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ABSTRACT

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

Objectives: We investigated whether nitrogen dioxide (NO_2), particulate matter (PM_{10}), temperature and humidity during pregnancy are associated with differences in genome-wide placental DNA methylation levels.

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

Results: The candidate genes approach identified nine CpGs mapped to 8 genes associated with prenatal NO_2, PM_{10} and humidity exposure [false discovery rate (FDR) $p<0.05$]. Among these, the methylation level of 2 CpGs located on *ADORA2B* remained significantly associated with NO_2 exposure during the 2^{nd} trimester exposure in the EWAS (FDR $p<0.05$). EWAS further revealed associations between exposure and variations of DNA methylation of 4 other CpGs. We further identified 27 DMRs significantly (FDR $p<0.05$) associated with air pollutants exposure and 13 DMRs with weather conditions exposure.

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

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

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

1. Introduction

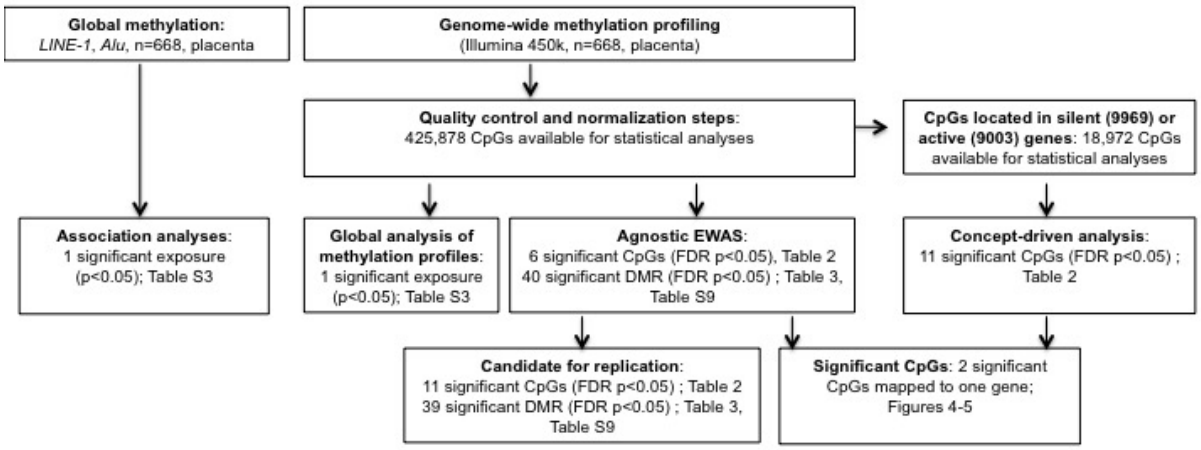
Despite significant improvements in air quality in past decades, 50% of the population in 2014 in Europe live in areas that do not meet the World Health Organisation guidelines (World Health Organization, 2006) for particulate matter less than 10 microns (PM_{10}), 85% for particulate matter less than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

The placenta plays a key role in foetal programming by supporting both the health of the mother and the development of the foetus. It conveys nutrients and oxygen to the foetus and regulates gas and waste exchanges as well as hormone interactions (Murphy et al., 2006). Alterations in placental physiology and function, potentially driven by epigenetic changes, may impact the health of the future individuals during their childhood as well as into their adulthood. Exposure to chemically and/or physically inappropriate environmental conditions during pregnancy can affect the placental function by modifying its epigenome (Nelissen et al., 2011). Indeed epigenetic mechanisms control the timing and levels of gene expression, by defining the extent of their activation or by maintaining them repressed. Hence in utero environmental exposures could result in epigenetic modifications of the placenta at birth, including changes in the DNA methylation profile. As a transient organ, the placenta may provide a unique record of exposures specifically occurring during pregnancy. While several studies have reported changes in global and gene-specific methylation patterns from adult blood associated with air pollution (Madrigano et al., 2011; Panni et al., 2016), temperature and relative humidity exposure (Bind et al., 2014), the role of maternal exposure to such environmental factors on new-borns DNA methylation patterns has received little attention so far. In cord blood, methylation in mitochondria-related genes was associated with NO_2 exposure during pregnancy in a recent meta–analysis (Gruzieva et al., 2017). In placenta, a few studies have linked global DNA methylation and gene candidate methylation to air pollution exposure during pregnancy (Cai et al., 2017; Janssen et al., 2013). However, no study has yet investigated the relationship between exposure to air pollutants and meteorological conditions during pregnancy and placental methylation patterns at a genome-wide scale.

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

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

Figure 1. Workflow of the study



2. Methods

2.1. Study population

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

2.2. Placental DNA methylation assessment

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

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

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

2.3. Cellular composition of placenta samples

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

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

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

2.5. Environmental exposures assessment

NO_2 and PM_{10} hourly concentrations were modelled at the women's home addresses during pregnancy using the quasi-Gaussian Atmospheric Dispersion Modelling System (ADMS) Urban 3.1 (Cambridge Environmental Research Consultants. Cambridge. United Kingdom) (Sellier et al., 2014). The model was implemented with the same methodology for both Poitiers and Nancy and provides hourly estimates on a 40x40 meters grid. Mean daily ambient temperature (T, in °C) and relative humidity (RH, in %) were obtained from the monitors of the French weather network *Météo France*. For each woman, the nearest monitor to her home address was used. Because the most biologically relevant time period for the effect of air pollution and weather conditions on methylation is unknown, we considered different exposure windows throughout pregnancy over which we averaged levels of NO_2, PM_{10}, T and RH: day 1, 2 and 3 before delivery, one week before delivery, one month before delivery, each trimester of pregnancy and the whole pregnancy.

2.6. Statistical analyses

2.6.1. Global methylation

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

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

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

Complementary to the repetitive elements analysis, we conducted a global analysis of methylation profiles (GAMP). This method, described by Zhao et al. (2015), allows to test whether exposure changes the overall profile or distribution of CpG measurements of the participants instead of examining the effect on individual CpGs. Each individual's methylation profile was characterized by approximating the density function and the cumulative distribution function of the methylation distribution using B-splines basis functions. The B-spline coefficients were then used to represent each individual overall methylation distribution. A variance component score test from the kernel machine framework, which naturally accommodates the correlation between B-spline coefficients, was computed to test the association of B-spline coefficients with each exposure of interest (temperature, humidity, NO_2, PM_{10}) adjusted for confounders. Meteorological exposures were investigated

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

2.6.2. Methylation at specific loci

We studied the association between environmental exposures (NO_2, PM_{10}, temperature, humidity) and CpG-specific methylation level using a robust linear regression model. We applied the same approach described for global methylation analysis by testing: the simultaneous effect of meteorological conditions (temperature and humidity) (1); the effect of each air pollutant adjusted for meteorological conditions (temperature and humidity) (2) using the following models:

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

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

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

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

2.6.3. Region-based analysis

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

3. Results

3.1. Population characteristics and environmental factors

On average (±SD), the participating mothers were 29 (± 5.1) years old, with a pre-pregnancy BMI of 22.9 (± 4.2) kg/m² (Table 1). Mean gestational duration was 40 (± 1.7) weeks and 32 babies (5%) were born preterm (<37 gestational weeks).

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

	mean±SE	n (%)	Median pregnancy levels			
Characteristics		**All**	**T**	**RH**	**NO_2**	**PM_{10}**
Center						
Poitiers		287 (43)	12.2	77.3	12.2	15.6
Nancy		381 (57)	9.8	77.2	23.4	23.4
Sex of offspring						

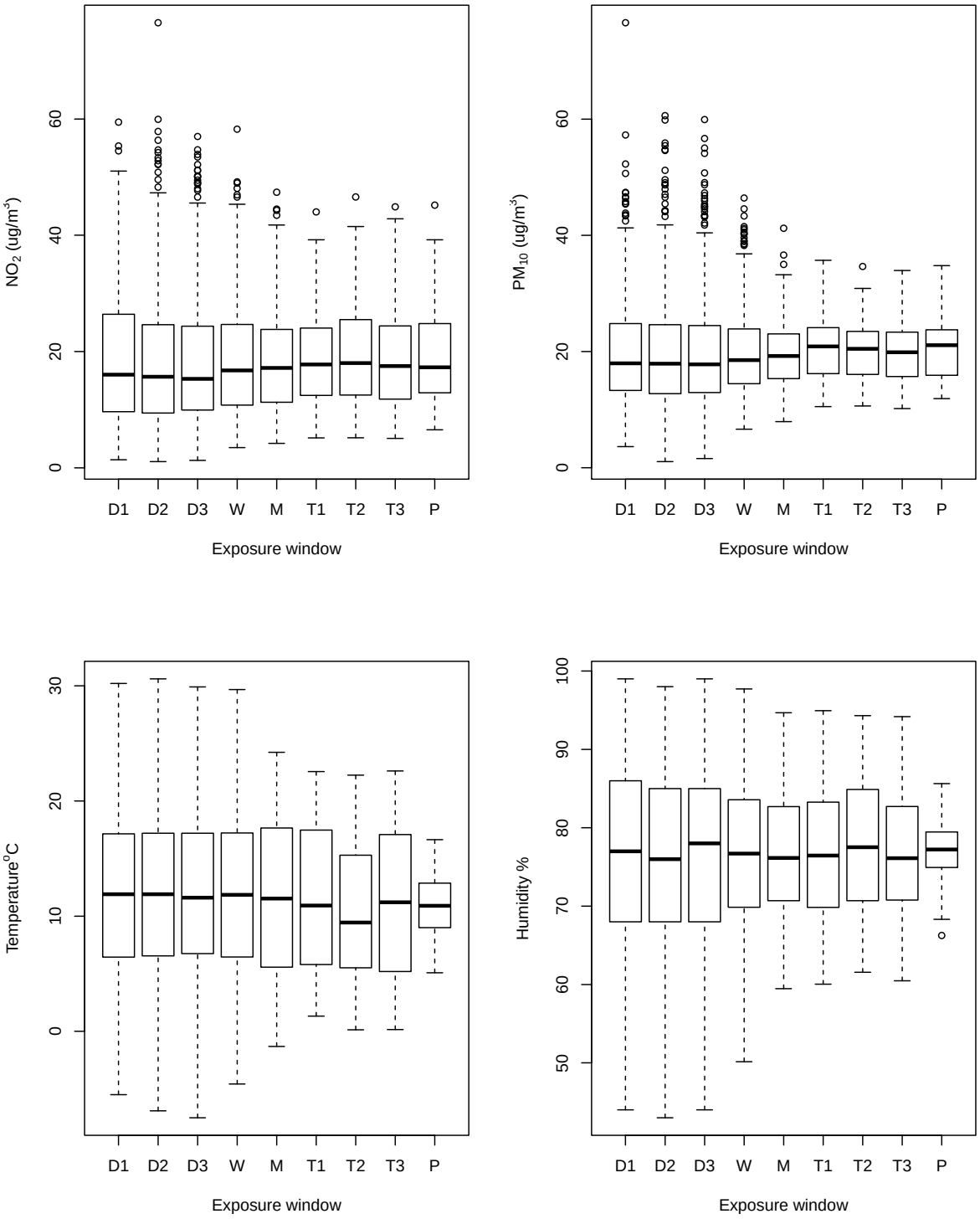
Male		349 (52)	10.7	77.4	17.1	20.6
Female		319 (48)	11.1	76.8	17.4	21.5
Parity						
0		301 (45)	11.3	77.3	17.0	20.8
1		252 (38)	10.5	77.4	17.3	21.2
≥2		115 (17)	11.0	76.9	17.4	21.0
Maternal age at end of education (year)						
≤18		132 (20)	10.9	77.2	17.4	20.7
19-20		114 (17)	11.1	77.5	15.8	17.5
21-22		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
Season of conception						
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
BMI (kg/m²)	22.9±4.2		-	-	-	-
Maternal age (year)	29.0±5.1		-	-	-	-
Maternal smoking[a]	1.7±3.5		-	-	-	-
Gestational duration (weeks)	39.8±1.7		-	-	-	-

T= Temperature (°C). RH= Relative humidity (%). NO_2= Nitrogen dioxide (µg/m^3). PM_{10}= Particulate matter smaller than 10 µm (µg/m^3). BMI= pre-pregnancy Body Mass Index.

[a]Average number of cigarette per day during pregnancy

The longer the exposure window was, the less variable was the exposure level (Figure 2). Mean pregnancy levels were 19 (± 7.7) µg/m^3 for NO_2 and 20 (± 4.5) µg/m^3 for PM_{10}.

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_2 and PM_{10} 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

positively correlated (correlation ranged from 0.52 to 0.78 for NO_2 and from 0.79 to 0.86 for PM_{10}). Average temperature exposure during pregnancy varied from 5 °C minimum to 16 °C maximum while relative humidity varied from 66% minimum to 86% maximum. Temperature and humidity were strongly and negatively correlated (range between -0.71 and -0.80, depending on averaging period, see Table S2). As for correlation between air pollutants and meteorological conditions, NO_2 levels were moderately correlated with temperature and humidity ($|r|=0.15$ to 0.61) and PM_{10} levels were weakly correlated with temperature and humidity ($|r|=0.04$ to 0.34).

3.2. Global DNA methylation

Average methylation level was 16.2 (± 1.0) for Alu and 26.1 (± 1.9) for LINE-1. The distribution of average methylation level by CpG site was bimodal, with a peak around 0.025 and another around 0.925 (Figure S1). Highest variability in methylation levels was observed for moderately methylated CpG sites (see Figure S2). PM_{10} exposure the day before birth was positively associated with Alu methylation with a 0.08 regression coefficient (p-value=0.01) (Table S3). Temperature exposure during the 1st trimester of pregnancy was significantly associated with the density of methylation distribution (p-value=0.03). However, analyses of Alu and LINE-1 as well as the global analysis of methylation profiles mainly yielded not significant differences with p-values higher than 0.05 (Table S3).

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

Among the list of placenta-predominantly expressed genes ("active genes"), 3 CpGs mapping to 3 genes, whose average methylation levels varied between 0.03 and 0.58, were significantly associated with NO_2 exposure (FDR p-values <0.05, Table 2). Two of these 3

CpGs are located in the body of *ADORA2B*, encoding the Adenosine A2B Receptor, one is in a region shared by the promoter regions of two genes, *PXT1* (Peroxisomal testis specific 1) and *KCTD20* (potassium channel teramerization domain containing 20). The methylation levels of the two CpGs of *ADORA2B* decreased on average with higher exposure to NO_2 during the 1st or 2nd trimester or during the whole pregnancy. Conversely, the methylation of the CpG located in the promoter of the *PXT1* and *KCTD20* genes was positively associated with NO_2 exposure during both the 2nd trimester and the whole pregnancy. Regarding exposure to PM_{10}, one CpG located in the promoter of *SLC44A5B* (solute carrier family 44 member 5) gene was positively associated with exposure the month before birth and another CpG located in the body of *KYNU* (kynureninase) was negatively associated with exposure 3 days before birth. No significant association with methylation was found for temperature and humidity levels (lowest FDR p-values, 1.00, 1.00, respectively).

Among the placenta DNA methylation-dependent repressed genes ("silent genes"), one CpG, located in the body of the *CAPN10* (Calcium-Activated Neutral Proteinase 10) gene, was positively associated with NO_2 exposure during the 3rd trimester. Exposure to PM_{10} the month before birth was negatively associated with mean methylation of one CpG located in the body of *TUBGCP2* (tubulin gamma complex associated protein 2) and was positively associated with mean methylation of two CpGs, one located in the body of *TGM6* (transglutaminase 6) and another located in the body of *ADCK5* (aarF domain containing kinase 5). Regarding exposure to meteorological conditions, no significant associations with methylation of active genes were found (lowest FDR p-values, 1.00 for temperature and humidity).

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

Table 2. CpGs associated (FDR p < 0.05) with prenatal exposure to air pollutants or meteorological conditions

Exposure	CpG	Chr	Position (built 37)	Gene	Location in gene	Location of CpG	β[a]	FDR p-value candidate	FDR p-value EWAS	Mean methylation level (SE)
CpGs located in placenta-predominantly expressed genes (n=9,003 sites)										
NO_2										
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	$<10^{-3}$	0.02	0.586 (0.138)
	cg07563400	chr17	15849556	ADORA2B	Body	S_Shore	-0.045	$<10^{-3}$		0.343 (0.125)
Trimester 1	cg17580614	chr17	15849512	ADORA2B	Body	Island	-0.037	$<10^{-3}$		0.586 (0.138)
	cg07563400	chr17	15849556	ADORA2B	Body	S_Shore	-0.042	$<10^{-3}$		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	$<10^{-4}$	$<10^{-3}$	0.586 (0.138)
	cg07563400	chr17	15849556	ADORA2B	Body	S_Shore	-0.047	$<10^{-4}$	$<10^{-3}$	0.343 (0.125)
PM_{10}										
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)
CpGs located in methylation-dependent repressed genes list (n=9,969 sites)										
NO_2										
Trimester 3	cg01712700	chr2	241535695	CAPN10	Body	Island	-0.004	0.02		0.948 (0.013)
PM_{10}										
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)
CpGs not in active and silent placenta genes list (i.e. EWAS approach)										
NO_2										
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)
PM_{10}										
Trimester 1	cg03215416	chr8	18823341	PSD3	Body	OpenSea	-0.020		0.03	0.913 (0.024)
Humidity										
Trimester 2	cg16917193	chr12	54089295			Island			0.04[b]	0.104 (0.114)
Month before birth	cg16075020	chr19	12606183	ZNF709	5'UTR	Island			$<10^{-3}$[b]	0.063 (0.077)

NO_2= Nitrogen dioxide ($\mu g/m^3$). PM_{10}= Particulate matter smaller than 10 µm ($\mu g/m^3$).

[a] Results per 10 units increase in prenatal exposure.

[b] p-value of the spline

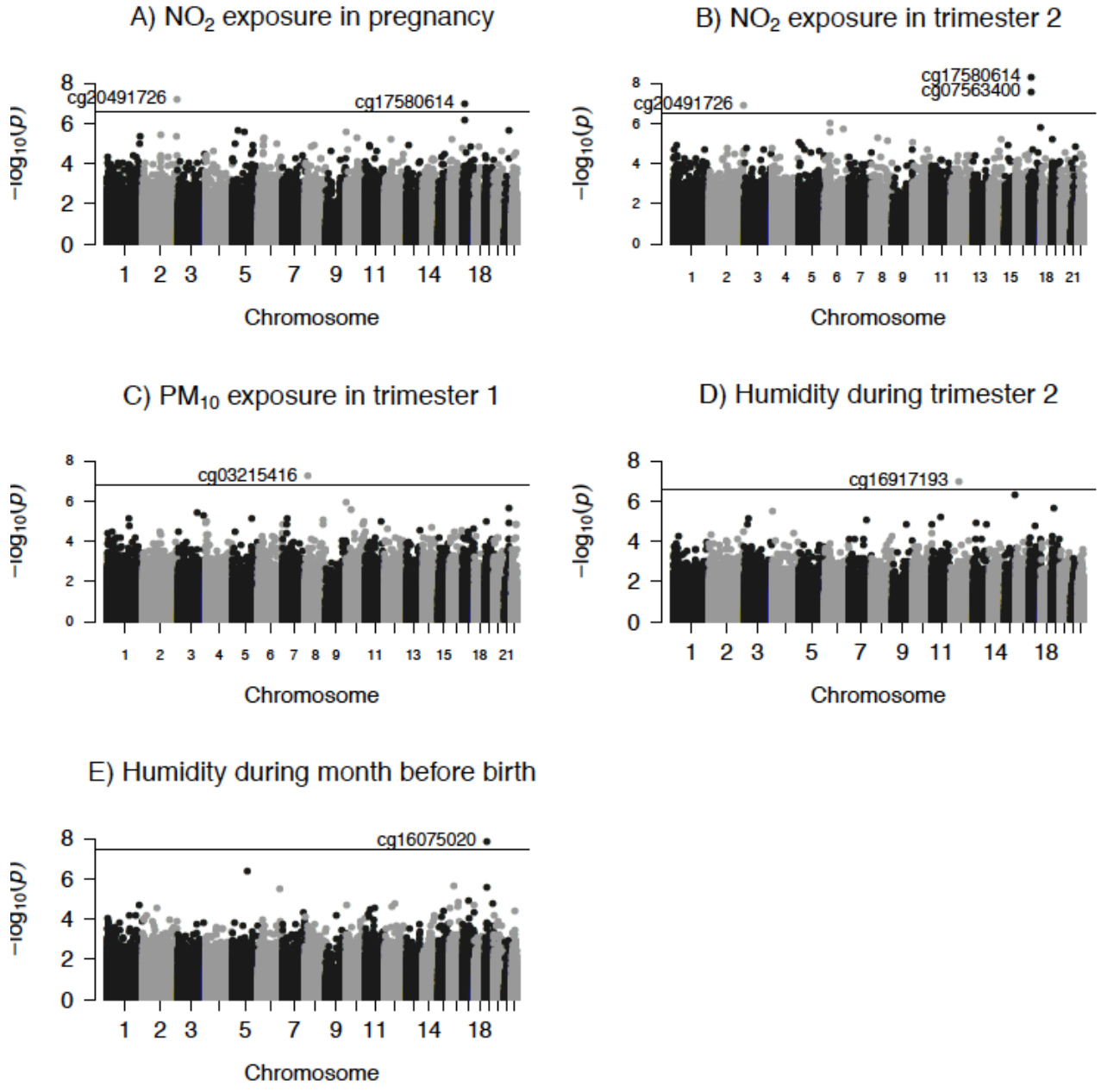
3.4. Agnostic approach: EWAS examining each CpG

When we independently tested the adjusted effect of exposures on each of the 425,878 CpGs, six showed significantly different methylation levels (Table 2). Among these, two CpGs had also been identified in the concept-driven analysis: cg07563400 and cg17580614, both located in *ADORA2B*, remained significantly associated with NO_2 exposure during the 2nd trimester of pregnancy after the genome-wide FDR correction of p-values (Table 2; Figure 3). Other CpGs identified as significantly associated with environmental exposures in the concept-driven approach did not achieve genome-wide statistical significance in the EWAS, but all except one of them (cg05142592 on gene *TUBGCP2*) were still in the top 20 CpGs with the lowest p-values for association with exposure (see Table S4 to S7).

The EWAS also revealed four new CpGs (not included in the concept-driven list of candidates) whose average methylation was significantly (FDR p-value <0.05) associated with humidity, NO_2 and PM_{10} exposure (Table 2). One CpG (cg20491726), mapped to a non genic region, was significantly associated with a 10 µg/m^3 increase in NO_2 exposure during both the second trimester (β=0.015 FDR p=0.02) and the whole pregnancy (β=0.015 FDR p=0.02). Another CpG mapped to *PSD3* (pleckstrin and Sec7 domain containing 3) was negatively associated with PM_{10} during the 1st trimester of pregnancy (Table 3). Two CpGs, one in the non genic region and one mapped to *ZNF709* (zinc finger protein 709), were non-linearly associated with humidity in the 2nd trimester of pregnancy and in the month before birth, respectively (Figure S7). The p-value distribution of the 425,878 CpGs was close to the theoretical distribution as indicated by the BIF values (range from 0.96 to 1.15) (Figure S8 to S11).

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

Figure 3. Manhattan plots of p-values showing the association between environmental exposure (NO_2 (A, B), PM_{10} (C), and humidity (D, E)) and 425,878 CpGs methylation using the agnostic EWAS. Each dot corresponds to the p-value of a CpG site and the horizontal lines indicates the level of statistical significance (FDR p < 0.05).



3.5. Regional analysis

Our regional analysis identified 27 DMRs significantly (Sidak-corrected P-value<0.05) associated with air pollutants exposure and 13 DMRs with weather conditions exposure (Table 3, Table S9). The *ADORA2B*-associated DMR was identified for the three trimesters and the whole pregnancy; the DMR included the two CpGs previously identified in the site-specific analyses. Among genes highly represented in the identified DMRs, several CpGs located in the *FAM38A* gene were

negatively associated with NO_2 exposure in each trimester, last month and pregnancy. The *FAM124B*-associated DMR was hypomethylated across the 7 CpGs included in women with increased exposure to NO_2 or PM_{10}, especially towards the end of pregnancy. NO_2 exposure, especially towards the end of pregnancy (3^{rd} trimester and last month) was negatively associated with two DMRs located in *HSP90AA1* and *LOC254559* and positively associated with two DMRs located in *LZTFL1*, and *P2RX4*. The top-ranked DMRs in terms of number of CpGs included were located in *CD81* (13 CpGs) and in a non-genic region on chromosome 6 (15 CpGs).

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

Gene[1]	Hg19	Exposure	Time window	No. of probes	Slk p-value	Sidak p-value	Direction of effect
ADORA2B	chr17:15849512-15849557	NO_2	p	2	4.93E-12	4.67E-08	-
		NO_2	t1	2	9.18E-12	8.69E-08	-
		NO_2	t2	2	1.29E-14	1.22E-10	-
		NO_2	t3	2	3.29E-09	3.11E-05	-
ANKRD45	chr1:173639044-173639136	NO_2	m	4	1.61E-08	7.43E-05	-
		pm10	m	4	2.99E-08	1.39E-04	-
B3GALT4	chr6:33245717-33245805	NO_2	p	6	1.04E-05	4.90E-02	-
C1orf86;LOC100128003	chr1:2125049-2125244	NO_2	p	7	7.43E-08	1.62E-04	-
C22orf9	chr22:45608345-45608517	PM_{10}	d2	7	4.92E-08	1.22E-04	+
CD81	chr11:2397486-2397832	PM_{10}	d1	13	1.37E-16	1.37E-13	+
DAXX	chr6:33288180-33288373	PM_{10}	d1	6	1.41E-10	3.10E-07	-
FAM124B	chr2:225266656-225266881	NO_2	p	7	1.41E-08	2.67E-05	-
		NO_2	t3	7	6.55E-11	1.24E-07	-
		NO_2	m	7	7.62E-10	1.44E-06	-
		PM_{10}	t3	7	4.80E-11	9.09E-08	-
FAM38A	chr16:88832476-88832573	NO_2	t3	4	1.05E-08	4.62E-05	-
		NO_2	m	4	1.51E-09	6.63E-06	-
FAM38A	chr16:88832476-88832701	NO_2	p	6	1.16E-09	2.20E-06	-
		NO_2	t1	6	6.15E-11	1.16E-07	-
FAM38A	chr16:88832532-88832573	NO_2	t2	2	4.61E-07	4.78E-03	-
GNAS;GNASAS	chr20:57426240-57426275	NO_2	p	3	1.64E-07	1.99E-03	+
GP1BB;SEPT5	chr22:19710880-19711052	PM_{10}	d2	6	1.96E-09	4.86E-06	-
HSP90AA1	chr14:102554826-102554978	NO_2	t3	4	8.60E-08	2.41E-04	-
		NO_2	m	4	6.66E-09	1.87E-05	-
IL32	chr16:3114986-3115134	NO_2	m	3	4.45E-09	1.28E-05	+
LIME1	chr20:62369445-62369606	PM_{10}	d2	4	1.38E-08	3.64E-05	-
LMF1	chr16:967929-968025	NO_2	t3	3	5.54E-08	2.46E-04	-
LOC254559	chr15:89921083-89921259	NO_2	t3	6	3.97E-08	1.64E-04	-
		NO_2	m	7	2.02E-09	4.90E-06	-
LZTFL1	chr3:45883529-45883735	NO_2	p	7	1.16E-07	2.39E-04	+
		NO_2	t3	8	2.75E-10	4.37E-07	+
		NO_2	m	10	1.51E-11	1.94E-08	+
NOTCH3	chr19:15281200-15281518	PM_{10}	d1	4	5.03E-12	6.73E-09	-
P2RX4	chr12:121647128-121647218	NO_2	p	5	7.16E-08	3.39E-04	+
		NO_2	t3	5	8.03E-08	3.80E-04	+
		NO_2	m	5	5.51E-11	2.61E-07	+
PRRT1	chr6:32116538-32116781	PM_{10}	d2	5	3.64E-09	6.38E-06	-
SOX2OT;SOX2	chr3:181428462-181428580	PM_{10}	m	5	6.05E-08	2.18E-04	+
TRIO	chr5:14359544-14359615	PM_{10}	m	3	1.44E-08	8.66E-05	+
ZNF563	chr19:12444484-12444742	PM_{10}	m	6	3.21E-11	5.29E-08	+
	chr3:194650099-194650198	NO_2	t3	2	6.78E-08	2.92E-04	+
	chr6:28583971-28584173	NO_2	m	15	6.42E-09	1.35E-05	-

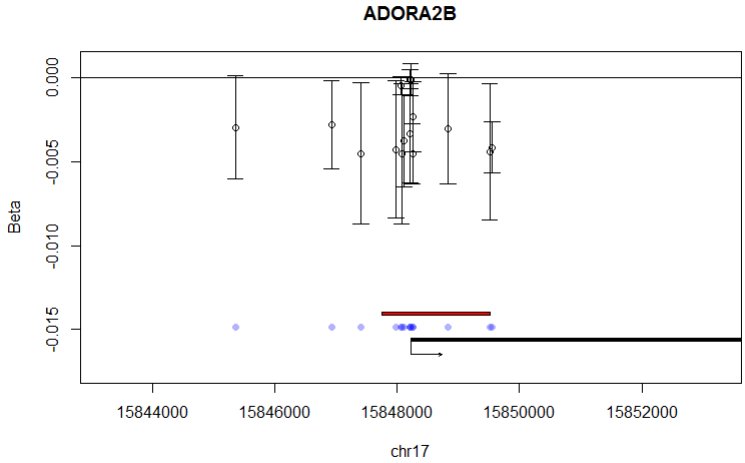
Hg19: Human Genome version 19; Slk: Stouffer-Liptak Kechris correction; NO_2= Nitrogen dioxide ($\mu g/m^3$). PM_{10}= Particulate matter smaller than 10 µm ($\mu g/m^3$). p: pregnancy. t1: trimester 1. t2:

trimester 2. t3: trimester 3. d1: day 1 before birth. d2: day 2 before birth. d3: day 3 before birth; [1] UCSC; [2] direction of effect was similar across all CpGs included in the DMR

3.6. Synthesis of the results

Our top finding was that one CpG (cg17580614) located in the body of *ADORA2B*, one of the placenta-predominantly expressed genes, showed a significantly lower methylation in placenta after NO_2 exposure during pregnancy. This result came out of the concept-driven analysis performed on 18,972 CpGs and was confirmed in the agnostic approach that used a more stringent FDR correction of the p-values on 425,878 CpGs and in the regional analysis. Furthermore, this CpG as well as another *ADORA2B* CpG (cg07563400) were in the top 20 of CpGs associated with NO_2 exposure during the 1st and 2nd trimesters and during the whole pregnancy (FDR p-values ≤0.1, Supplemental material, Table S6). Figure 4 shows the regression coefficient for each of the *ADORA2B* CpGs against its uncorrected p-value regarding the association with NO_2 exposure during the 2nd trimester and the whole pregnancy. Among the 19 CpGs of this gene, 16 were negatively associated with NO_2 exposure during the 2nd trimester and the whole pregnancy, and 11 (10, respectively) were in the top 1000 of the CpGs associated with placenta-predominantly expressed genes (Figure 4).

Figure 4: Visualisation of regional associations between NO_2 exposure during pregnancy and *ADORA2B* methylation indicating the direction of the effect (coefficient) and confidence interval for each CpG .



5. Discussion

To the best of our knowledge, the present study shows the results of the first genome-wide analysis of differentially methylated probes and regions in placenta in relation to in utero exposure to PM_{10}, NO_2, temperature and humidity. Associating a concept-driven analysis with an agnostic EWAS, we showed lower methylation levels in two CpGs located in the body of *ADORA2B* associated with increased exposure to NO_2 during pregnancy, especially in trimesters 1 and 2. The agnostic EWAS further revealed new significant associations of prenatal exposure to PM_{10}, NO_2, humidity and temperature during various time-windows and methylation of 4 CpGs (mapped to 2 genes (*PSD3*, *ZNF709*) and 2 non genic regions) and 39 regions (Table 3, Table S9), which are candidates for replication in future studies. Among these, several DMR were located in genes pointing towards hypertensive and metabolic disorders.

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

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

et al., 2007) and hypomethylated in preeclamptic placentas (Chu et al., 2014). In our study, NO_2 exposure in pregnancy, 3rd trimester and last month of pregnancy was positively associated with *P2RX4* methylation. In accordance with our result, a CpG of *P2RX4* was found hypermethylated in blood from adults exposed to $PM_{2.5}$ in the previous month (Panni et al., 2016). In addition, our results indicated a positive association of PM_{10} exposure the day before birth with methylation in a DMR located in *CD81* and a negative association with DMRs in *DAXX* and *NOTCH3*. *CD81*, a gene encoding a member of the transmembrane 4 superfamily, was shown to be mainly expressed in normal first trimester placentas and gradually downregulated with gestational age (Shen et al., 2017). In early-onset (≤34 weeks) severe preeclamptic placentas, *CD81* was up-regulated in syncytiotrophoblasts and extravillous cytotrophoblasts, which play a major role in maternal–fetal exchanges (Malassiné and Cronier, 2002) and placentation (Pijnenborg et al., 2006). The overexpression of *CD81* was also demonstrated in a PE-like phenotype in rats (Shen et al., 2017). A recent study demonstrated that *DAXX* methylation was involved in trophoblast differentiation, PE, and response to hypoxia, suggesting an important role of *DAXX* in placentation (Novakovic et al., 2017). Similarly, *NOTCH3* placental expression has been shown down regulated in PE (Fragkiadaki et al., 2015) and up regulated in early onset PE (Zhao et al., 2014). Notch signaling has been suggested to play a key role in cytotrophoblasts motility and differentiation (Haider et al., 2014). In contrast to our finding, *NOTCH3* methylation in blood form adults has been positively associated with $PM_{2.5}$ exposure the previous month (Panni et al., 2016). Further studies are required in order to determine the functional relationship between these genes and their placental methylation. Hypertensive disorders, among which preeclampsia in pregnant women (M. Pedersen et al., 2014), are increasingly suspected as a potential mechanism to explain health effects of air pollutants (Shah et al., 2013).

Among air pollutants associated DMR in our study, four were located in *LMF1*, *GNAS*, *GNASAS*, and *IL-32* genes. These genes have been previously involved in metabolic syndrome and related phenotypes (Hong et al., 2017). Single nucleotide polymorphisms of *LMF1* is involved in severe

hypertriglyceridemia (Péterfy, 2012). *LMF1* blood methylation in adults has been positively associated with $PM_{2.5}$ exposure in the previous month (Panni et al., 2016), while our results indicated a negative association of NO_2 exposure in the 3rd trimester with placental methylation of this gene. *GNAS* is an imprinted gene with a complex imprinted pattern ; decreased placental expression has been associated with intra uterine growth retardation (Dunk et al., 2012; McMinn et al., 2006) and cord blood hypermetylation has been associated with gestational diabetes (D. Chen et al., 2014). *GNASAS*, the GNAS antisense RNA 1, placental expression was related to newborns neurobehavioral profile (Green et al., 2016), while its methylation in adult blood has been linked to prenatal exposure to famine (Tobi et al., 2009). In rabbits, pregnancy exposure to diesel engine exhaust has been associated with changes in triglyceride and cholesterol levels in the offspring exposed *in utero* (Valentino et al., 2015) but further investigations are required to identify if this association could be to some extent mediated by epigenetic changes. Taken together our findings point to PE- and metabolic syndrome-associated genes, which is in line with the increasing evidence on the relationship between air pollution and the metabolic syndrome (B.-Y. Yang et al., 2018) and with the hypothesis that such effects might start early in life (Park, 2017).

Significant variations of the methylation levels of other CpGs and DMR, identified either from the concept-driven analysis or from the agnostic EWAS approach, have occasionally been found associated with NO_2, PM_{10}, temperature or humidity exposure at specific time windows of pregnancy. However, the biological significance of the differential methylation observed for these genes is unclear (Supplemental material, Table S10). These genes have not been associated with NO_2 or proximity to traffic in the two recent EWAS conducted in newborns (Gruzieva et al., 2017; Kingsley et al., 2016). Noteworthy, some of these results were in agreement with air pollution association studies conducted on blood DNA methylation. This was the case for the positive association of methylation in *LZTFL1*-DMR with average NO_2 exposure during pregnancy, 3rd trimester and last month of pregnancy which was consistent with the relationship between *LZTFL1* blood methylation and chronic $PM_{2.5}$ exposure observed in an elderly population (Nwanaji-

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

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

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

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

A novel feature of our study is the focus on genes with tissue specific patterns of expression, in which we identified first a category of genes that are mostly active in the placenta and silent in other tissues ("active genes") and second a category of genes with a methylation-dependent repressed state ("silent genes") in the placenta. The definition of silent and active chromatin points to regions with different dynamics and hence different capacity to respond to signals and to be reprogrammed. In order to identify these regions, we used available transcriptomic data (for selecting genes with specific patterns of expression in placenta) and the present methylome data (list of silent genes with high methylation levels). This original approach not only allowed us to identify potential target CpGs through the activity of their regulatory regions, but also to limit the number of CpG sites to the most relevant ones to be tested in relation to environmental exposures, without limiting the analysis to particular pathways previously identified in the literature. In the context of a constantly expanding number of CpGs interrogated by microarrays and sequencing techniques, which usually exceeds the sample size of the study, replication is often used to validate the findings and limit the risk of false positives. Our strategy was rather to restrict our analysis to the most biologically relevant genes and chromatin regions, which we defined as those prone to be affected by alterations in methylation. Noteworthy, the results of the EWAS, which used a more 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_{10} and NO_2 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. $PM_{2.5}$ are of great concern because they can penetrate deep into the
593 lungs and enter the blood circulation. While our results identified mainly NO_2 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.

6. Conclusions

Among the placenta methylated silent genes, we identified four CpGs, mapped to four genes, whose methylation levels in the foetal placenta were significantly associated with maternal exposures to NO_2 and PM_{10}. Among placenta active genes, we found five CpGs, mapped to five genes, whose methylation levels in the foetal placenta were significantly associated with maternal exposures to NO_2 and PM_{10}. One of these active genes, *ADORA2B*, had 2 CpGs whose methylation levels were also significantly associated with NO_2 exposure during the second trimester of pregnancy in the EWAS approach after FDR correction of the p-value. The DMRs analyses showed ADORA2B methylation as associated with NO_2 exposure in pregnancy and at each trimester of pregnancy. *ADORA2B* has been implicated in placental development, hypoxia and preeclampsia, a pathology which has been previously associated with air pollution exposure during pregnancy. This latter observation, together with our results, supports the hypothesis that placental DNA methylation is involved in the exposure-disease relationships during pregnancy. Whether the observed associations potentially bear long-term consequences for the health of the child and adult individual, or even possibly for future generations, remains to be investigated.

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