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Peer-reviewed author version

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>

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

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20

21

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

24 **Abstract**

25 BACKGROUND: Prenatal air pollution exposure can affect postnatal growth, but this association has  
26 hardly been explored. Mitochondrial DNA (mtDNA), as a marker of oxidative stress, and growth at  
27 birth can play an intermediate role in this association.

28 OBJECTIVE: In a subset of the Spanish birth cohort INMA we assessed first whether prenatal nitrogen  
29 dioxide (NO<sub>2</sub>) exposure is associated with infant growth. Secondly, we evaluated whether growth at  
30 birth (length and weight) could play a mediating role in this association. Finally, the mediation role of  
31 placental mitochondrial DNA content in this association was assessed.

32 METHODS: In 336 INMA children, relative placental mtDNA content was measured. Land-use  
33 regression models were used to estimate prenatal NO<sub>2</sub> exposure. Infant growth (height and weight)  
34 was assessed at birth, at 6 months of age, and at 1 year of age. We used multiple linear regression  
35 models and performed mediation analyses.

36 RESULTS: Prenatal NO<sub>2</sub> exposure was inversely associated with all infant growth parameters. A 10  
37 µg/m<sup>3</sup> increment in prenatal NO<sub>2</sub> exposure during trimester 1 of pregnancy was significantly  
38 inversely associated with height at 6 months of age (-6.6%; 95%CI: -11.4, -1.9) and weight at 1 year of  
39 age (-4.2%; 95%CI: -8.3, -0.1). These associations were mediated by birth length (31.7%; 95%CI: 34.5,  
40 14.3) and weight (53.7%; 95%CI: 65.3, -0.3), respectively. Furthermore, 5.5% (95%CI: 10.0, -0.2) of  
41 the association between trimester 1 NO<sub>2</sub> exposure and length at 6 months of age and 8.3% (95%CI:  
42 12.4, 5.0) of the association between trimester 2 NO<sub>2</sub> exposure and length at 6 months could be  
43 mediated by placental mtDNA content.

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

48

49

50

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

53 **Introduction**

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

68 The placenta is a metabolically active organ that connects and separates two genetically  
69 distinct individuals: the mother and the fetus. It plays an essential role in nutrient transfer, growth  
70 and organ development. The placenta is a unique vascular organ that requires a constant source of  
71 energy. This energy provision is regulated by mitochondrial function of placental cells (Myllynen *et al.*  
72 2005). Mitochondria, the energy producers of the cells, are the major intracellular sources of reactive  
73 oxygen species (ROS), which are generated under normal conditions as by-product of oxidative  
74 phosphorylation. Mitochondria are the primary targets of oxidative stress because mitochondrial  
75 DNA (mtDNA) lacks protective strategies associated with nuclear DNA. Consequently, mitochondria  
76 are uniquely sensitive to environmental toxicants (Lee and Wei 2000). Furthermore, mtDNA content  
77 is correlated with the size and number of mitochondria, which have been shown to change under  
78 different energy demands, as well as different physiological or environmental conditions (Clay  
79 Montier 2009). Fetus adapt their mitochondrial structure and metabolism when the supply of  
80 nutrients is limited (Gemma *et al.* 2006). Changes in placental mtDNA content may represent a  
81 biological effect along the path linking air pollution to effects on the infant.

82 Recently, it was shown that placental mtDNA content was influenced by prenatal particulate  
83 matter  $<10\mu\text{m}$  (PM<sub>10</sub>) and nitrogen dioxide (NO<sub>2</sub>) exposure (Clemente *et al.* 2016; Janssen *et al.*  
84 2012). Furthermore, in our previous study we showed that placental mtDNA content was significantly  
85 associated with birth weight and that it could be one of the mediators of the inverse association  
86 between prenatal NO<sub>2</sub> exposure and birth weight (Clemente *et al.* 2016). These findings raise the

87 question of whether prenatal air pollution exposure may also result in subsequent changes in infant  
88 growth and whether placental mtDNA alterations can be linked to these outcomes in later life.

89 In the current study we therefore evaluated firstly whether prenatal NO<sub>2</sub> exposure is associated with  
90 infant growth (height and weight) at 6 months and 1 year of age. Secondly, we evaluated whether  
91 growth deficits at birth (length and weight) play a mediating role in this association. Finally, the  
92 mediating role of placental mtDNA content in the association between prenatal air pollution  
93 exposure and infant growth was assessed.

94

## 95 **Methods**

### 96 ***Study design and population***

97 INMA (INfancia y Medio Ambiente; Environment and Childhood) is a birth cohort study that recruited  
98 pregnant women in seven regions, following a common protocol (Guxens *et al.* 2012). In this study  
99 we used participants with singleton live-born infants from three INMA regions (Valencia, Sabadell  
100 and Gipuzkoa). Pregnant women were enrolled between 2004 and 2008 during the first trimester of  
101 pregnancy at primary health care centers public hospitals if they fulfilled the inclusion criteria:  
102 singleton pregnancy, intention to deliver at the reference hospital, ≥ 16 years of age, no problems of  
103 communication, and no assisted conception. Of all eligible women, 57% agreed to participate. The  
104 present analysis included 336 mother-newborn pairs from whom placentas were collected and  
105 placental mtDNA content measured.

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

108

### 109 ***Ambient air pollution assessment***

110 Ambient concentrations of nitrogen dioxide (NO<sub>2</sub>) were measured with the aid of passive samplers  
111 (Radiello, Fondazione Salvatore Maugeri, Padua, Italy) installed in several sampling campaigns each  
112 lasting seven days and distributed across the study areas according to geographic criteria, taking into  
113 account the expected pollution gradients and the distribution of the residences of the participating  
114 women.

115 The methodology has been described in detail elsewhere (Aguilera *et al.* 2008; Iniguez *et al.*  
116 2009). Briefly, area-specific land use regression (LUR) models were used to predict NO<sub>2</sub> levels at  
117 women's residential addresses, using the average of the NO<sub>2</sub> levels registered across campaigns to  
118 represent an annual mean level, together with land use (agricultural, industrial or urban), traffic-  
119 related variables, and altitude. Residential NO<sub>2</sub> estimations from LUR were then adjusted to time of  
120 pregnancy for each woman, using daily records from the monitoring network stations covering the

121 study area. This model also took into account residential changes if women lived at least 2 months of  
122 pregnancy in the new residence. The validation statistics gave a spatial explained variance ( $R^2$ ) for  
123 annual mean  $\text{NO}_2$  from 0.51 to 0.75 in the three INMA regions (Aguilera *et al.* 2008).

124 In order to explore potentially critical exposures during pregnancy, individual  $\text{NO}_2$   
125 concentrations were calculated for different periods of pregnancy: trimester 1 (1-13 weeks),  
126 trimester 2 (14-28 weeks), trimester 3 (29 weeks to delivery), and for the entire pregnancy.

127

### 128 **Placental mtDNA content**

129 As previously described (Clemente *et al.* 2016), a total of 502 placentas were entirely frozen after  
130 delivery at  $-20^\circ\text{C}$  and afterwards at  $-86^\circ\text{C}$ . Placentas were thawed minimally to obtain tissue biopsies  
131 for DNA extractions. To minimize the impact of within-placental variability, biopsies were all taken 1-  
132 1.5 cm below the chorio amniotic membrane at a fixed location and preserved at  $-80^\circ\text{C}$  (Janssen *et al.*  
133 2012). MtDNA content was measured in 336 out of the 502 placentas. Briefly, DNA was extracted  
134 from placental tissue cells and quantified. MtDNA content was measured in placental tissue cells by  
135 determining the ratio of two mitochondrial gene copy numbers (mitochondrial encoded NADH  
136 dehydrogenase subunit 1 (*MT-ND1*) and mitochondrial forward primer for nucleotide 3212 and  
137 reverse primer from nucleotide 3319 (*MTF3212/R3319*)) to two single-copy nuclear control genes  
138 (acidic ribosomal phosphoprotein P0 (*RPLP0*), and beta-actin (*ACTB*)) using the 7900HT Fast Real-  
139 Time PCR System (Life Technologies, Foster City, CA, United States) (Janssen *et al.* 2012). Samples  
140 were run in triplicate. qBase software (Biogazelle, Zwijnaarde, Belgium) automatically averaged  
141 triplicate measurements that pass quality control and normalizes the data to nuclear reference genes  
142 while correcting for run-to-run differences (Hellemans *et al.* 2007).

143

### 144 **Infant growth**

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

147 Repeated height and weight measures from birth to 6 months of age were extracted from  
148 medical records. For infants without weight measures available within  $\pm 14$  days of their exact 6-  
149 month anniversary, we used the 2<sup>nd</sup>-order Reed sex-specific early infancy growth models to predict  
150 the weight of children as described previously (Valvi *et al.* 2013). Child height and weight were  
151 measured at 1 year of age using standard protocols, with light clothing and without shoes. Age- and  
152 sex-specific z-scores for height and weight at 6 months and 1 year of age were calculated using the  
153 World Health Organization (WHO) referent (de Onis *et al.* 2009).

154

### 155 **Covariates**

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

159

### 160 **Statistical analysis**

161 Continuous data were checked for normality using the Shapiro-Wilk test statistic. Continuous data  
162 were presented as mean  $\pm$  SD and categorical data as frequencies and percentages. Average  
163 placental mtDNA was log10-transformed to improve the normality of the distributions and described  
164 by geometric mean and 25th-75th percentile. Multiple linear regression models were used to assess  
165 the association between (i) prenatal NO<sub>2</sub> exposure and infant growth (height and weight at 6 months  
166 and at 1 year of age) and between (ii) placental mtDNA content and infant growth.

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

174 Several mediation analyses were performed. Firstly, we investigated if birth length mediated  
175 the association between prenatal NO<sub>2</sub> exposure and length in the infants at 6 months and 1 year of  
176 age. Secondly, we assessed whether birth weight mediated the association between prenatal NO<sub>2</sub>  
177 exposure and weight in the infants at 6 months and 1 year of age. Thirdly, we investigated whether  
178 placental mtDNA content was a mediator of the association between prenatal NO<sub>2</sub> exposure and the  
179 different infant growth characteristics (height and weight at 6 months and 1 year of age). We only  
180 performed the mediation analysis when there was a significant association between the outcome  
181 and the exposure, a significant association between the exposure and the mediator, and a significant  
182 association between the outcome and the mediator. To perform these mediation analyses we used  
183 the SAS macro developed by Valeri and VanderWeele (Valeri and VanderWeele 2013). In this macro,  
184 the direct effect (DE), indirect effect (IE) and total effect (TE) were determined. The DE represents  
185 the effect of exposure on the outcome after controlling for the mediator whereas the IE is the effect  
186 of exposure operating through the mediator. The proportion of mediation was calculated as the ratio  
187 of IE to TE.

188

189

### 190 **Results**

191 **Characteristics and exposure levels of the study population**

192 Table 1 summarizes the characteristics of the 336 mother-newborn pairs. Briefly, mean maternal age  
 193 was 32.2 years. Pre-gestational BMI of the participating mothers averaged 23.5 kg/m<sup>2</sup> and 44.6% of  
 194 the mothers never smoked cigarettes. The newborns, 168 of which were girls (50.0%), had a mean  
 195 gestational age of 39.9 weeks. More than 90% of the newborns were European.

196

197 **Table 1.** Study population characteristics (n=336)

Characteristics	Mean ± SD range or number (%)
<b>Maternal</b>	
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 <sup>2</sup>	23.5 ± 4.3
Region	
Gipuzkoa	154 (45.8)
Sabadell	121 (36.0)
Valencia	61 (18.2)
<b>Newborn</b>	
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)

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

201

202 Table 2 displays the daily outdoor NO<sub>2</sub> exposure levels averaged for the different exposure  
 203 periods. Average (25<sup>th</sup>-75<sup>th</sup> percentile) period-specific NO<sub>2</sub> exposure was 27.0 (16.8-34.7) µg/m<sup>3</sup> for



204 trimester 1, 26.0 (16.7-32.6)  $\mu\text{g}/\text{m}^3$  for trimester 2, 26.4 (17.0-33.4)  $\mu\text{g}/\text{m}^3$  for trimester 3, and 26.2  
 205 (17.4-33.3)  $\mu\text{g}/\text{m}^3$  for the entire pregnancy.

206

207 **Table 2.** Descriptive statistics of prenatal NO<sub>2</sub> exposure ( $\mu\text{g}/\text{m}^3$ ) in the INMA study (N=336)

NO <sub>2</sub> exposure ( $\mu\text{g}/\text{m}^3$ )	Mean $\pm$ SD	P5	P25	P50	P75	P95	Correlation <sup>a</sup>			
							Trimester 1	Trimester 2	Trimester 3	Entire pregnancy
<b>Trimester 1</b>	27.0 $\pm$ 13.0	5.6	16.8	24.8	34.7	74.2	1			
<b>Trimester 2</b>	26.0 $\pm$ 11.9	5.7	16.7	24.7	32.6	74.7	0.85*	1		
<b>Trimester 3</b>	26.4 $\pm$ 12.5	5.7	17.0	24.0	33.4	74.4	0.78*	0.85*	1	
<b>Entire pregnancy</b>	26.2 $\pm$ 11.6	5.7	17.4	24.6	33.3	66.7	0.91*	0.92*	0.94*	1

<sup>a</sup>Spearman correlation coefficients between different exposure periods

\*P-value <0.0001

208

209 **Association between prenatal NO<sub>2</sub> exposure and infant growth**

210 Table 3 displays the percent change in z-scores for height and weight at 6 months and at 1 year of  
 211 age for every 10  $\mu\text{g}/\text{m}^3$  increment in NO<sub>2</sub> exposure during the different exposure windows of  
 212 pregnancy. A 10  $\mu\text{g}/\text{m}^3$  increment in prenatal NO<sub>2</sub> exposure was inversely and significantly  
 213 associated with height at 6 months, especially during trimester (-6.64%; 95%CI: -11.38, -1.90) and  
 214 trimester 2 (-5.56%; 95%CI: -10.86, -0.26). Furthermore, each 10 $\mu\text{g}/\text{m}^3$  increment in NO<sub>2</sub> levels  
 215 during trimester 1 of pregnancy was inversely and significantly associated with weight at 1 year (-  
 216 4.21%; 95%CI: -8.34, -0.09). Prenatal NO<sub>2</sub> exposure was negatively but not significantly associated  
 217 with weight at 6 months, and height at 1 year of age.

218

219 **Table 3.** Association between maternal NO<sub>2</sub> exposure in different exposure periods of pregnancy and infant  
 220 growth in the INMA study

	N	Change (%)	95% CI	P-value
<b>zLength at 6 months</b>				
NO <sub>2</sub> Trimester 1	286	-6.64	-11.38, -1.90	<0.01
NO <sub>2</sub> Trimester 2	286	-5.56	-10.86, -0.26	0.04
NO <sub>2</sub> Trimester 3	286	-2.22	-7.13, 2.69	0.37
NO <sub>2</sub> Entire pregnancy	286	-5.20	-10.8, 0.41	0.07
<b>zWeight at 6 months</b>				
NO <sub>2</sub> Trimester 1	289	-3.32	-7.32, 0.67	0.10
NO <sub>2</sub> Trimester 2	289	-3.26	-7.6, 1.07	0.14
NO <sub>2</sub> Trimester 3	289	-2.16	-6.23, 1.92	0.30
NO <sub>2</sub> Entire pregnancy	289	-2.94	-7.62, 1.75	0.22
<b>zLength at 1 year</b>				
NO <sub>2</sub> Trimester 1	286	-3.70	-9.58, 2.19	0.22
NO <sub>2</sub> Trimester 2	286	-3.53	-9.86, 2.54	0.24
NO <sub>2</sub> Trimester 3	286	-3.71	-9.96, 2.54	0.24
NO <sub>2</sub> Entire pregnancy	286	-4.51	-11.42, 5.97	0.20
<b>zWeight at 1 year</b>				
NO <sub>2</sub> Trimester 1	289	-4.21	-8.34, -0.09	0.04
NO <sub>2</sub> Trimester 2	289	-3.45	-7.99, 1.08	0.13
NO <sub>2</sub> Trimester 3	289	-2.47	-6.71, 1.77	0.25
NO <sub>2</sub> Entire pregnancy	289	-3.97	-8.84, 0.89	0.11

221 Effect size was estimated for each 10  $\mu\text{g}/\text{m}^3$  increment in exposure to NO<sub>2</sub> at each mother's residence during the  
 222 corresponding period;

223 Models were adjusted for newborn's sex, maternal age, smoking status, gestational age, gestational age<sup>2</sup>,  
 224 maternal pre-pregnancy BMI, parity, ethnicity, season, and education;

225

226 **Association between placental mtDNA content and infant growth**

227 Table 4 shows that placental mtDNA content was significantly and positively associated with length  
228 at birth (0.29g; 95%CI: 0.04, 0.55). Furthermore, placental mtDNA content was significantly and  
229 positively associated with height at 6 months (5.90%; 95%CI: 0.60, 13.24) (Table 4). Placental mtDNA  
230 content was positively but not significantly associated with weight at 6 months, and with weight and  
231 height at 1 year of age.

232

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

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

234 Effect size was estimated for each IQR (0.18) increase in placental mtDNA content;  
235 Models were adjusted for newborn’s sex, maternal age, smoking status, gestational age, gestational age<sup>2</sup>,  
236 maternal pre-pregnancy BMI, parity, ethnicity, season, and education;  
237 <sup>a</sup>These results were presented in a previous paper based on the same cohorts (Clemente *et al.* 2016)

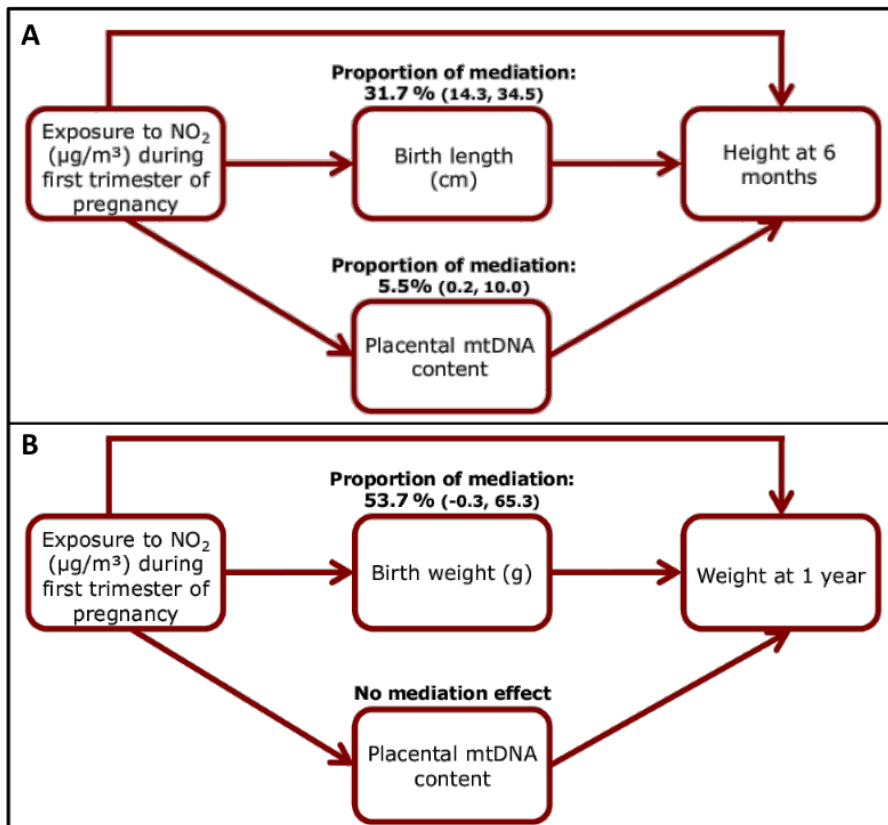
238

239 **Mediation analyses**

240 We tested whether birth outcomes (birth length and weight) and mtDNA content could be  
241 mediators of the association between prenatal NO<sub>2</sub> exposure and infant growth. Prenatal NO<sub>2</sub>  
242 exposure during all the three trimesters of pregnancy and during the entire pregnancy was  
243 associated with birth weight, birth length, and mtDNA content (previous study (Clemente *et al.* 2016)  
244 except for birth length and Supplemental Material, Table S1). Mediation analysis showed that birth  
245 length mediated 31.7% (95%CI: 14.3, 34.5) and placental mtDNA content 5.5% (95%CI: 0.2, 10.0) of  
246 the inverse association between prenatal NO<sub>2</sub> exposure during trimester 1 and infant length at age 6  
247 months (Figure 1A). Additionally, 8.3% (95%CI: 5.0, 12.4) of the association between prenatal NO<sub>2</sub>  
248 exposure during trimester 2 and infant length at 6 months could be explained via placental mtDNA  
249 content. Furthermore, the mediation analysis showed that birth weight mediated 53.7% (95%CI: -0.3,  
250 65.3) of the negative association between prenatal NO<sub>2</sub> exposure during trimester 1 and infant  
251 weight at 1 year of age (Figure 1B). Since mtDNA content was not associated with infant weight at 1  
252 year we did not perform mediation analysis for this association.

253

254



255

256 **Figure 1. Mediation analyses.** This figure shows the estimated proportion of the association between  
 257 a 10 μg/m<sup>3</sup> increment in NO<sub>2</sub> exposure during the first trimester of pregnancy and height at 6 months  
 258 of age (A), and weight at 1 year of age, mediated through placental mtDNA content. Furthermore, it  
 259 also includes mediation analysis showing the estimated proportion of the association between NO<sub>2</sub>  
 260 exposure during the first trimester of pregnancy and both height at 6 months, mediated through birth  
 261 length (A), and weight at 1 year of age, mediated through birth weight (B).

262

263

## 264 Discussion

265 The present study indicates that prenatal air pollution exposure during early pregnancy  
 266 results in significant growth deficits in newborns, and shows that these deficits continue to be seen  
 267 at 6 months and 1 year of age. Furthermore, this study shows that growth at birth mediated the  
 268 effect of prenatal air pollution exposure on postnatal growth. Additionally, we showed that the  
 269 association between prenatal NO<sub>2</sub> exposure and length at 6 months of age could be mediated by  
 270 placental mtDNA content.

271 The fetus and the infant are especially vulnerable to ambient air pollutants due to their  
 272 differences in exposure, physiological immaturity, and long life expectancy after exposure, compared  
 273 to adults (Lacasana *et al.* 2005). The analysis of birth outcomes in our study documented significant  
 274 inverse associations between prenatal exposure to NO<sub>2</sub> and length of the newborn. Additionally, in a  
 275 previous study we showed that birth weight was significantly inversely associated with prenatal

276 exposure to NO<sub>2</sub> (Clemente *et al.* 2016). Several studies have estimated the impact of air pollution on  
277 anthropometric parameters at birth such as length, and weight (Glinianaia *et al.* 2004; Lacasana *et al.*  
278 2005; Maisonet *et al.* 2004; Proietti *et al.* 2013; Sram *et al.* 2005). An earlier observation in INMA on  
279 the participants from the region of Valencia reported a significant decrease in birth length of 0.27 cm  
280 (95% CI: -0.51, -0.03) (Iniguez *et al.* 2012). Our birth outcome results are also consistent with the  
281 previous reported findings based on the same regions (Estarlich *et al.* 2011).

282 Exposure to air pollution during pregnancy may have long-term implications: Impaired fetal  
283 growth is believed to negatively influence infant growth and is also a risk factor for a number of adult  
284 chronic diseases such as cardiovascular diseases, and diabetes (Barker 2004). The results of this study  
285 show that air pollution exposure during the beginning of pregnancy is significantly negatively  
286 associated with height at 6 months of age and weight at 1 year of age. Our observation that prenatal  
287 NO<sub>2</sub> exposure during pregnancy appears to affect early postnatal growth in a negative manner is also  
288 consistent with two studies on the effect of smoking during pregnancy. A study in Brazil  
289 demonstrated that children exposed to maternal smoking during pregnancy showed persistent lower  
290 height-for-age from birth to adolescence compared to non-exposed (Muraro *et al.* 2014). Another  
291 study in Turkey showed that infants of mothers that smoked during pregnancy had significant weight  
292 and length deficits at birth compared with nonsmokers' infants. Moreover, those infants continued  
293 to show significant deficits in height and weight at 6 months of age. So far, only one study has  
294 focused on the association between prenatal ambient air pollution and infant growth. This study in  
295 South Korea reported that prenatal PM<sub>10</sub> exposure significantly lowered children's weight at 1 year of  
296 age (Kim *et al.* 2016). The results of this study are consistent with our results that showed that  
297 prenatal NO<sub>2</sub> exposure is significantly negatively associated with infant weight at 1 year of age. As  
298 already mentioned, infant growth is believed to be a continuation of *in utero* growth (Hindmarsh *et*  
299 *al.* 2008); this present study showed a mediation effect of fetal growth on the association between  
300 prenatal NO<sub>2</sub> and infant growth. This indicates that infant growth can be influenced by factors  
301 determining intra-uterine growth and nutrition. Nonetheless, a study with long-term follow-up  
302 observations is required to enhance the understanding of the association between prenatal ambient  
303 air pollution exposure and child's growth and to determine if intra-uterine effects may translate into  
304 variations in growth patterns during childhood.

305 The biological mechanisms whereby air pollutants might cause adverse growth effects are  
306 still unclear. Hypothesis are that oxidative stress and inflammation are important mechanisms in  
307 which air pollutants could cause adverse health outcomes (Kannan *et al.* 2006). Mitochondria are  
308 uniquely sensitive to environmental toxicants that induce oxidative stress, such as air pollutants.  
309 MtDNA is particularly vulnerable to ROS-induced damage and has been described as a proxy of air  
310 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 ENVIRONAGE 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<sub>2</sub> 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<sub>2</sub> 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<sub>2</sub> 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<sub>2</sub> 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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340 **References**

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