth and nutrition. Nonetheless, a study with long-term follow-up observations is required to enhance the understanding of the association between

prenatal ambient air pollution exposure and child's growth and to determine if intra-uterine effects may translate into variations in growth patterns during childhood.

The biological mechanisms whereby air pollutants might cause adverse growth effects are still unclear. Hypothesis are that oxidative stress and inflammation are important mechanisms in which air pollutants could cause adverse health outcomes (Kannan et al. 2006). Mitochondria are uniquely sensitive to environmental toxicants that induce oxidative stress, such as air pollutants. MtDNA is particularly vulnerable to ROS-induced damage and has been described as a proxy of air pollution-induced damage (Byun and Baccarelli 2014; Hou et al. 2010). MtDNA has a high mutation rate (Linnane et al. 1989) and mitochondria compensate for these mutations by altering their mtDNA content (each mitochondria carries 2-10 copies of mtDNA) (Bouhours-Nouet et al. 2005; Hou et al. 2010). In a subsequent study combining the Belgian ENVIR*ON*AGE birth cohort with the INMA birth cohort, we demonstrated that mtDNA content was one of the potential mediators between the association of prenatal air pollution exposure and birth weight (Clemente et al. 2016). This current study adds information by showing that 5.5% of the association between NO_2 exposure during the first trimesters of pregnancy and length at 6 months of age could be mediated through placental mtDNA content. This may indicate that a decrease in mtDNA content in early life could lead to impaired growth trajectories up to six months of age, which might have health consequences in later life. Furthermore, mitochondrial dysfunction can be caused by a change in mtDNA content and a decrease in mtDNA content has been related to the development of multiple forms of disease as type 2 diabetes (Gianotti et al. 2008; Xia et al. 2015), breast cancer (Yu et al. 2007), and low birth weight (Clemente et al. 2016; Gemma et al. 2006).

Cross-sectional human studies on the association between ambient air pollution exposure and mtDNA content are still limited with inconsistent results. PM air pollution exposure was associated with an increase (Hou et al. 2010), a decrease (Hou et al. 2013; Pieters et al. 2016), and no change in mtDNA content (Xia et al. 2015) in adults and elderly. Additionally, increased mtDNA content in adults has been associated with short-to moderate-term ambient black carbon (BC) levels (Zhong et al. 2016) and benzene exposure (Shen et al. 2008). Studies investigating the effect of ambient air pollution exposure during pregnancy on placental mtDNA content are limited to maternal tobacco smoke exposure (Bouhours-Nouet et al. 2005; Garrabou et al. 2016). Although, recently a significant inverse association between prenatal $PM_{2.5}$ exposure and lower mtDNA content in cord blood was found (Rosa et al. 2017). The previously mentioned discrepancy in the mtDNA content results, can be explained by the very dynamic nature of mtDNA. MtDNA content most importantly depends on oxidative stress level, type of environmental factor, cell antioxidant capacity and dose of exposure, additionally, mtDNA content also fluctuates under the influence of age, ethnicity, and the tissue investigated (Castegna et al. 2015; Shaughnessy et al. 2014). The current hypothesis of this

discrepancy is that mild oxidative stress may stimulate synthesis of mtDNA copy number and abundance as a compensatory mechanism. As a result, oxidative stress levels will increase and may result in decreased or no synthesis of mitochondria due to severe oxidative damage in cells (Lee and Wei 2005). Taken this hypothesis into account, a study in smokers found that the relative mtDNA content was increased in the lung tissues of light smokers but significantly decreased in heavy smokers (Lee et al. 1998).

In the present study we have found that birth length mediates the association between prenatal NO_2 exposure and infant length at 6 months. Additionally, we also showed that mtDNA content mediates the association between prenatal NO_2 exposure and birth length (6.20%; 95%CI: 9.5, 0.4). Consequently, this can mean that the mediating role of placental mtDNA content could be partially independent by mediating the association between prenatal NO_2 exposure and infant length at 6 months of age and partially dependent through its mediating effect of prenatal NO2 exposure on birth length. Furthermore, no significant association could be found between prenatal NO_2 exposure and length at 1 year of age. However, we found that prenatal NO_2 exposure was significantly positively associated with a change in length z-score between 6 months and 1 year of age. This could lead to the hypothesis that the child will undergo a height catch-up during this period which could explain the non-significant effect of prenatal NO_2 exposure on height at 1 year of age observed in this study. According to the literature, the period of life between 6 months and 2 years had been noted as a period of critical height development (Martorell 1995). Height growth rather than fat storage may be stimulated preferentially during this period (McCarthy et al. 2007). This could explain why prenatal NO_2 exposure was associated with length and not with weight at 6 months of age.

We acknowledge several limitations in the present study. Our placental mtDNA content associations should be interpreted cautiously within the context of cellular heterogeneity. All tissues are composed of multiple cell types and the mtDNA content in a tissue is an average of the mtDNA content in all existing cell types. The placenta is composed of a complex population of cells [mesenchymal cells, mesenchymal derived macrophages (Hofbauer cells), fibroblasts, smooth muscle cells, perivascular cells (pericytes), and endothelial cells] (Yuen et al. 2009). There is a possibility that placental mtDNA content data is confounded by variation in cell type distributions and may not reflect true mtDNA content differences, but only differences in cell type composition. To overcome this caveat, it is necessary to characterize and explore the effects of cellular heterogeneity in heterogeneous tissues such as the placenta (Jaffe and Irizarry 2014). In whole blood, it is possible to correct for differences in blood composition using algorithms that estimate cell type proportions (Houseman et al. 2012; Jaffe and Irizarry 2014). Currently, there is no algorithm available to estimate the cell type proportions in placental tissue. However, to minimize the impact of within placental variability, biopsies used for mtDNA content assays were all taken 1-1.5 cm below the chorio-

amniotic membrane at a fixed location. Care was taken by visual examination and dissection to avoid the chorio-amniotic membrane contamination. Histological confirmation of cell type showed no difference in cell type composition between the fetal samples taken at four standardized sites across the middle region of the placenta (approximately 4 cm away from the umbilical cord), nor between the four placentas. Furthermore, although our results were consistent after multiple adjustments, we cannot exclude that our findings were caused by some unknown factor that is associated with prenatal air pollution exposure, placental mtDNA content and infant growth. Thirdly, although we used a recently developed statistical mediation method (Valeri and VanderWeele 2013), this method cannot prove the biological direction (causality); nevertheless, our formal mediation analysis is based on a predefined hypothesis and is in line with experimental evidence. Fourthly, as discussed previously, other studies showed an effect of other air pollutants (such as PM and BC) on mtDNA content. In INMA, NO_2 was the only ambient exposure that was available for all regions during pregnancy and we were not able to account for other exposures. Therefore, confounding due to co-pollutants (PM, BC, and others) may have introduced bias in the present study. Nonetheless, NO_2 is frequently used as a surrogate for traffic related air pollution because it is considered to be a good proxy of other pollutants originating from the same sources (WHO 2005). Finally, we need to consider that the prenatal NO_2 exposure assessment was limited to the residential address of the mothers and did not consider the individual's time based activity patterns; therefore, the measure of NO_2 exposure could be inaccurate for the mothers that stayed outside their living area for a longer time period. However, there was no significant difference in the associations when we restricted our analysis to mother who spend > 15 hr/day at home (data not shown).

In conclusion, this study suggests that prenatal air pollution exposure can lead to impaired infant growth that is determined by intra-uterine growth. Additionally, air pollution induced alterations in placental mtDNA, indicating a biological oxidative stress pathway involving the placenta, might have consequences to growth up to six months of age.

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Supplemental Material

Prenatal ambient air pollution exposure, infant growth and placental mitochondrial DNA content in the INMA birth cohort

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Supplemental Material, Table S1. Comparison of the characteristics of INMA participants that provided placenta samples included in this study (n = 336) with those that were not included in this study (n = 166).

	INMA participants that provided placenta samples included in this study (n = 336)	**INMA participants that provided placenta samples, but were not included in this study (n = 166)**
Characteristics	**Mean ± SD range percentage (%)**	**Mean ± SD range or percentage (%)**
Maternal		
Age, years	32.2 ± 3.9	32.1 ± 4.3
Smoking		
Never	44.6	46.1
Quit smoking before week 12	38.1	33.9
During entire pregnancy	17.3	20.0
Education		
Primary school or none	20.2	21.1
Secondary school	45.8	40.7
University	33.9	38.1
Parity		
1	54.8	54.5
2	38.1	37.2
≥3	7.1	8.3
Pre-pregnancy BMI, kg/m^2	23.5 ± 4.3	23.7 ± 4.2
Newborn		
Gestational age, weeks	39.9 ± 1.4	39.8 ± 1.3
Sex		
Male	50.0	56.2
Female	50.0	43.8
Ethnicity		
European	91.1	91.5
Non-European	8.9	8.5

Continuous covariates expressed by mean and standard deviation (SD) (normally distributed); categorical covariates described by numbers and frequencies (%).

Supplemental Material, Table S2. Comparison of the prenatal NO_2 exposure (µg/m³) during the different exposure windows of pregnancy and the entire pregnancy of INMA participants that provided placenta samples included in this study (n = 336) with those that were not included in this study (n = 166).

	INMA participants that provided placenta samples included in this study (n = 336)						**INMA participants that provided placenta samples, but were not included in this study (n = 166)**					
NO_2 exposure (µg/m^3)	**Mean ± SD**	**P5**	**P25**	**P50**	**P75**	**P95**	**Mean ± SD**	**P5**	**P25**	**P50**	**P75**	**P95**
Trimester 1	27.0 ± 13.0	5.6	16.8	24.8	34.7	74.2	24.6 ± 11.4	9.6	16.5	21.5	32.0	45.7
Trimester 2	26.0 ± 11.9	5.7	16.7	24.7	32.6	74.7	25.2 ± 9.4	11.1	17.7	25.9	30.2	40.2
Trimester 3	26.4 ± 12.5	5.7	17.0	24.0	33.4	74.4	25.7 ± 10.6	11.4	17.9	24.0	29.9	46.5
Entire pregnancy	26.2 ± 11.6	5.7	17.4	24.6	33.3	66.7	25.1 ± 9.4	11.1	18.5	24.4	29.7	41.9

Supplemental Material, Table S3. Association between maternal NO_2 exposure in different exposure periods of pregnancy and infant growth in a multi trimester model

	N	Change (%)	95% CI	P-value
zHeight at 6 months				
NO_2 Trimester 1	286	-7.65	-15.62, 0.31	0.06
NO_2 Trimester 2	286	-2.48	-13.05, 8.1	0.64
NO_2 Trimester 3	286	-4.5	-12.03, 3.03	0.24
zWeight at 6 months				
NO_2 Trimester 1	289	-2.58	-9.19, 4.02	0.44
NO_2 Trimester 2	289	-1.48	-9.78, 6.83	0.73
NO_2 Trimester 3	289	0.54	-5.62, 6.70	0.86
zHeight at 1 year				
NO_2 Trimester 1	286	-0.28	-10.81, 10.25	0.96
NO_2 Trimester 2	286	-1.87	-14.23, 10.48	0.77
NO_2 Trimester 3	286	-1.09	-11.16, 8.98	0.83
zWeight at 1 year				
NO_2 Trimester 1	289	-3.97	-10.88, -2.94	0.26
NO_2 Trimester 2	289	-0.5	-9.19, 8.19	0.91
NO_2 Trimester 3	289	0.67	-5.78, 7.11	0.84

Effect size was estimated for each 10 $\mu g/m^3$ increment in exposure to NO_2 at each mother's residence during the corresponding period;

Models were adjusted for newborn's sex, maternal age, smoking status, gestational age, gestational age^2, maternal pre-pregnancy BMI, parity, ethnicity, season, education, and NO_2 exposure during the other two trimesters.

Supplemental Material, Table S4. Association between prenatal NO_2 in different exposure periods of pregnancy and change in weight/length Z-scores between birth and 6months/ 1 year of age and between 6 months and 1 year of age.

	N	Change (%)	95% CI	P-value
Change in weight z-scores between birth and 6 months of age				
NO_2 Trimester 1	286	0.38	-0.40, 1.59	0.34
NO_2 Trimester 2	286	0.47	-0.39, 1.33	0.28
NO_2 Trimester 3	286	0.24	-0.57, 1.04	0.56
NO_2 Entire pregnancy	286	0.51	-0.42, 1.43	0.28
Change in weight z-scores between birth and 1 year of age				
NO_2 Trimester 1	289	0.13	-0.28, 0.55	0.52
NO_2 Trimester 2	289	0.22	-0.23, 0.68	0.33
NO_2 Trimester 3	289	0.11	-0.31, 0.53	0.61
NO_2 Entire pregnancy	289	0.19	-0.30, -0.68	0.45
Change in weight z-scores between 6 months and 1 year of age				
NO_2 Trimester 1	281	-0.11	-0.45, 0.23	0.52
NO_2 Trimester 2	281	-0.02	-0.39, 0.36	0.92
NO_2 Trimester 3	281	-0.01	-0.37, 0.34	0.93
NO_2 Entire pregnancy	281	-0.13	-0.53, 0.28	0.54
Change in length z-scores between birth and 6 months of age				
NO_2 Trimester 1	286	-0.23	-1.16, 0.71	0.63
NO_2 Trimester 2	286	0.32	-0.73, 1.37	0.55
NO_2 Trimester 3	286	0.19	-0.77, 1.14	0.70
NO_2 Entire pregnancy	286	0.15	-0.95, 1.25	0.79
Change in length z-scores between birth and 1 year of age				
NO_2 Trimester 1	289	0.30	-0.25, 0.95	0.28
NO_2 Trimester 2	289	0.35	-0.25, 0.95	0.25
NO_2 Trimester 3	289	0.14	-0.46, 0.73	0.66
NO_2 Entire pregnancy	289	0.31	-0.35, 0.97	0.36
Change in length z-scores between 6 months and 1 year of age				
NO_2 Trimester 1	281	0.80	0.17, 1.43	0.01
NO_2 Trimester 2	281	0.45	-0.26, 1.16	0.21
NO_2 Trimester 3	281	0.11	-0.59, 0.8	0.76
NO_2 Entire pregnancy	281	0.36	-0.41, 1.13	0.36

Effect size was estimated for each 10 $\mu g/m^3$ increment in exposure to NO_2 at each mother's residence during the corresponding period;

Models were adjusted for newborn's sex, maternal age, smoking status, gestational age, gestational age^2, maternal pre-pregnancy BMI, parity, ethnicity, season, and education;

Supplemental Material, Table S5. Association between prenatal NO_2 in different exposure periods of pregnancy and birth length, birth weight and placental mtDNA content

	Change	95% CI	P-value
Birth length, cm[a]			
NO_2 Trimester 1	-0.27	-0.46, -0.08	<0.01
NO_2 Trimester 2	-0.21	-0.40, -0.01	0.04
NO_2 Trimester 3	-0.21	-0.40, -0.01	0.03
NO_2 Entire pregnancy	-0.27	-0.49, -0.05	0.01
Birth weight, g			
NO_2 Trimester 1	-61.9	-102.2, -21.7	<0.01
NO_2 Trimester 2	-61.2	-103.1, -19.3	<0.01
NO_2 Trimester 3	-58.2	-100.2, -16.1	<0.01
NO_2 Entire pregnancy	-73.4	-120.4, -26.4	<0.01
Placental mtDNA content, %[a]			
NO_2 Trimester 1	-3.5	-6.5, -0.4	0.03
NO_2 Trimester 2	-3.9	-7.0, -0.7	0.02
NO_2 Trimester 3	-3.9	-7.1, -0.7	0.02
NO_2 Entire pregnancy	-4.5	-7.9, -0.9	0.02

Effect size was estimated for each 10 $\mu g/m^3$ increment in exposure to NO_2 at each mother's residence during the corresponding period;

Models were adjusted for newborn's sex, maternal age, smoking status, gestational age, gestational age^2, maternal pre-pregnancy BMI, parity, ethnicity, season, and education;

[a]These results were presented in a previous paper based on the same cohorts (Clemente et al. 2016)