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2 **Pregnancy exposure to atmospheric pollution and**  
3 **meteorological conditions and placental DNA methylation**

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44

45 **ABSTRACT**

46 **Background:** *In utero* air pollution exposure represents a major health threat to the  
47 developing foetus. DNA methylation is one of the most well-known molecular determinants  
48 of the epigenetic status of cells. Blood DNA methylation has been proven sensitive to air  
49 pollutants, but the molecular impact on new-borns has so far received little attention.

50 **Objectives:** We investigated whether nitrogen dioxide (NO<sub>2</sub>), particulate matter (PM<sub>10</sub>),  
51 temperature and humidity during pregnancy are associated with differences in genome-wide  
52 placental DNA methylation levels.

53 **Methods:** Whole-genome DNA-methylation was measured using the Illumina's Infinium  
54 HumanMethylation450 BeadChip in the placenta of in 668 individuals enrolled in the EDEN  
55 cohort. We designed an original strategy using *a priori* biological information to focus on  
56 candidate genes with a specific expression pattern in placenta (active or silent) combined with  
57 an agnostic epigenome-wide association study (EWAS). We used robust linear regression to  
58 identify CpGs and differentially methylated regions (DMR) associated with each exposure  
59 during short- and long-term time-windows.

60 **Results:** The candidate genes approach identified nine CpGs mapped to 8 genes associated  
61 with prenatal NO<sub>2</sub>, PM<sub>10</sub> and humidity exposure [false discovery rate (FDR) p<0.05]. Among  
62 these, the methylation level of 2 CpGs located on *ADORA2B* remained significantly  
63 associated with NO<sub>2</sub> exposure during the 2<sup>nd</sup> trimester exposure in the EWAS (FDR p<0.05).  
64 EWAS further revealed associations between exposure and variations of DNA methylation of  
65 4 other CpGs. We further identified 27 DMRs significantly (FDR p<0.05) associated with air  
66 pollutants exposure and 13 DMRs with weather conditions exposure.

67 **Conclusions:** These findings demonstrate that air pollutants exposure at levels commonly  
68 experienced in the European population are associated with placental gene methylation and  
69 provide some mechanistic insight into some of the reported effect of air pollutants on pre-

70 eclampsia. Indeed, the methylation of *ADORA2B*, a gene whose expression was previously  
71 associated with hypoxia and pre-eclampsia, was consistently found here sensitive to  
72 atmospheric pollutants. In addition, a number of air pollutants associated DMRs pointed to  
73 genes previously implicated in preeclampsia and metabolic syndrome.

74 **Key words:** mother-child cohort, placenta, air pollution, epigenetics, temperature, humidity

75

## 76 **1. Introduction**

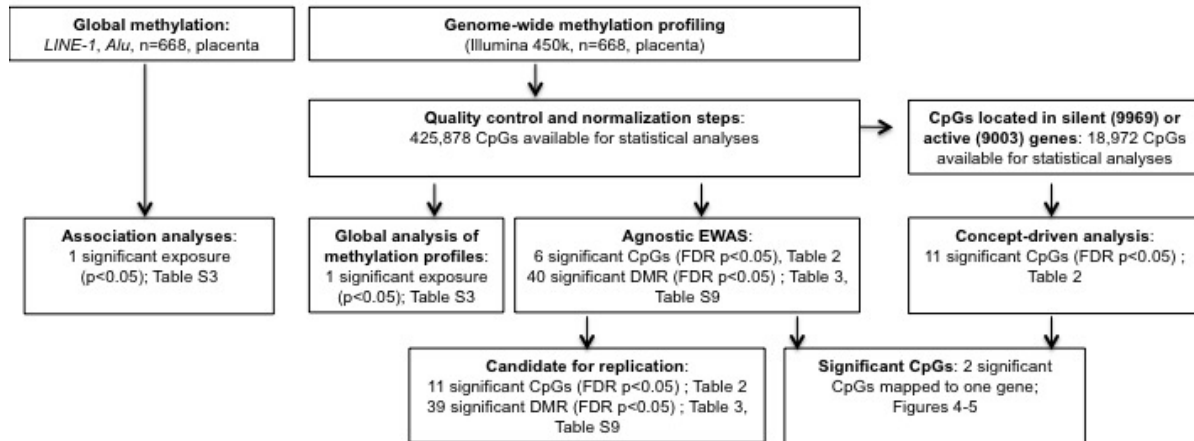
77 Despite significant improvements in air quality in past decades, 50% of the population in  
78 2014 in Europe live in areas that do not meet the World Health Organisation guidelines  
79 (World Health Organization, 2006) for particulate matter less than 10 microns ( $PM_{10}$ ), 85%  
80 for particulate matter less than 2.5 microns ( $PM_{2.5}$ ), and 7% for nitrogen dioxide ( $NO_2$ ) (Ortiz,  
81 2017). Ambient air pollution includes gaseous pollutants, such as nitrogen oxides, sulphur  
82 dioxide, ozone, benzene, as well as particulate matter of various sizes, which are a mixture of  
83 solid and liquid droplets including black carbon, metals, and polycyclic aromatic  
84 hydrocarbons. Air pollutants exposure during pregnancy is a major health threat to children as  
85 it can cross the placenta and expose the developing foetus (Valentino et al., 2015; Wick et al.,  
86 2010). Exposure to air pollutants during pregnancy has been associated to a range of adverse  
87 health outcomes both in the short-term, including low birth weight and preterm birth and in  
88 the long term, including infant reduced lung function and neurodevelopmental disorders (Chiu  
89 et al., 2016; Clifford et al., 2016; Jedrychowski et al., 2010; Stieb et al., 2012). Air pollution  
90 pregnancy exposure is also a threat to the pregnant woman and is likely a risk factor of  
91 preeclampsia (M. Pedersen et al., 2014). More recently, high- or low-ambient temperatures  
92 have also been suggested to play a role in adverse pregnancy outcomes (Beltran et al., 2014;  
93 Giorgis-Allemand et al., 2017; Kloog et al., 2015).

94 The placenta plays a key role in foetal programming by supporting both the health of the  
95 mother and the development of the foetus. It conveys nutrients and oxygen to the foetus and  
96 regulates gas and waste exchanges as well as hormone interactions (Murphy et al., 2006).  
97 Alterations in placental physiology and function, potentially driven by epigenetic changes,  
98 may impact the health of the future individuals during their childhood as well as into their  
99 adulthood. Exposure to chemically and/or physically inappropriate environmental conditions  
100 during pregnancy can affect the placental function by modifying its epigenome (Nelissen et  
101 al., 2011). Indeed epigenetic mechanisms control the timing and levels of gene expression, by  
102 defining the extent of their activation or by maintaining them repressed. Hence in utero  
103 environmental exposures could result in epigenetic modifications of the placenta at birth,  
104 including changes in the DNA methylation profile. As a transient organ, the placenta may  
105 provide a unique record of exposures specifically occurring during pregnancy. While several  
106 studies have reported changes in global and gene-specific methylation patterns from adult  
107 blood associated with air pollution (Madrigano et al., 2011; Panni et al., 2016), temperature  
108 and relative humidity exposure (Bind et al., 2014), the role of maternal exposure to such  
109 environmental factors on new-borns DNA methylation patterns has received little attention so  
110 far. In cord blood, methylation in mitochondria-related genes was associated with NO<sub>2</sub>  
111 exposure during pregnancy in a recent meta-analysis (Gruzieva et al., 2017). In placenta, a  
112 few studies have linked global DNA methylation and gene candidate methylation to air  
113 pollution exposure during pregnancy (Cai et al., 2017; Janssen et al., 2013). However, no  
114 study has yet investigated the relationship between exposure to air pollutants and  
115 meteorological conditions during pregnancy and placental methylation patterns at a genome-  
116 wide scale.

117 We hypothesized that maternal exposure to air pollutants and meteorological conditions could  
118 alter the placental function through modifications of DNA methylation. Therefore we

119 investigated the relationship of air pollutants and meteorological conditions with global DNA  
120 methylation and gene-specific methylation in placentas at birth using the Illumina 450k array.  
121 In order to identify potentially relevant changes in genomic methylation sites related to  
122 environmental exposures, we used two complementary approaches combining a concept-  
123 driven analysis with an Epigenome Wide Association Study (EWAS) (Figure 1). The  
124 concept-driven analysis is an original strategy relying on the hypothesis that sensitivity to  
125 DNA methylation variations induced by environmental factors may depend on the activity of  
126 the genomic region considered (Rousseaux et al., 2013). Indeed, a change in the DNA  
127 methylation profile affecting a region involved in the epigenetic control of the expression  
128 level of a gene actively repressed or activated during pregnancy is more likely to be  
129 biologically relevant than a change occurring in another region of the genome. Therefore,  
130 based on available expression data in placenta, we identified genes whose expression is either  
131 predominant in full-term placenta or undergoes significant variations during placenta  
132 development. In addition, using our methylation data, we also established a list of genes with  
133 highly methylated CpG-rich promoters. Focussing on these genes with a specific status or  
134 pattern of expression in the placenta, enabled us to explore the “basal epigenome dynamics –  
135 epigenome response” relationship in the placenta. We then used an agnostic EWAS design, in  
136 an attempt to confirm the findings of the concept-driven analysis and to identify new regions  
137 associated with maternal air pollutants exposure.

138 Figure 1. Workflow of the study



139

## 140 2. Methods

### 141 2.1. Study population

142 The EDEN mother-child cohort included 2002 pregnant women, mainly Caucasian, enrolled  
 143 before 24 weeks of gestation in Nancy and Poitiers university hospitals, France, between 2003  
 144 and 2006 (Heude et al., 2015). Exclusion criteria were multiple pregnancies, pre-pregnancy  
 145 diabetes, French illiteracy and planned move outside the region in the following 3 years.  
 146 Residential addresses, lifestyle, demographic and medical data were collected by  
 147 questionnaires and interviews during pregnancy and after delivery. Among the 1301 women  
 148 for whom placenta samples were collected, we focused on 668 women. Placenta samples  
 149 were collected at delivery by the midwife or the technician of the study using a standardized  
 150 procedure. Samples of around 5mm x 5mm were carried out in the centre of the placenta on  
 151 the foetal side and were immediately frozen at -80°C. The EDEN cohort received approval  
 152 from the ethics committee (CCPPRB) of Kremlin Bicêtre and from the French data privacy  
 153 institution “*Commission Nationale de l’Informatique et des Libertés*” (CNIL). Written  
 154 consent was obtained from the mother for herself and for the offspring.

### 155 2.2. Placental DNA methylation assessment



156 DNA from placental samples was extracted using the QIA Symphony instrument (Qiagen,  
157 Germany). The DNA methylation analysis was performed by The Centre National de  
158 Génomique (CNG, Evry, France). The DNA samples were plated onto 96-well or 48-well  
159 plates. In total, nine plates including 64 chips were used. These plates were analyzed in 4  
160 batches. The ratios for sex (boy/girl) and recruitment centre (Poitiers/Nancy) were balanced  
161 for each chip. The Illumina's Infinium HumanMethylation450 BeadChip, representing over  
162 485,000 individual CpG sites, was used to assess levels of methylation in placenta samples  
163 following the manufacturer's instructions (Illumina, San Diego, CA, USA). Raw signals of  
164 450K BeadChips were extracted using the GenomeStudio<sup>®</sup> software (v2011.1, Illumina). The  
165 DNA methylation level of each CpG was calculated as the ratio of the intensity of fluorescent  
166 signals of the methylated alleles over the sum of methylated and unmethylated alleles ( $\beta$   
167 value). All samples passed initial quality control and had on average more than 98 % of valid  
168 data points (detection p-value <0.01). A refined version of the Subset Quantile Normalization  
169 (SQN) pipeline (Touleimat and Tost, 2012) including a revised annotation file (Price et al.,  
170 2013) was used for data processing, correction and normalization. Intensity values were  
171 corrected for potential biases in fluorescent dye intensity and background corrected using the  
172 *lumi* R package (Du et al., 2008) as implemented in the SQN pipeline. Probes potentially  
173 influenced by SNPs underlying the entire sequence of the probe (+1 or +2 bases depending  
174 on the Infinium probe type) that are present in the EUR population of the 1000 Genome  
175 project (<http://www.1000genomes.org>) at a frequency of more than 5% were removed from  
176 the analysis. Probes previously reported to map to several genomic regions were removed  
177 (Y.-A. Chen et al., 2013). The SQN pipeline uses the intensity signals of high-quality (i.e. low  
178 detection p-value) Infinium I probes as "anchors" to estimate a reference distribution of  
179 quantiles for probes in a biologically similar context based on the annotation file (Touleimat  
180 and Tost, 2012). This reference was then used to estimate a target distribution of quantiles for

181 InfII probes as a means to provide an accurate normalization of InfI/InfII probes and correct  
182 for the shift. SQN is performed for each individual separately. A principal component  
183 analysis as well as a hierarchical clustering were applied and showed no overall difference in  
184 the methylation patterns across participants samples and control samples, so that a quantile  
185 normalization was performed for between sample normalization. After quality control and  
186 normalization steps, there were 426,049 CpG sites left. Methylation beta values ranged from 0  
187 to 1. Data points with a detection p-value  $>0.01$  were excluded from subsequent analyses. To  
188 reduce the influence of potential outliers, we excluded data points below the 25<sup>th</sup> percentile -  
189  $3 \times$  interquartile ranges or above the 75<sup>th</sup> percentile +  $3 \times$  interquartile ranges for each probe,  
190 which removed 0.4% of all methylation beta values across participants. CpGs with more than  
191 25% of missing data were removed, leaving 425,878 CpG sites for statistical analyses.

192 Global methylation was also evaluated by measuring methylation in four CpG sites of  
193 repetitive Alu elements (*Alu*) and long interspersed nucleotide elements 1 (*LINE-1*) using a  
194 previously published pyrosequencing methylation assay (A. S. Yang et al., 2004). We then  
195 used the median percent methylation of the four CpG sites.

### 196 **2.3. Cellular composition of placenta samples**

197 Cellular composition of biological samples is a potential confounder in epigenetic  
198 epidemiology studies. In the absence of reference methylomes for placental tissue, we used a  
199 reference-free method, the RefFreeEWAS package available in R (Houseman et al., 2016), to  
200 deconvolute cell-type proportions from DNA methylation array data. The method relies on  
201 the identification of latent variables as surrogates for cell-type mixture. From the 10,000 most  
202 variable CpGs, we identified the optimal number of cell-types to be 6. We then used the  
203 425,878 CpGs to estimate the proportion of each cell-type per sample.

204

#### 205 **2.4. Identification of genes with specific patterns of expression**

206 We developed an approach relying on the integration of biological knowledge on the  
207 “epigenome-expression” relationship in the placenta. We hypothesised that genes and  
208 chromatin regions whose activity is controlled by DNA methylation as well as dynamically  
209 changing chromatin regions should be more prone to be affected by alterations of DNA  
210 methylation following environmental insults. We therefore focused on two categories of  
211 genes defined below: genes that are particularly *active* in the placenta and genes that are *silent*  
212 in the placenta but poised to be activated in response to external stimuli.

213 We defined placenta-predominantly expressed (“active”) genes as genes highly expressed in  
214 the placenta at the end of pregnancy, and with little or no expression in other tissues. This list  
215 was defined through the exploitation of public transcriptomic data  
216 (<http://www.ncbi.nlm.nih.gov/geo/>: GSE3526, GSE18809, GSE7434, GSE9984 studies) in  
217 which 575 genes showed a predominant expression in placentas at birth after normal  
218 pregnancies. A placenta predominant expression was defined by a mean level of expression  
219 value in placenta samples being above a threshold defined as the mean expression values + 2  
220 standard deviations of a series of 112 adult non-germline/non-placenta tissues of various  
221 origins. The pre-treatment of transcriptomic data and the strategy to identify genes with a  
222 predominant expression in specific tissue types have been described previously (Rousseaux et  
223 al., 2013; Wang et al., 2015). These 575 placenta-predominantly expressed genes  
224 corresponded to 9,003 CpGs measured by the Illumina beadchip (Table S1).

225 Placenta DNA methylation-dependent repressed (“silent”) genes included genes whose  
226 regulatory region is heavily methylated in placenta, based on the assumption that a high level  
227 of methylation in CpG-rich regions (or CpG islands) in the regulatory elements of these genes  
228 could be associated with a methylation dependent repression. We then hypothesized that these  
229 genes could potentially be reactivated upon demethylation. There were 9,969 CpGs located in

230 CpG islands (and which were not in the CpGs list corresponding to placenta-predominantly  
231 expressed genes) and whose mean methylation level in our dataset was above 80% (Table  
232 S1). Annotation of CpGs was based on information available in the R/Bioconductor  
233 package *IlluminaHumanMethylation450kanno.ilmn12.hg19*, version 0.6.0.

234

## 235 **2.5. Environmental exposures assessment**

236 NO<sub>2</sub> and PM<sub>10</sub> hourly concentrations were modelled at the women's home addresses during  
237 pregnancy using the quasi-Gaussian Atmospheric Dispersion Modelling System (ADMS)  
238 Urban 3.1 (Cambridge Environmental Research Consultants. Cambridge. United Kingdom)  
239 (Sellier et al., 2014). The model was implemented with the same methodology for both  
240 Poitiers and Nancy and provides hourly estimates on a 40x40 meters grid. Mean daily  
241 ambient temperature (T, in °C) and relative humidity (RH, in %) were obtained from the  
242 monitors of the French weather network *Météo France*. For each woman, the nearest monitor  
243 to her home address was used. Because the most biologically relevant time period for the  
244 effect of air pollution and weather conditions on methylation is unknown, we considered  
245 different exposure windows throughout pregnancy over which we averaged levels of NO<sub>2</sub>,  
246 PM<sub>10</sub>, T and RH: day 1, 2 and 3 before delivery, one week before delivery, one month before  
247 delivery, each trimester of pregnancy and the whole pregnancy.

248

## 249 **2.6. Statistical analyses**

### 250 **2.6.1. Global methylation**

251 We examined the impact of environmental exposures on the overall methylation level across  
252 the entire genome using two approaches, one relying on repetitive elements *Alu* and *LINE-1*

253 and the other relying on the global profile of methylation levels estimated for the 425,878  
254 CpGs.

255 Association of *Alu* and *LINE-1* methylation levels with air pollutants and meteorological  
256 exposures were investigated using robust linear regression, which accounts for potential  
257 outliers and heteroscedasticity in the data. We adjusted for the following *a priori* selected  
258 covariates: child sex, parity (0, 1,  $\geq 2$  children), maternal age at end of education ( $\leq 18$ , 19-20,  
259 21-22, 23-24,  $\geq 25$  years), season of conception, study centre (Poitiers and Nancy), maternal  
260 body mass index before pregnancy (continuous), maternal age at delivery (linear and  
261 quadratic terms), maternal smoking during pregnancy (continuous) and gestational duration  
262 (linear and quadratic terms). We additionally adjusted for technical factors related to the  
263 methylation measurements (batch, plate and chip) and for the estimated cell-type proportions.  
264 Meteorological exposures - temperature and humidity (restricted cubic splines) - were  
265 investigated simultaneously. Each air pollutant was studied separately and further adjusted for  
266 meteorological exposures (temperature and humidity, with restricted cubic splines).

267 Complementary to the repetitive elements analysis, we conducted a global analysis of  
268 methylation profiles (GAMP). This method, described by Zhao et al. (2015), allows to test  
269 whether exposure changes the overall profile or distribution of CpG measurements of the  
270 participants instead of examining the effect on individual CpGs. Each individual's methylation  
271 profile was characterized by approximating the density function and the cumulative  
272 distribution function of the methylation distribution using B-splines basis functions. The B-  
273 spline coefficients were then used to represent each individual overall methylation  
274 distribution. A variance component score test from the kernel machine framework, which  
275 naturally accommodates the correlation between B-spline coefficients, was computed to test  
276 the association of B-spline coefficients with each exposure of interest (temperature, humidity,  
277  $\text{NO}_2$ ,  $\text{PM}_{10}$ ) adjusted for confounders. Meteorological exposures were investigated

278 simultaneously. Each air pollutant was studied separately in models further adjusted for  
 279 meteorological exposures (temperature and humidity).

280

### 281 **2.6.2. Methylation at specific loci**

282 We studied the association between environmental exposures (NO<sub>2</sub>, PM<sub>10</sub>, temperature,  
 283 humidity) and CpG-specific methylation level using a robust linear regression model. We  
 284 applied the same approach described for global methylation analysis by testing: the  
 285 simultaneous effect of meteorological conditions (temperature and humidity) (1); the effect of  
 286 each air pollutant adjusted for meteorological conditions (temperature and humidity) (2) using  
 287 the following models:

$$288 \quad (1) Y_{ij} = \beta_0 + f(\beta_1 \cdot \text{Temperature}_i) + f(\beta_2 \cdot \text{Humidity}_i) + \beta_Z^T Z_i + \varepsilon_{ij}.$$

$$289 \quad (2) Y_{ij} = \beta_0 + \beta_1 \text{Pollutant}_i + f(\beta_2 \cdot \text{Temperature}_i) + f(\beta_3 \cdot \text{Humidity}_i) + \beta_Z^T Z_i + \varepsilon_{ij}.$$

290 Where  $Y_{ij}$  is the methylation measurement for CpG  $j$  in subject  $i$ ,  
 291  $f(\beta_2 \cdot \text{Temperature}_i)$  and  $f(\beta_3 \cdot \text{Humidity}_i)$  are restricted cubic spline functions of  
 292 temperature and humidity,  $Z_i$  is the set of adjustment factors similar to the one used for the  
 293 global methylation analysis and  $\varepsilon_{ij}$  is the random error. Each exposure window was tested in  
 294 a separate model. Multiple testing was accounted for by applying a Benjamini and Hochberg  
 295 False Discovery Rate (FDR) correction to the p-values that were obtained (Benjamini and  
 296 Hochberg, 1995). The FDR corrected p-values were calculated for the 18,972 CpGs included  
 297 in the *active* and *silent* genes lists for the concept-driven analysis and for the 425,878 CpGs  
 298 for the agnostic EWAS. An FDR-corrected p-value <0.05 was considered statistically  
 299 significant. For all significant CpGs (FDR-corrected p-value <0.05), we further tested whether  
 300 the association with the exposures differed by child sex by adding an interaction term in the

301 model. The genomic inflation factor ( $\lambda$ ) has been widely used in GWAS to assess  
 302 inflation/deflation of p-values (Devlin et al., 2001). A recent study demonstrated that the  
 303 Bayesian Inflation Factor (BIF) was a more relevant approach for EWAS (van Iterson et al.,  
 304 2017). We computed the  $\lambda$  and BIF for both the concept-driven analysis and the  
 305 agnostic EWAS.

306

### 307 **2.6.3. Region-based analysis**

308 To identify Differentially Methylated Regions (DMRs) in our data, we used Comb-p, a  
 309 method relying on the Stouffer-Liptak-Kechris correction that combines specific CpG p-  
 310 values using sliding windows and accounting for correlation between CpGs (B. S. Pedersen et  
 311 al., 2012). DMR p-values were adjusted for multiple testing by Šidák correction (Šidák,  
 312 1967). Significant DMRs (p-value<0.05) included at least 2 probes (p-value<0.001) at a  
 313 maximum distance of 500 bp. All analyses were performed using the statistical software R  
 314 (version 3.0.1) and Python (version 2.7.14).

315

## 316 **3. Results**

### 317 **3.1. Population characteristics and environmental factors**

318 On average ( $\pm$ SD), the participating mothers were 29 ( $\pm$  5.1) years old, with a pre-pregnancy  
 319 BMI of 22.9 ( $\pm$  4.2) kg/m<sup>2</sup> (Table 1). Mean gestational duration was 40 ( $\pm$  1.7) weeks and 32  
 320 babies (5%) were born preterm (<37 gestational weeks).

321 Table 1. Characteristics of the EDEN study population (n=668) and environmental exposures  
 322 over the whole pregnancy period.

Characteristics	mean $\pm$ SE	n (%)	Median pregnancy levels			
			All	T	RH	NO <sub>2</sub>
<b>Center</b>						
Poitiers		287 (43)	12.2	77.3	12.2	15.6
Nancy		381 (57)	9.8	77.2	23.4	23.4
<b>Sex of offspring</b>						

Male	349 (52)	10.7	77.4	17.1	20.6
Female	319 (48)	11.1	76.8	17.4	21.5
<b>Parity</b>					
0	301 (45)	11.3	77.3	17.0	20.8
1	252 (38)	10.5	77.4	17.3	21.2
≥2	115 (17)	11.0	76.9	17.4	21.0
<b>Maternal age at end of education (year)</b>					
≤18	132 (20)	10.9	77.2	17.4	20.7
19-20	114 (17)	11.1	77.5	15.8	17.5
21-22	151 (23)	10.9	77.1	16.9	21.3
23-24	145 (22)	11.0	76.7	18.2	21.3
≥25	126 (19)	10.8	77.8	18.8	21.4
<b>Season of conception</b>					
January – March	143 (21)	13.5	73.8	14.6	17.2
April – June	153 (23)	12.3	77.3	18.6	21.8
July – September	189 (28)	8.7	80.1	19.3	22.0
October – December	183 (27)	10.1	76.7	16.9	20.5
<b>BMI (kg/m<sup>2</sup>)</b>	22.9±4.2	-	-	-	-
<b>Maternal age (year)</b>	29.0±5.1	-	-	-	-
<b>Maternal smoking<sup>a</sup></b>	1.7±3.5	-	-	-	-
<b>Gestational duration (weeks)</b>	39.8±1.7	-	-	-	-

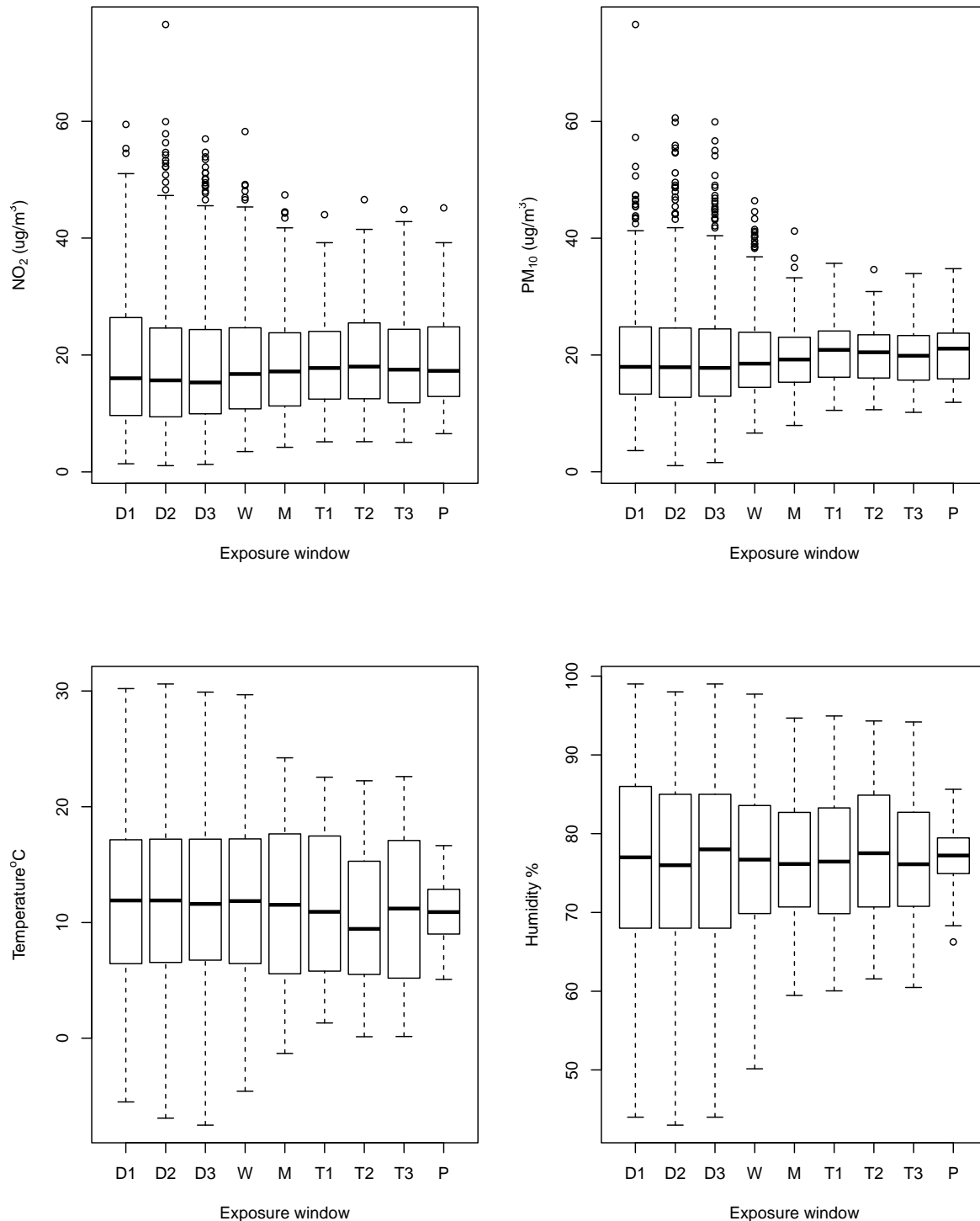
323 T= Temperature (°C). RH= Relative humidity (%). NO<sub>2</sub>= Nitrogen dioxide (µg/m<sup>3</sup>). PM<sub>10</sub>= Particulate matter  
324 smaller than 10 µm (µg/m<sup>3</sup>). BMI= pre-pregnancy Body Mass Index.  
325 <sup>a</sup>Average number of cigarette per day during pregnancy  
326

327 The longer the exposure window was, the less variable was the exposure level (Figure 2).

328 Mean pregnancy levels were 19 (± 7.7) µg/m<sup>3</sup> for NO<sub>2</sub> and 20 (± 4.5) µg/m<sup>3</sup> for PM<sub>10</sub>.



329 Figure 2. Environmental exposure across time window (d1, 2, 3 = day 1, 2, 3 before delivery;  
 330 w = week before delivery; m = month before delivery; t1, 2, 3 = trimester 1, 2, 3 and P =  
 331 whole pregnancy).



332

333 NO<sub>2</sub> and PM<sub>10</sub> levels were highly correlated (range, 0.73 to 0.84 depending on averaging  
 334 period, see Table S2). For each air pollutant, trimester-specific levels were strongly and

335 positively correlated (correlation ranged from 0.52 to 0.78 for NO<sub>2</sub> and from 0.79 to 0.86 for  
336 PM<sub>10</sub>). Average temperature exposure during pregnancy varied from 5 °C minimum to 16 °C  
337 maximum while relative humidity varied from 66% minimum to 86% maximum.  
338 Temperature and humidity were strongly and negatively correlated (range between -0.71 and -  
339 0.80, depending on averaging period, see Table S2). As for correlation between air pollutants  
340 and meteorological conditions, NO<sub>2</sub> levels were moderately correlated with temperature and  
341 humidity ( $|r|=0.15$  to 0.61) and PM<sub>10</sub> levels were weakly correlated with temperature and  
342 humidity ( $|r|=0.04$  to 0.34).

343

### 344 **3.2. Global DNA methylation**

345 Average methylation level was 16.2 ( $\pm$  1.0) for Alu and 26.1 ( $\pm$  1.9) for LINE-1. The  
346 distribution of average methylation level by CpG site was bimodal, with a peak around 0.025  
347 and another around 0.925 (Figure S1). Highest variability in methylation levels was observed  
348 for moderately methylated CpG sites (see Figure S2). PM<sub>10</sub> exposure the day before birth was  
349 positively associated with Alu methylation with a 0.08 regression coefficient (p-value=0.01)  
350 (Table S3). Temperature exposure during the 1<sup>st</sup> trimester of pregnancy was significantly  
351 associated with the density of methylation distribution (p-value=0.03). However, analyses of  
352 Alu and LINE-1 as well as the global analysis of methylation profiles mainly yielded not  
353 significant differences with p-values higher than 0.05 (Table S3).

354

### 355 **3.3. Concept-driven approach: *silent* and *active* genes in placenta**

356 Among the list of placenta-predominantly expressed genes (“active genes”), 3 CpGs mapping  
357 to 3 genes, whose average methylation levels varied between 0.03 and 0.58, were  
358 significantly associated with NO<sub>2</sub> exposure (FDR p-values <0.05, Table 2). Two of these 3

359 CpGs are located in the body of *ADORA2B*, encoding the Adenosine A2B Receptor, one is in  
360 a region shared by the promoter regions of two genes, *PXT1* (Peroxisomal testis specific 1)  
361 and *KCTD20* (potassium channel tetramerization domain containing 20). The methylation  
362 levels of the two CpGs of *ADORA2B* decreased on average with higher exposure to NO<sub>2</sub>  
363 during the 1<sup>st</sup> or 2<sup>nd</sup> trimester or during the whole pregnancy. Conversely, the methylation of  
364 the CpG located in the promoter of the *PXT1* and *KCTD20* genes was positively associated  
365 with NO<sub>2</sub> exposure during both the 2<sup>nd</sup> trimester and the whole pregnancy. Regarding  
366 exposure to PM<sub>10</sub>, one CpG located in the promoter of *SLC44A5B* (solute carrier family 44  
367 member 5) gene was positively associated with exposure the month before birth and another  
368 CpG located in the body of *KYNU* (kynureninase) was negatively associated with exposure 3  
369 days before birth. No significant association with methylation was found for temperature and  
370 humidity levels (lowest FDR p-values, 1.00, 1.00, respectively).

371 Among the placenta DNA methylation-dependent repressed genes (“silent genes”), one CpG,  
372 located in the body of the *CAPN10* (Calcium-Activated Neutral Proteinase 10) gene, was  
373 positively associated with NO<sub>2</sub> exposure during the 3<sup>rd</sup> trimester. Exposure to PM<sub>10</sub> the month  
374 before birth was negatively associated with mean methylation of one CpG located in the body  
375 of *TUBGCP2* (tubulin gamma complex associated protein 2) and was positively associated  
376 with mean methylation of two CpGs, one located in the body of *TGM6* (transglutaminase 6)  
377 and another located in the body of *ADCK5* (aarF domain containing kinase 5). Regarding  
378 exposure to meteorological conditions, no significant associations with methylation of active  
379 genes were found (lowest FDR p-values, 1.00 for temperature and humidity).

380 The p-value distribution of the 18,972 CpGs included in the concept-driven analysis was close  
381 to the theoretical distribution as indicated by the BIF values (range from 0.95 to 1.1) which  
382 were substantially smaller compared to the lambda values (figures S3-S6).

383 Table 2. CpGs associated (FDR p &lt; 0.05) with prenatal exposure to air pollutants or meteorological conditions

Exposure	CpG	Chr	Position (built 37)	Gene	Location in gene	Location of CpG	$\beta^a$	FDR p-value candidate	EWAS	Mean methylation level (SE)
<i>CpGs located in placenta-predominantly expressed genes (n=9,003 sites)</i>										
<b>NO<sub>2</sub></b>										
Pregnancy	cg10984505	chr6	36410951	PXT1;KCTD20	TSS1500;5'UTR	Island	0.002	0.04		0.031 (0.006)
	cg17580614	chr17	15849512	ADORA2B	Body	Island	-0.042	<10 <sup>-3</sup>	0.02	0.586 (0.138)
	cg07563400	chr17	15849556	ADORA2B	Body	S_Shore	-0.045	<10 <sup>-3</sup>		0.343 (0.125)
Trimester 1	cg17580614	chr17	15849512	ADORA2B	Body	Island	-0.037	<10 <sup>-3</sup>		0.586 (0.138)
	cg07563400	chr17	15849556	ADORA2B	Body	S_Shore	-0.042	<10 <sup>-3</sup>		0.343 (0.125)
Trimester 2	cg10984505	chr6	36410951	PXT1;KCTD20	TSS1500;5'UTR	Island	0.002	0.02		0.031 (0.006)
	cg17580614	chr17	15849512	ADORA2B	Body	Island	-0.044	<10 <sup>-4</sup>	<10 <sup>-3</sup>	0.586 (0.138)
	cg07563400	chr17	15849556	ADORA2B	Body	S_Shore	-0.047	<10 <sup>-4</sup>	<10 <sup>-3</sup>	0.343 (0.125)
<b>PM<sub>10</sub></b>										
Month before birth	cg12659128	chr1	76078176	SLC44A5	TSS1500	N_Shelf	0.037	0.03		0.587 (0.070)
Day 3 before birth	cg04112100	chr2	143701758	KYNU	Body	OpenSea	-0.012	0.04		0.257 (0.068)
<i>CpGs located in methylation-dependent repressed genes list (n=9,969 sites)</i>										
<b>NO<sub>2</sub></b>										
Trimester 3	cg01712700	chr2	241535695	CAPN10	Body	Island	-0.004	0.02		0.948 (0.013)
<b>PM<sub>10</sub></b>										
Month before birth	cg23075260	chr8	145617435	ADCK5	Body	Island	0.018	0.03		0.878 (0.034)
	cg05142592	chr10	135097800	TUBGCP2	Body	Island	-0.008	0.03		0.953 (0.018)
	cg06967014	chr20	2384240	TGM6	Body	Island	0.007	0.03		0.924 (0.015)
<i>CpGs not in active and silent placenta genes list (i.e. EWAS approach)</i>										
<b>NO<sub>2</sub></b>										
Pregnancy	cg20491726	chr2	242820622			N_Shelf	0.015		0.02	0.715 (0.051)
Trimester 2	cg20491726	chr2	242820622			N_Shelf	0.015		0.02	0.715 (0.051)
<b>PM<sub>10</sub></b>										
Trimester 1	cg03215416	chr8	18823341	PSD3	Body	OpenSea	-0.020		0.03	0.913 (0.024)
<b>Humidity</b>										
Trimester 2	cg16917193	chr12	54089295			Island			0.04 <sup>b</sup>	0.104 (0.114)
Month before birth	cg16075020	chr19	12606183	ZNF709	5'UTR	Island			<10 <sup>-3b</sup>	0.063 (0.077)

384 NO<sub>2</sub>= Nitrogen dioxide (µg/m<sup>3</sup>). PM<sub>10</sub>= Particulate matter smaller than 10 µm (µg/m<sup>3</sup>).385 <sup>a</sup> Results per 10 units increase in prenatal exposure.386 <sup>b</sup> p-value of the spline

### 387 3.4. Agnostic approach: EWAS examining each CpG

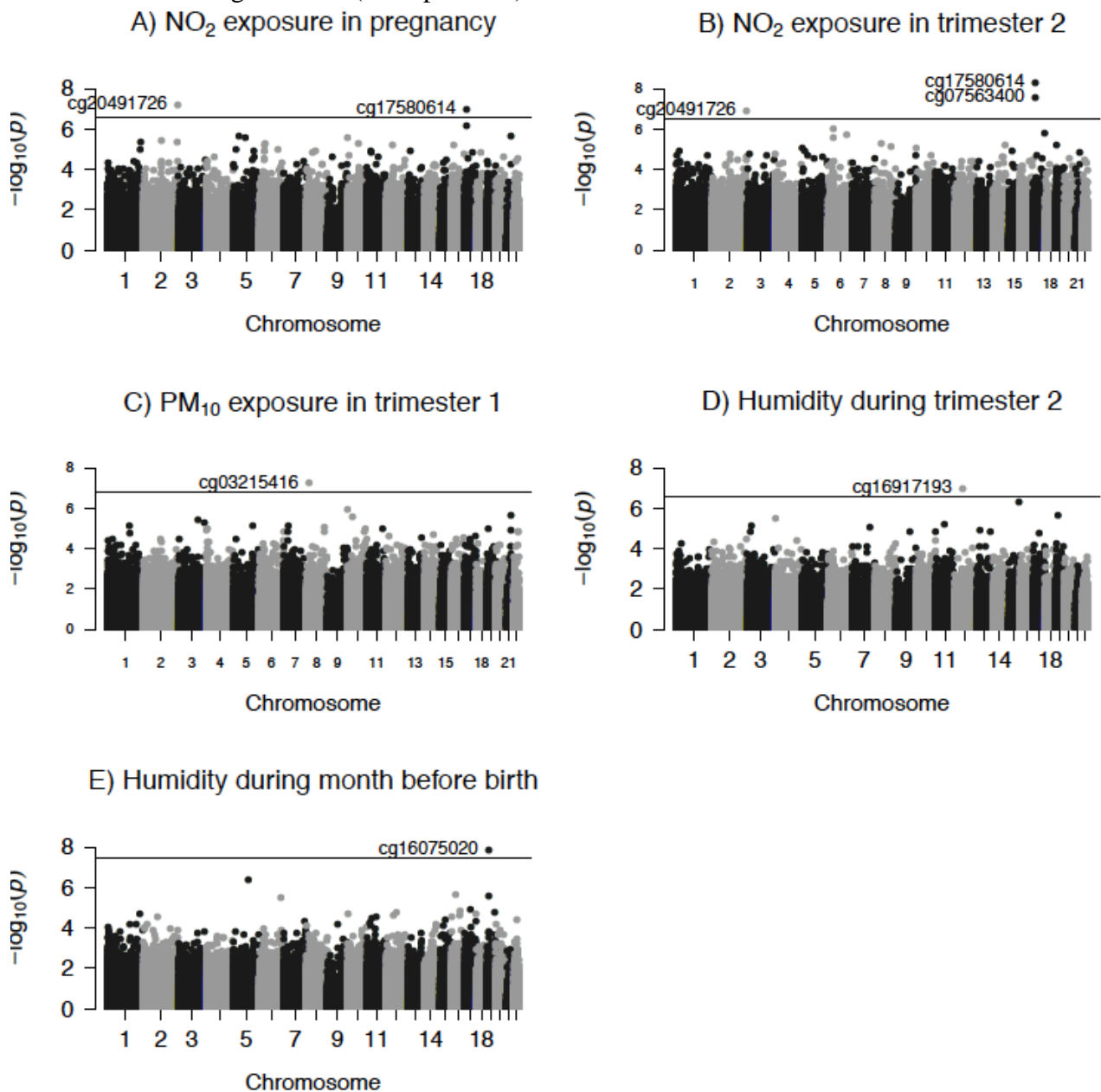
388 When we independently tested the adjusted effect of exposures on each of the 425,878 CpGs, six  
389 showed significantly different methylation levels (Table 2). Among these, two CpGs had also been  
390 identified in the concept-driven analysis: cg07563400 and cg17580614, both located in *ADORA2B*,  
391 remained significantly associated with NO<sub>2</sub> exposure during the 2<sup>nd</sup> trimester of pregnancy after the  
392 genome-wide FDR correction of p-values (Table 2; Figure 3). Other CpGs identified as  
393 significantly associated with environmental exposures in the concept-driven approach did not  
394 achieve genome-wide statistical significance in the EWAS, but all except one of them (cg05142592  
395 on gene *TUBGCP2*) were still in the top 20 CpGs with the lowest p-values for association with  
396 exposure (see Table S4 to S7).

397 The EWAS also revealed four new CpGs (not included in the concept-driven list of candidates)  
398 whose average methylation was significantly (FDR p-value <0.05) associated with humidity, NO<sub>2</sub>  
399 and PM<sub>10</sub> exposure (Table 2). One CpG (cg20491726), mapped to a non genic region, was  
400 significantly associated with a 10 µg/m<sup>3</sup> increase in NO<sub>2</sub> exposure during both the second trimester  
401 ( $\beta=0.015$  FDR p=0.02) and the whole pregnancy ( $\beta=0.015$  FDR p=0.02). Another CpG mapped to  
402 *PSD3* (pleckstrin and Sec7 domain containing 3) was negatively associated with PM<sub>10</sub> during the 1<sup>st</sup>  
403 trimester of pregnancy (Table 3). Two CpGs, one in the non genic region and one mapped to  
404 *ZNF709* (zinc finger protein 709), were non-linearly associated with humidity in the 2<sup>nd</sup> trimester of  
405 pregnancy and in the month before birth, respectively (Figure S7). The p-value distribution of the  
406 425,878 CpGs was close to the theoretical distribution as indicated by the BIF values (range from  
407 0.96 to 1.15) (Figure S8 to S11).

408 Although interaction terms with sex were significant for two CpGs (cg06967014, cg16917193), the  
409 effect estimates (Table 8) and dose-response relationship (Figure S7) did not appear substantially  
410 different between boys and girls.

411

412 Figure 3. Manhattan plots of p-values showing the association between environmental exposure  
413 (NO<sub>2</sub> (A, B), PM<sub>10</sub> (C), and humidity (D, E)) and 425,878 CpGs methylation using the agnostic  
414 EWAS. Each dot corresponds to the p-value of a CpG site and the horizontal lines indicates the  
415 level of statistical significance (FDR p < 0.05).



416

### 417 3.5. Regional analysis

418 Our regional analysis identified 27 DMRs significantly (Sidak-corrected P-value<0.05) associated  
419 with air pollutants exposure and 13 DMRs with weather conditions exposure (Table 3, Table S9).

420 The *ADORA2B*-associated DMR was identified for the three trimesters and the whole pregnancy;  
421 the DMR included the two CpGs previously identified in the site-specific analyses. Among genes  
422 highly represented in the identified DMRs, several CpGs located in the *FAM38A* gene were

423 negatively associated with NO<sub>2</sub> exposure in each trimester, last month and pregnancy. The  
424 *FAM124B*-associated DMR was hypomethylated across the 7 CpGs included in women with  
425 increased exposure to NO<sub>2</sub> or PM<sub>10</sub>, especially towards the end of pregnancy. NO<sub>2</sub> exposure,  
426 especially towards the end of pregnancy (3<sup>rd</sup> trimester and last month) was negatively associated  
427 with two DMRs located in *HSP90AA1* and *LOC254559* and positively associated with two DMRs  
428 located in *LZTFL1*, and *P2RX4*. The top-ranked DMRs in terms of number of CpGs included were  
429 located in *CD81* (13 CpGs) and in a non-genic region on chromosome 6 (15 CpGs).

430

431

432  
433

Table 3. Regions identified using the Comb-P method as differentially methylated in the placenta in association with air pollutants exposure in pregnancy.

Gene <sup>1</sup>	Hg19	Exposure	Time window	No. of probes	Slk p-value	Sidak p-value	Direction of effect
ADORA2B	chr17:15849512-15849557	NO <sub>2</sub>	p	2	4.93E-12	4.67E-08	-
		NO <sub>2</sub>	t1	2	9.18E-12	8.69E-08	-
		NO <sub>2</sub>	t2	2	1.29E-14	1.22E-10	-
		NO <sub>2</sub>	t3	2	3.29E-09	3.11E-05	-
ANKRD45	chr1:173639044-173639136	NO <sub>2</sub>	m	4	1.61E-08	7.43E-05	-
		pm10	m	4	2.99E-08	1.39E-04	-
B3GALT4	chr6:33245717-33245805	NO <sub>2</sub>	p	6	1.04E-05	4.90E-02	-
C1orf86;LOC100128003	chr1:2125049-2125244	NO <sub>2</sub>	p	7	7.43E-08	1.62E-04	-
C22orf9	chr22:45608345-45608517	PM <sub>10</sub>	d2	7	4.92E-08	1.22E-04	+
CD81	chr11:2397486-2397832	PM <sub>10</sub>	d1	13	1.37E-16	1.37E-13	+
DAXX	chr6:33288180-33288373	PM <sub>10</sub>	d1	6	1.41E-10	3.10E-07	-
FAM124B	chr2:225266656-225266881	NO <sub>2</sub>	p	7	1.41E-08	2.67E-05	-
		NO <sub>2</sub>	t3	7	6.55E-11	1.24E-07	-
		NO <sub>2</sub>	m	7	7.62E-10	1.44E-06	-
		PM <sub>10</sub>	t3	7	4.80E-11	9.09E-08	-
FAM38A	chr16:88832476-88832573	NO <sub>2</sub>	t3	4	1.05E-08	4.62E-05	-
		NO <sub>2</sub>	m	4	1.51E-09	6.63E-06	-
FAM38A	chr16:88832476-88832701	NO <sub>2</sub>	p	6	1.16E-09	2.20E-06	-
		NO <sub>2</sub>	t1	6	6.15E-11	1.16E-07	-
FAM38A	chr16:88832532-88832573	NO <sub>2</sub>	t2	2	4.61E-07	4.78E-03	-
GNAS;GNASAS	chr20:57426240-57426275	NO <sub>2</sub>	p	3	1.64E-07	1.99E-03	+
GP1BB;SEPT5	chr22:19710880-19711052	PM <sub>10</sub>	d2	6	1.96E-09	4.86E-06	-
HSP90AA1	chr14:102554826-102554978	NO <sub>2</sub>	t3	4	8.60E-08	2.41E-04	-
		NO <sub>2</sub>	m	4	6.66E-09	1.87E-05	-
IL32	chr16:3114986-3115134	NO <sub>2</sub>	m	3	4.45E-09	1.28E-05	+
LIME1	chr20:62369445-62369606	PM <sub>10</sub>	d2	4	1.38E-08	3.64E-05	-
LMF1	chr16:967929-968025	NO <sub>2</sub>	t3	3	5.54E-08	2.46E-04	-
LOC254559	chr15:89921083-89921259	NO <sub>2</sub>	t3	6	3.97E-08	1.64E-04	-
		NO <sub>2</sub>	m	7	2.02E-09	4.90E-06	-
LZTFL1	chr3:45883529-45883735	NO <sub>2</sub>	p	7	1.16E-07	2.39E-04	+
		NO <sub>2</sub>	t3	8	2.75E-10	4.37E-07	+
		NO <sub>2</sub>	m	10	1.51E-11	1.94E-08	+
NOTCH3	chr19:15281200-15281518	PM <sub>10</sub>	d1	4	5.03E-12	6.73E-09	-
P2RX4	chr12:121647128-121647218	NO <sub>2</sub>	p	5	7.16E-08	3.39E-04	+
		NO <sub>2</sub>	t3	5	8.03E-08	3.80E-04	+
		NO <sub>2</sub>	m	5	5.51E-11	2.61E-07	+
PRRT1	chr6:32116538-32116781	PM <sub>10</sub>	d2	5	3.64E-09	6.38E-06	-
SOX2OT;SOX2	chr3:181428462-181428580	PM <sub>10</sub>	m	5	6.05E-08	2.18E-04	+
TRIO	chr5:14359544-14359615	PM <sub>10</sub>	m	3	1.44E-08	8.66E-05	+
ZNF563	chr19:12444484-12444742	PM <sub>10</sub>	m	6	3.21E-11	5.29E-08	+
	chr3:194650099-194650198	NO <sub>2</sub>	t3	2	6.78E-08	2.92E-04	+
	chr6:28583971-28584173	NO <sub>2</sub>	m	15	6.42E-09	1.35E-05	-

434  
435

Hg19: Human Genome version 19; Slk: Stouffer-Liptak Kechris correction; NO<sub>2</sub>= Nitrogen dioxide (µg/m<sup>3</sup>). PM<sub>10</sub>= Particulate matter smaller than 10 µm (µg/m<sup>3</sup>). p: pregnancy. t1: trimester 1. t2:

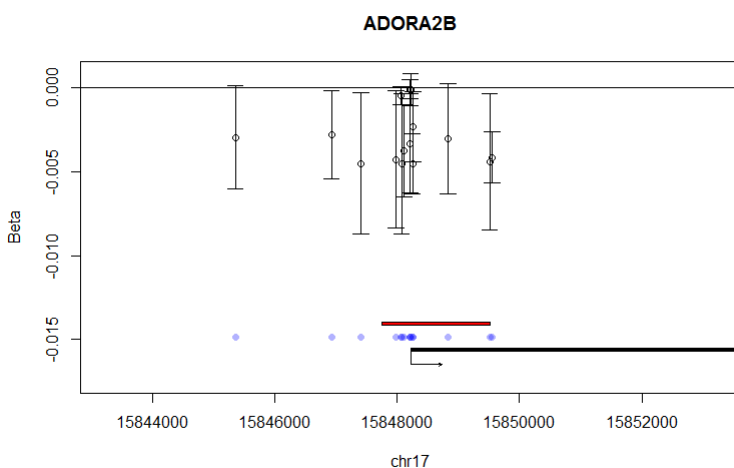


436 trimester 2. t3: trimester 3. d1: day 1 before birth. d2: day 2 before birth. d3: day 3 before birth; <sup>1</sup>  
437 UCSC; <sup>2</sup> direction of effect was similar across all CpGs included in the DMR  
438

### 439 3.6. Synthesis of the results

440 Our top finding was that one CpG (cg17580614) located in the body of *ADORA2B*, one of the  
441 placenta-predominantly expressed genes, showed a significantly lower methylation in placenta after  
442 NO<sub>2</sub> exposure during pregnancy. This result came out of the concept-driven analysis performed on  
443 18,972 CpGs and was confirmed in the agnostic approach that used a more stringent FDR  
444 correction of the p-values on 425,878 CpGs and in the regional analysis. Furthermore, this CpG as  
445 well as another *ADORA2B* CpG (cg07563400) were in the top 20 of CpGs associated with NO<sub>2</sub>  
446 exposure during the 1<sup>st</sup> and 2<sup>nd</sup> trimesters and during the whole pregnancy (FDR p-values  $\leq 0.1$ ,  
447 Supplemental material, Table S6). Figure 4 shows the regression coefficient for each of the  
448 *ADORA2B* CpGs against its uncorrected p-value regarding the association with NO<sub>2</sub> exposure  
449 during the 2<sup>nd</sup> trimester and the whole pregnancy. Among the 19 CpGs of this gene, 16 were  
450 negatively associated with NO<sub>2</sub> exposure during the 2nd trimester and the whole pregnancy, and 11  
451 (10, respectively) were in the top 1000 of the CpGs associated with placenta-predominantly  
452 expressed genes (Figure 4).

453 Figure 4: Visualisation of regional associations between NO<sub>2</sub> exposure during pregnancy and  
454 *ADORA2B* methylation indicating the direction of the effect (coefficient) and confidence interval  
455 for each CpG .



456

## 457 **5. Discussion**

458 To the best of our knowledge, the present study shows the results of the first genome-wide analysis  
459 of differentially methylated probes and regions in placenta in relation to in utero exposure to PM<sub>10</sub>,  
460 NO<sub>2</sub>, temperature and humidity. Associating a concept-driven analysis with an agnostic EWAS, we  
461 showed lower methylation levels in two CpGs located in the body of *ADORA2B* associated with  
462 increased exposure to NO<sub>2</sub> during pregnancy, especially in trimesters 1 and 2. The agnostic EWAS  
463 further revealed new significant associations of prenatal exposure to PM<sub>10</sub>, NO<sub>2</sub>, humidity and  
464 temperature during various time-windows and methylation of 4 CpGs (mapped to 2 genes (*PSD3*,  
465 *ZNF709*) and 2 non genic regions) and 39 regions (Table 3, Table S9), which are candidates for  
466 replication in future studies. Among these, several DMR were located in genes pointing towards  
467 hypertensive and metabolic disorders.

468 *ADORA2B*, encoding the Adenosine A2B receptor, was shown to play a role in placental  
469 development and possibly in the pathophysiology of hypoxia and preeclampsia (PE) in pregnant  
470 women (Acurio et al., 2014; Darashchonak et al., 2014; Jia et al., 2012), a pathology which has also  
471 been associated with air pollution exposure during pregnancy (M. Pedersen et al., 2014). In both  
472 mice and humans, it has been demonstrated that activation of ADORA2B signalling contributes to  
473 the pathogenesis of PE (Huang et al., 2017), to small foetuses, small placentas and foetal growth  
474 restriction (Iriyama et al., 2015). The molecular basis of the functional link and relationship  
475 between *ADORA2B* gene and placenta DNA methylation, and the involvement of the ADORA2B  
476 protein in PE and birth outcomes are questions to be addressed in future investigations. Additional  
477 work will also be required to assess the relevance of *ADORA2B* as a candidate marker and/or target  
478 for the diagnosis, prevention and treatment of PE.

479 Of interest, four DMR were located in *CD81*, *DAXX*, *NOTCH3* and *P2RX4* genes, which have all  
480 been implicated in PE phenotypes. *P2RX4*, a purinergic receptor highly expressed in normal  
481 placentas, was shown to be overexpressed in both term and preterm preeclamptic placentas (Roberts

482 et al., 2007) and hypomethylated in preeclamptic placentas (Chu et al., 2014). In our study, NO<sub>2</sub>  
483 exposure in pregnancy, 3<sup>rd</sup> trimester and last month of pregnancy was positively associated with  
484 *P2RX4* methylation. In accordance with our result, a CpG of *P2RX4* was found hypermethylated in  
485 blood from adults exposed to PM<sub>2.5</sub> in the previous month (Panni et al., 2016). In addition, our  
486 results indicated a positive association of PM<sub>10</sub> exposure the day before birth with methylation in a  
487 DMR located in *CD81* and a negative association with DMRs in *DAXX* and *NOTCH3*. *CD81*, a  
488 gene encoding a member of the transmembrane 4 superfamily, was shown to be mainly expressed  
489 in normal first trimester placentas and gradually downregulated with gestational age (Shen et al.,  
490 2017). In early-onset ( $\leq 34$  weeks) severe preeclamptic placentas, *CD81* was up-regulated in  
491 syncytiotrophoblasts and extravillous cytotrophoblasts, which play a major role in maternal–fetal  
492 exchanges (Malassiné and Cronier, 2002) and placentation (Pijnenborg et al., 2006). The  
493 overexpression of *CD81* was also demonstrated in a PE-like phenotype in rats (Shen et al., 2017). A  
494 recent study demonstrated that *DAXX* methylation was involved in trophoblast differentiation, PE,  
495 and response to hypoxia, suggesting an important role of *DAXX* in placentation (Novakovic et al.,  
496 2017). Similarly, *NOTCH3* placental expression has been shown down regulated in PE (Fragkiadaki  
497 et al., 2015) and up regulated in early onset PE (Zhao et al., 2014). Notch signaling has been  
498 suggested to play a key role in cytotrophoblasts motility and differentiation (Haider et al., 2014). In  
499 contrast to our finding, *NOTCH3* methylation in blood form adults has been positively associated  
500 with PM<sub>2.5</sub> exposure the previous month (Panni et al., 2016). Further studies are required in order to  
501 determine the functional relationship between these genes and their placental methylation.  
502 Hypertensive disorders, among which preeclampsia in pregnant women (M. Pedersen et al., 2014),  
503 are increasingly suspected as a potential mechanism to explain health effects of air pollutants (Shah  
504 et al., 2013).

505 Among air pollutants associated DMR in our study, four were located in *LMFI*, *GNAS*, *GNASAS*,  
506 and *IL-32* genes. These genes have been previously involved in metabolic syndrome and related  
507 phenotypes (Hong et al., 2017). Single nucleotide polymorphisms of *LMFI* is involved in severe

508 hypertriglyceridemia (Péterfy, 2012). *LMF1* blood methylation in adults has been positively  
509 associated with PM<sub>2.5</sub> exposure in the previous month (Panni et al., 2016), while our results  
510 indicated a negative association of NO<sub>2</sub> exposure in the 3<sup>rd</sup> trimester with placental methylation of  
511 this gene. *GNAS* is an imprinted gene with a complex imprinted pattern ; decreased placental  
512 expression has been associated with intra uterine growth retardation (Dunk et al., 2012; McMinn et  
513 al., 2006) and cord blood hypermethylation has been associated with gestational diabetes (D. Chen  
514 et al., 2014). *GNASAS*, the *GNAS* antisense RNA 1, placental expression was related to newborns  
515 neurobehavioral profile (Green et al., 2016), while its methylation in adult blood has been linked to  
516 prenatal exposure to famine (Tobi et al., 2009). In rabbits, pregnancy exposure to diesel engine  
517 exhaust has been associated with changes in triglyceride and cholesterol levels in the offspring  
518 exposed *in utero* (Valentino et al., 2015) but further investigations are required to identify if this  
519 association could be to some extent mediated by epigenetic changes. Taken together our findings  
520 point to PE- and metabolic syndrome-associated genes, which is in line with the increasing  
521 evidence on the relationship between air pollution and the metabolic syndrome (B.-Y. Yang et al.,  
522 2018) and with the hypothesis that such effects might start early in life (Park, 2017).

523 Significant variations of the methylation levels of other CpGs and DMR, identified either from the  
524 concept-driven analysis or from the agnostic EWAS approach, have occasionally been found  
525 associated with NO<sub>2</sub>, PM<sub>10</sub>, temperature or humidity exposure at specific time windows of  
526 pregnancy. However, the biological significance of the differential methylation observed for these  
527 genes is unclear (Supplemental material, Table S10). These genes have not been associated with  
528 NO<sub>2</sub> or proximity to traffic in the two recent EWAS conducted in newborns (Gruzieva et al., 2017;  
529 Kingsley et al., 2016). Noteworthy, some of these results were in agreement with air pollution  
530 association studies conducted on blood DNA methylation. This was the case for the positive  
531 association of methylation in *LZTFL1*-DMR with average NO<sub>2</sub> exposure during pregnancy, 3<sup>rd</sup>  
532 trimester and last month of pregnancy which was consistent with the relationship between *LZTFL1*  
533 blood methylation and chronic PM<sub>2.5</sub> exposure observed in an elderly population (Nwanaji-

534 Enwerem et al., 2016). The significant (FDR corrected) associations of PM<sub>2.5</sub> exposure in the past  
535 month and decreased blood methylation in *ANKRD45* and *LOC254559* and increased blood  
536 methylation in *TRIO* found by Panni (Panni et al., 2016) was in agreement with our findings in the  
537 placenta. However, the negative relationship they found between PM<sub>2.5</sub> exposure and *ZNF563*  
538 methylation disagree with our results. The small overlap between our results and those of previous  
539 published EWAS can be explained by the fact that these studies relied on different tissues. Gruzieva  
540 et al. investigated cord blood (Gruzieva et al., 2017) and Kingsley et al. examined placenta samples  
541 from the maternal side (Kingsley et al., 2016). DNA methylation is tissue-specific (Ollikainen et  
542 al., 2010) and it is likely that different tissue will respond differently to similar environmental  
543 stimuli. The fact that DNA methylation is a dynamic mark also explains this absence of overlap  
544 between the present results obtained in our population of newborns and other data obtained for  
545 different populations and exposure levels.

546 Breton et al. (Breton et al., 2016) found that prenatal exposure to PM<sub>10</sub> in the first trimester was  
547 associated with lower LINE-1 methylation in 459 newborn bloodspots. A similar result was found  
548 in 471 placenta samples where LINE-1 was associated with distance to traffic (Kingsley et al.,  
549 2016) and in 240 placenta samples where global methylation was linked to first trimester exposure  
550 to PM<sub>2.5</sub> (Janssen et al., 2013). These studies did not investigate shorter time-windows of exposure.  
551 In our study, we also observed a negative relationship between PM<sub>10</sub> exposure during the first  
552 trimester and LINE-1 and Alu CpG methylation, but our results did not reach statistical  
553 significance. In the short-term, PM<sub>10</sub> exposure the day before birth was significantly and positively  
554 associated with Alu repetitive elements methylation.

555 As for meteorological conditions, we observed a trend for temperature in the first trimester of  
556 pregnancy being associated with the methylation distribution profiles (p-value for density function  
557 = 0.03, p-value for cumulative distribution function = 0.11). We are aware of only one previous  
558 study investigating the effects of meteorological conditions on DNA methylation in blood cells of  
559 elderly men (Bind et al., 2014). The authors reported associations of ambient temperature with

560 DNA methylation in genes related to coagulation, inflammation, cortisol, and metabolic pathway  
561 and associations of relative humidity with *LINE-1* hypomethylation and Alu hypermethylation. In  
562 our study, we found two loci, one in *ZNF709* and one in a non genic region whose methylation was  
563 associated with humidity exposure during the second trimester and the month preceding birth,  
564 respectively. We also identified 13 DMRs mapping to 8 genes associated with meteorological  
565 exposures. These genes do not seem involved in the inflammation or metabolic pathways  
566 previously highlighted with blood methylation, and their association with prenatal meteorological  
567 conditions had not been examined so far.

568 A novel feature of our study is the focus on genes with tissue specific patterns of expression, in  
569 which we identified first a category of genes that are mostly active in the placenta and silent in  
570 other tissues (“active genes”) and second a category of genes with a methylation-dependent  
571 repressed state (“silent genes”) in the placenta. The definition of silent and active chromatin points  
572 to regions with different dynamics and hence different capacity to respond to signals and to be  
573 reprogrammed. In order to identify these regions, we used available transcriptomic data (for  
574 selecting genes with specific patterns of expression in placenta) and the present methylome data  
575 (list of silent genes with high methylation levels). This original approach not only allowed us to  
576 identify potential target CpGs through the activity of their regulatory regions, but also to limit the  
577 number of CpG sites to the most relevant ones to be tested in relation to environmental exposures,  
578 without limiting the analysis to particular pathways previously identified in the literature. In the  
579 context of a constantly expanding number of CpGs interrogated by microarrays and sequencing  
580 techniques, which usually exceeds the sample size of the study, replication is often used to validate  
581 the findings and limit the risk of false positives. Our strategy was rather to restrict our analysis to  
582 the most biologically relevant genes and chromatin regions, which we defined as those prone to be  
583 affected by alterations in methylation. Noteworthy, the results of the EWAS, which used a more  
584 stringent correction of the p-value, and the DMR analyses strongly supports our finding from the

585 concept-driven analysis that *ADORA2B* placental methylation could be sensitive to prenatal air  
586 pollution exposure.

587 We acknowledge several strengths and limitations to this study. First, our results demonstrate that  
588 placental DNA methylation is associated with air pollutants exposure, but we cannot draw  
589 conclusions on how gene expression relates to these methylation differences. Second, our study  
590 focused on PM<sub>10</sub> and NO<sub>2</sub> air pollutants exposure, for which high temporally and spatially resolved  
591 exposures were available. Air pollution is a complex mixture of compounds which are correlated  
592 with the assessed pollutants. PM<sub>2.5</sub> are of great concern because they can penetrate deep into the  
593 lungs and enter the blood circulation. While our results identified mainly NO<sub>2</sub> exposure as  
594 associated with placental DNA methylation, we rather interpret this association as an indication of  
595 traffic-pollutants association with DNA methylation. Another limitation is the use of residential  
596 address to estimate air pollution exposure, which is prone to misclassification due to the lack of  
597 consideration of time activity patterns and of indoor exposures. However, we used a high spatially  
598 and temporally resolved ambient dispersion model. Third, although we attempted to account for  
599 many potential confounders including technical factors and cell heterogeneity, we cannot rule out  
600 confounding from unaccounted factors such as physical activity, diet, or genetics. Moreover, since  
601 our cohort consists of highly educated mainly Caucasian women, our findings may not be general-  
602 izable to other populations. An important strength is that the sample size is one of the largest for a  
603 study on placental DNA methylation and more generally for an environmental epigenetic study.  
604 Samples were collected from the foetal side of the placenta, which is relatively easy to collect, non-  
605 invasive and may allow a good reproducibility of results since the placenta lifetime is limited to the  
606 pregnancy. In perinatal studies, the placenta represents a relevant tissue considered as an accurate  
607 ‘record’ of children’s in-utero exposures (Maccani and Marsit, 2009) and as a health biomarker due  
608 to its master regulator function of the fetal hormonal and endocrine milieu. EDEN is a well  
609 characterized mother-child cohort, which allowed the consideration of a large range of potential  
610 confounders, including data collected early in pregnancy.

611

612

## 613 **6. Conclusions**

614 Among the placenta methylated silent genes, we identified four CpGs, mapped to four genes, whose  
615 methylation levels in the foetal placenta were significantly associated with maternal exposures to  
616 NO<sub>2</sub> and PM<sub>10</sub>. Among placenta active genes, we found five CpGs, mapped to five genes, whose  
617 methylation levels in the foetal placenta were significantly associated with maternal exposures to  
618 NO<sub>2</sub> and PM<sub>10</sub>. One of these active genes, *ADORA2B*, had 2 CpGs whose methylation levels were  
619 also significantly associated with NO<sub>2</sub> exposure during the second trimester of pregnancy in the  
620 EWAS approach after FDR correction of the p-value. The DMRs analyses showed *ADORA2B*  
621 methylation as associated with NO<sub>2</sub> exposure in pregnancy and at each trimester of pregnancy.  
622 *ADORA2B* has been implicated in placental development, hypoxia and preeclampsia, a pathology  
623 which has been previously associated with air pollution exposure during pregnancy. This latter  
624 observation, together with our results, supports the hypothesis that placental DNA methylation is  
625 involved in the exposure-disease relationships during pregnancy. Whether the observed associations  
626 potentially bear long-term consequences for the health of the child and adult individual, or even  
627 possibly for future generations, remains to be investigated.

628

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