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CLEMENTE BATALHA PARDAL, Diana; Casas, Maribel; JANSSEN, Bram; Lertxundi, Aitana; Santa-Marina, Loreto; Iniguez, Carmen; Llop, Sabrina; Sunyer, Jordi; Guxens, Monica; NAWROT, Tim & Vrijheid, Martine (2017) Prenatal ambient air pollution exposure, infant growth and placental mitochondrial DNA content in the INMA birth cohort. In: ENVIRONMENTAL RESEARCH, 157, p. 96-102.

DOI: 10.1016/j.envres.2017.05.018
Handle: http://hdl.handle.net/1942/24332

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

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**Acknowledgment:** The research leading to these results was funded by the Spanish Ministry of Health (FIS-PI11/00610).

**Abstract**

BACKGROUND: Prenatal air pollution exposure can affect postnatal growth, but this association 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.

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 and 8.3% (95%CI: 12.4, 5.0) of the association between trimester 2 $NO_2$ exposure and length at 6 months could be mediated by placental mtDNA content.

CONCLUSIONS: Our results suggest that impaired fetal growth caused by prenatal air pollution exposure leads 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

## Introduction

In the last decade, numerous studies have reported an association between prenatal ambient air pollution exposure and adverse birth outcomes, such as low birth weight, intra-uterine growth retardation, and preterm birth, even at low levels of air pollution (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 ambient air pollution 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 air pollutants 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). Variations in growth patterns after birth may be important determinants 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 physiological or environmental conditions (Clay Montier 2009). 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.

## Methods

### *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 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. The present analysis included 336 mother-newborn pairs from whom placentas were collected and placental mtDNA content measured.

Study approval was obtained from the ethics committees of each participating center and informed consents were obtained from the mothers.

### *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.

***Placental mtDNA content***

As previously described (Clemente *et al.* 2016), a total of 502 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). MtDNA content was measured in 336 out of the 502 placentas. 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 in triplicate. 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).

***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, 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 6 months and 1 year of age were calculated using the World Health Organization (WHO) referent (de Onis *et al.* 2009).

***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 1st and 3rd trimester of pregnancy. Child sex and date of birth was obtained from clinical records.

***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. 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.

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.

**Results**

***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– 75$^{th}$ 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 (25$^{th}$-75$^{th}$ 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)

| $NO_2$ exposure (µg/m³) | Mean ± SD | P5 | P25 | P50 | P75 | P95 | Correlation[a] 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

### *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 (-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.

**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 |
|---|---|---|---|---|
| **zLength 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 |
| **zLength 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/m³ 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;

### *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.

**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 |
| **zLength 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 |
| **zLength 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)

### *Mediation analyses*

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 S1). Mediation analysis showed that birth length mediated 31.7% (95%CI: 14.3, 34.5) and placental mtDNA content 5.5% (95%CI: 0.2, 10.0) of the inverse association between prenatal $NO_2$ exposure during trimester 1 and infant length at age 6 months (Figure 1A). Additionally, 8.3% (95%CI: 5.0, 12.4) of the association between prenatal $NO_2$ exposure during trimester 2 and infant length at 6 months could be explained via placental mtDNA content. Furthermore, the mediation analysis showed that birth weight mediated 53.7% (95%CI: -0.3, 65.3) of the negative association between prenatal $NO_2$ exposure during trimester 1 and infant weight at 1 year of age (Figure 1B). Since mtDNA content was not associated with infant weight at 1 year we did not perform mediation analysis for this association.

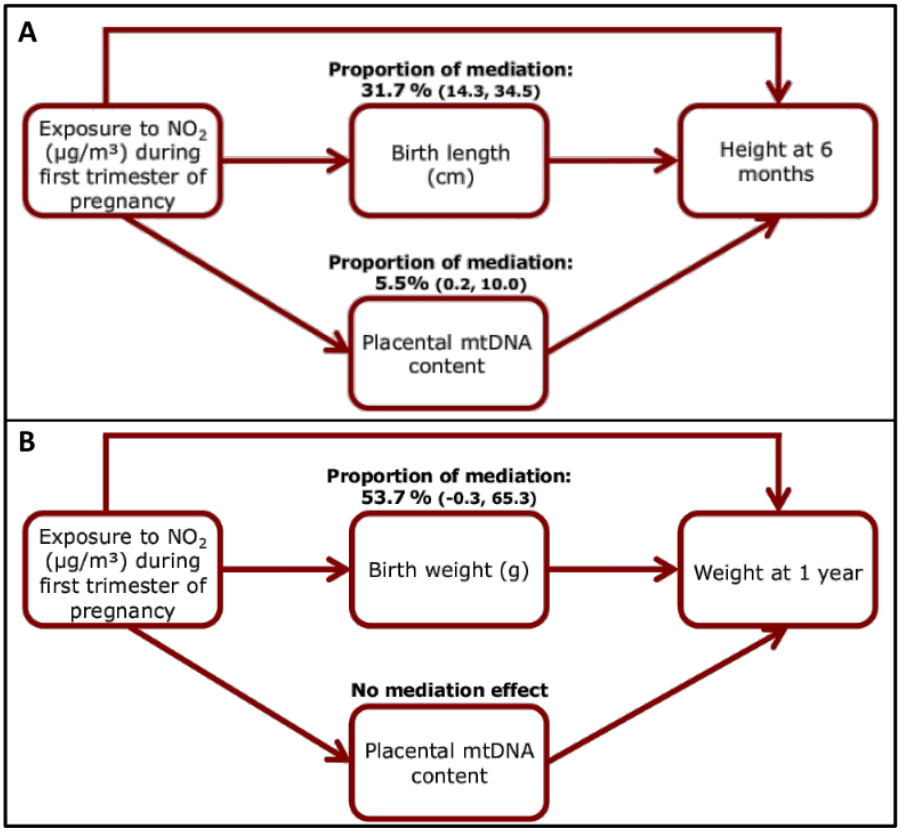


**Figure 1. Mediation analyses.** This figure shows the estimated proportion of the association between a 10 µg/m³ increment in $NO_2$ exposure during the first trimester of pregnancy and height at 6 months of age (A), and weight at 1 year of age, mediated through placental mtDNA content. Furthermore, 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 trough birth length (A), and weight at 1 year of age, mediated through birth weight (B).

## 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 mediated 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. 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

311 rate (Lamson and Plaza 2002); mitochondria compensate for these mutations by altering the mtDNA
312 content (each mitochondria carries 2-10 copies of mtDNA) (Bouhours-Nouet *et al.* 2005). In a
313 subsequent study combining the Belgian ENVIR*ON*AGE birth cohort with the INMA birth cohort, we
314 demonstrated that mtDNA content was one of the potential mediators between the association of
315 prenatal air pollution exposure and birth weight (Clemente *et al.* 2016). This current study adds
316 information by showing that 5.5% of the association between $NO_2$ exposure during the first
317 trimesters of pregnancy and length at 6 months of age and 8.3% of the association between
318 trimester 2 $NO_2$ exposure and length at 6 months of age could be mediated through placental mtDNA
319 content. This may indicate that a decrease in mtDNA content in early life could lead to impaired
320 growth trajectories up to six months of age.
321 We acknowledge several limitations in the present study. Although our results were
322 consistent after multiple adjustments, we cannot exclude that our findings were caused by some
323 unknown factor that is associated with prenatal air pollution exposure, placental mtDNA content and
324 infant growth. Secondly, although we used a recently developed statistical mediation method (Valeri
325 and VanderWeele 2013), this method cannot prove the biological direction (causality); nevertheless,
326 our formal mediation analysis is based on a predefined hypothesis and is in line with experimental
327 evidence. Thirdly, we acknowledge the fact that variability within the placenta exists; therefore, to
328 minimize variation of placental mtDNA content attributed to differences across placental regions, we
329 took biopsies at a fixed location. Finally, we need to consider that the prenatal $NO_2$ exposure
330 assessment was limited to the residential address of the mothers and did not consider the
331 individual's time based activity patterns; therefore, the measure of $NO_2$ exposure could be inaccurate
332 for the mothers that stayed outside their living area for a longer time period. However, there was no
333 significant difference in the associations when we restricted our analysis to mother who spend > 15
334 hr/day at home (data not shown).
335 In conclusion, this study suggests that prenatal air pollution exposure can lead to an impaired
336 infant growth that is determined by intra-uterine growth. Additionally, air pollution induced
337 alterations in placental mtDNA, indicating a biological oxidative stress pathway involving the
338 placenta, might have consequences to growth trajectories up to six months of age.
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