

1 **Supplement**

2 **Pregnancy exposure to synthetic phenols and placental DNA methylation — An epigenome-**  
3 **wide association study in male infants from the EDEN cohort**

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## 47 **Supplementary methods**

### 48 **1. Study exclusion criteria**

49 Women with multiple fetuses, maternal diabetes before pregnancy, intention to deliver outside the  
50 university hospital or to move out of the study region within the next three years, and unable to speak  
51 French were excluded from the study.

### 52 **2. Quantification of maternal concentrations of creatinine and phenols**

53 Quantification of maternal concentrations of creatinine and nine phenols (free plus conjugated  
54 species): bisphenol A, benzophenone-3, triclosan, 2,4- and 2,5-dichlorophenol and butyl-, ethyl-,  
55 methyl-, propylparaben was performed at the Centers for Disease Control and Prevention (CDC). In  
56 short, 0.1 mL of urine spiked with the appropriate reagents and standards were incubated to hydrolyze  
57 the biomarkers urinary conjugates. The procedure for extracting the deconjugated biomarkers from  
58 the urine involved concurrent online solid phase extraction and high performance liquid  
59 chromatography followed by isotope dilution tandem mass spectrometry as detailed before (Ye et al.  
60 2005).

### 61 **3. Quality control/quality assurance for the assessment of synthetic phenols in maternal** 62 **urine**

63 The CDC laboratory quantified the phenols following the strict quality control/quality assurance  
64 requirements set forth in the Clinical Laboratory Improvement Amendments of 1988  
65 (<https://www.cms.gov/Regulations-and-Guidance/Legislation/CLIA/index.html>). These requirements  
66 include evaluation of calibrators and successfully analyzing twice per year proficiency testing samples  
67 such as those provided by the University of Erlangen-Nuremberg's German External Quality  
68 Assessment Scheme (G-EQUAS) and the Centre de Toxicologie du Québec's External Quality  
69 Assessment Scheme for Organic Substances in Urine (OSEQAS). Furthermore, along with study  
70 samples, each analytical run included a set of calibrators, high- and low-concentration quality control

71 materials, and reagent blanks to assure the accuracy and reliability of the data. CDC has used the  
72 method to quantify phenols since the early 2000s for the analyses of tens of thousands of biological  
73 specimens, including those collected as part of the ongoing U.S. National Health and Nutrition  
74 Examination Survey (NHANES). Precision (relative standard deviation from repeated measurements  
75 of quality control materials) varied from 4% to 10%, depending on the analyte.

#### 76 **4. Phenol concentrations standardization**

77 Phenol concentrations were standardized on sampling conditions (hour of sampling, day of  
78 sampling, year of sample analysis, gestational age at collection, duration of storage at room  
79 temperature before freezing) and creatinine concentration using a method based on regression  
80 residuals (Mortamais et al. 2012).

#### 81 **5. Placental tissue collection and DNA extraction**

82 Placental tissue from the fetal side, a few centimeters from the insertion of the cord, was sampled  
83 at delivery by the EDEN midwife or a technician of the study using a standardized procedure.  
84 Samples of around 5mm<sup>3</sup> were collected and immediately frozen at -80°C. DNA was extracted by  
85 QIAGEN Genomic Services using the QIASymphony instrument (QIAGEN GmbH, Germany) with  
86 the QIASymphony DSP DNA Mini Kit according to the instruction. DNA concentration was  
87 determined by Nanodrop (ThermoFisher Scientific, USA) measurement and fluorescent quantification  
88 using PicoGreen (ThermoFisher Scientific, France). No samples were discarded due to low DNA  
89 concentration.

#### 90 **6. Placental DNA methylation quality control, normalization and filtering of outliers**

91 To reduce the influence of technical factors such as batch effects and to ensure the balance of  
92 individuals from each gender and each recruitment center (Poitiers/ Nancy), DNA samples from the  
93 main experiment (n = 668 containing children of both genders) were randomly allocated to assay  
94 chips. 24 samples were measured in replicates (from two to five replicates) across batches, sample

95 plates and chips to detect technical issues, if any. Raw intensities of fluorescent signals were  
96 processed with the Chip Analysis Methylation Pipeline (ChAMP) V2.14 (Morris et al. 2014; Tian et  
97 al. 2017). All samples but one passed initial quality control with an average of >98% valid data points  
98 (p-value <0.01). Among the 667 samples that passed quality control, 202 were included in our study.  
99 Filtering included removal of probes with detection p-values above 0.01 (52,692 probes), low  
100 numbers of measured events [beadcount <3 in at least 5% of samples (44 probes)], probes not  
101 targeting a CpG (2,034 probes), probes associated with SNPs (50,829 probes) or unspecific probes (9  
102 probes) (Nordlund et al. 2013). Methylation levels of individual CpGs were reported as continuous  
103 averaged  $\beta$ -values, representing the proportion of methylated alleles for each methylation site ranging  
104 from 0 (indicating that the site is completely unmethylated) to 1 (completely methylated), and were  
105 normalized in ChAMP via the Beta Mixture Quantile (BMIQ) normalization (Teschendorff et al.  
106 2013) (Appendix Figure 5). To reduce the influence of outliers, methylation beta values above the  
107 75th percentile + three interquartile ranges (IQRs) or below the 25th percentile - three IQRs for a CpG  
108 were removed (in total 0.39% of all methylation values in our subsample of 202 participants). 379,904  
109 methylation sites remained after quality control, normalization and filtering of outliers.

## 110 **7. Placental tissue heterogeneity estimation**

111 To estimate placental tissue heterogeneity, we followed the pipeline recommended by Decamps et  
112 al. (Decamps et al. 2020). First, we filtered out the probes correlated with factors affecting the DNA  
113 methylation but unlikely to affect the placental cell proportions, such as center of recruitment,  
114 delivery mode and technical factors (batch, plate and chip). To assess the associations between these  
115 factors and each probe we ran univariate linear regressions (*CF\_detection* function from the *medepir*  
116 R package, <https://bcm-uga.github.io/medepir>) with default settings. CpGs associated with at least one  
117 of these factors (p-value <0.15) were excluded from the cell mix estimation. For the 3,645 remaining  
118 CpGs we identified six putative constituent cell types using Cattell's rule applied to the scree plot  
119 (Cattell 1966) and then estimated tissue heterogeneity for the same set of probes using a reference-

120 free based method via the *RefFreeCellMix* function from the *RefFreeEWAS* package in R (Houseman  
121 et al. 2016).

## 122 **8. Mediation analysis**

123 To explore whether placental cell heterogeneity could mediate the association between phenol  
124 exposure and DNA methylation levels, we first estimated the difference between regression estimates  
125 obtained for the models unadjusted and adjusted for the estimated placental tissue heterogeneity. The  
126 percentage difference was calculated using the following formula:  $[(\beta_{\text{adjusted}} - \beta_{\text{unadjusted}}) / \beta_{\text{unadjusted}}] \times$   
127 100%. Regression coefficient values showing  $\geq 20\%$  absolute difference between adjusted and  
128 unadjusted effect estimates were further tested for mediation. The six estimated placental cell types  
129 were reduced to the first principal component (PC1) explaining  $>50\%$  of the variance of the cell  
130 heterogeneity. PC1 was then used in the mediation analysis using the *mediation* R package (Tingley et  
131 al. 2014).

132 **Appendix Tables**

133 **Appendix Table 1:** Population characteristics for the mother-son pairs included in the study and  
 134 recruited between 2003 and 2006 (n = 202).

<b>Characteristics</b>	<b>Distribution</b>	
	<b>n (%)</b>	<b>Median [Q1, Q3]</b>
<b>Center of recruitment</b>		
Nancy	103 (51.0%)	
Poitiers	99 (49.0%)	
<b>Season of conception</b>		
January-March	44 (21.8%)	
April-June	41 (20.3%)	
July-September	57 (28.2%)	
October-December	60 (29.7%)	
<b>Maternal active smoking in the 3 months preceding pregnancy and during pregnancy</b>		
Did not smoke	127 (62.9%)	
Smoked before pregnancy	19 (9.4%)	
Smoked before and during pregnancy	26 (12.9%)	
Other	30 (14.9%)	
<b>Parity</b>		
Nulliparous	88 (43.6%)	
≥ 1 child	114 (56.4%)	
<b>Maternal level of education</b>		
<2 years after high school	93 (46.0%)	
high school + 2 years	43 (21.3%)	
≥high school + 3 years	66 (32.7%)	
<b>Maternal age (years)</b>		29.1 [25.6;33.0]
<b>Gestational age at delivery (weeks)<sup>a</sup></b>		40.0 [38.9;41.0]

135 <sup>a</sup> Based on the date of the LMP or gestational duration assessed by the obstetrician if it differed from  
 136 the LMP-based estimate by more than 2 weeks. Abbreviations: LMP = last menstrual period. Q =  
 137 quantile.  
 138

139 **Appendix Table 2:** Maternal urinary phenol concentrations assessed between 22 and 29 gestational  
 140 weeks in spot urine samples, measured and standardized on sampling conditions (n = 202).

Phenol	LOD (µg/L)	% > LOD	Measured concentrations			Standardized concentrations <sup>a</sup>		
			Percentiles (µg/L)			Percentiles (µg/L)		
			5th	50th	95th	5th	50th	95th
<b>2,4-dichlorophenol</b>	0.2	98	0.3	1.1	7.9	0.3	1.1	5.9
<b>2,5-dichlorophenol</b>	0.2	100	2.3	10.3	187.5	2.0	10.3	161.6
<b>∑ dichlorophenols</b>	NA	NA	0.0	0.1	1.2	0.0	0.1	1.0
<b>Bisphenol A</b>	0.4	100	0.7	2.7	11.4	0.9	2.2	9.6
<b>Benzophenone-3</b>	0.4	89.1	< LOD	2.2	65.2	< LOD	2.0	53.2
<b>Triclosan</b>	2.3	84.2	< LOD	38.0	889.1	< LOD	43.1	758.6
<b>Butylparaben</b>	0.2	86.1	< LOD	1.9	77.8	< LOD	1.9	72.4
<b>Ethylparaben</b>	1.0	72.8	< LOD	4.1	61.9	< LOD	2.9	47.8
<b>Methylparaben</b>	1.0	100	8.4	98.8	1135.5	10.3	101.6	980.2
<b>Propylparaben</b>	0.2	98	0.6	15.4	304.4	0.5	12.2	249.8

141

142 Phenol concentrations are displayed in µg/L for all compounds except for the ∑ dichlorophenols for  
 143 which the concentrations are presented as µmol/L.

144 <sup>a</sup> Measured concentrations were standardized on sampling conditions using a method based on  
 145 regression residuals (Mortamais et al. 2012).

146 Abbreviations: LOD = limit of detection.

147



148 **Appendix Table 3:** Differentially methylated CpGs associated with pregnancy concentrations of  
149 phenols in EWAS unadjusted and adjusted for placental cell heterogeneity (FDR p-value <0.05, n =  
150 202, 379,904 CpGs).

151 [PLEASE REFER TO THE EXTERNAL APPENDIX TABLE 3](#)

152  
153 EWAS regression models were adjusted for recruitment center, maternal active smoking in the three  
154 months preceding pregnancy and during pregnancy, maternal age, parity, maternal education level,  
155 season of conception, batch, plate, and chip. Sensitivity analysis was additionally adjusted for  
156 placental cell heterogeneity and the percentage difference between regression coefficient estimates  
157 was calculated using the following formula:  $[(\beta_{\text{adjusted}} - \beta_{\text{unadjusted}}) / \beta_{\text{unadjusted}}] \times 100\%$ . ACME p-value  
158 was calculated using the first principal component representing six reference-free estimated placental  
159 cell types.

160 <sup>a</sup> UCSC.

161 <sup>b</sup> Mediation analysis was performed for CpGs associated with triclosan for which the absolute  
162 percentage difference between regression coefficient estimates obtained in models unadjusted and  
163 adjusted for placental tissue heterogeneity was  $\geq 20\%$ .

164 Abbreviations: ACME = average causal mediation effect. Chr = chromosome. CI = confidence  
165 interval. FDR = false discovery rate. UCSC = University of California, Santa Cruz.

166

167 **Appendix Table 4:** DMRs associated with pregnancy concentrations of phenols unadjusted and  
168 adjusted for placental cell heterogeneity (Šidák-corrected p-value <0.05, n = 202, 379,904 CpGs).

169 [PLEASE REFER TO THE EXTERNAL APPENDIX TABLE 4](#)

170  
171 EWAS regression models on which the DMR analysis was based were adjusted for recruitment  
172 center, maternal active smoking in the three months preceding pregnancy and during pregnancy,  
173 maternal age, parity, maternal education level, season of conception, batch, plate, and chip. Sensitivity  
174 analysis was additionally adjusted for placental cell heterogeneity. DMRs with less than five CpGs are  
175 highlighted in grey.

176 <sup>a</sup> UCSC.

177 Abbreviations: Chr = chromosome; DMR = differentially methylated region. NS = not significant.

178 SLK = Stouffer-Liptak-Kechris correction. UCSC = University of California, Santa Cruz.

179

180 **Appendix Table 5:** Data on the function of the genes associated with phenol concentrations.

181 [PLEASE REFER TO THE EXTERNAL APPENDIX TABLE 5](#)

182 Information on genes encompassed by the DMRs identified as associated with phenol concentrations  
183 were retrieved from the GeneCards Human Gene Database (Stelzer et al. 2016).

184

185 **Appendix Table 6:** Adjusted global analysis of methylation profiles (GAMP) associated with  
 186 pregnancy concentrations of phenols (n = 202, 379,904 CpGs).

Phenol	CDF <sup>a</sup> p-value	Density <sup>b</sup> p-value
<b>2,4-dichlorophenol</b>	0.32	0.46
<b>2,5-dichlorophenol</b>	0.27	0.48
<b>∑ dichlorophenols</b>	0.25	0.47
<b>Bisphenol A</b>	0.67	0.84
<b>Benzophenone-3</b>	0.14	0.6
<b>Triclosan</b>	0.55	0.67
<b>Butylparaben</b>	0.57	0.9
<b>Ethylparaben</b>	0.24	1.00
<b>Methylparaben</b>	0.13	0.24
<b>Propylparaben</b>	0.56	0.51

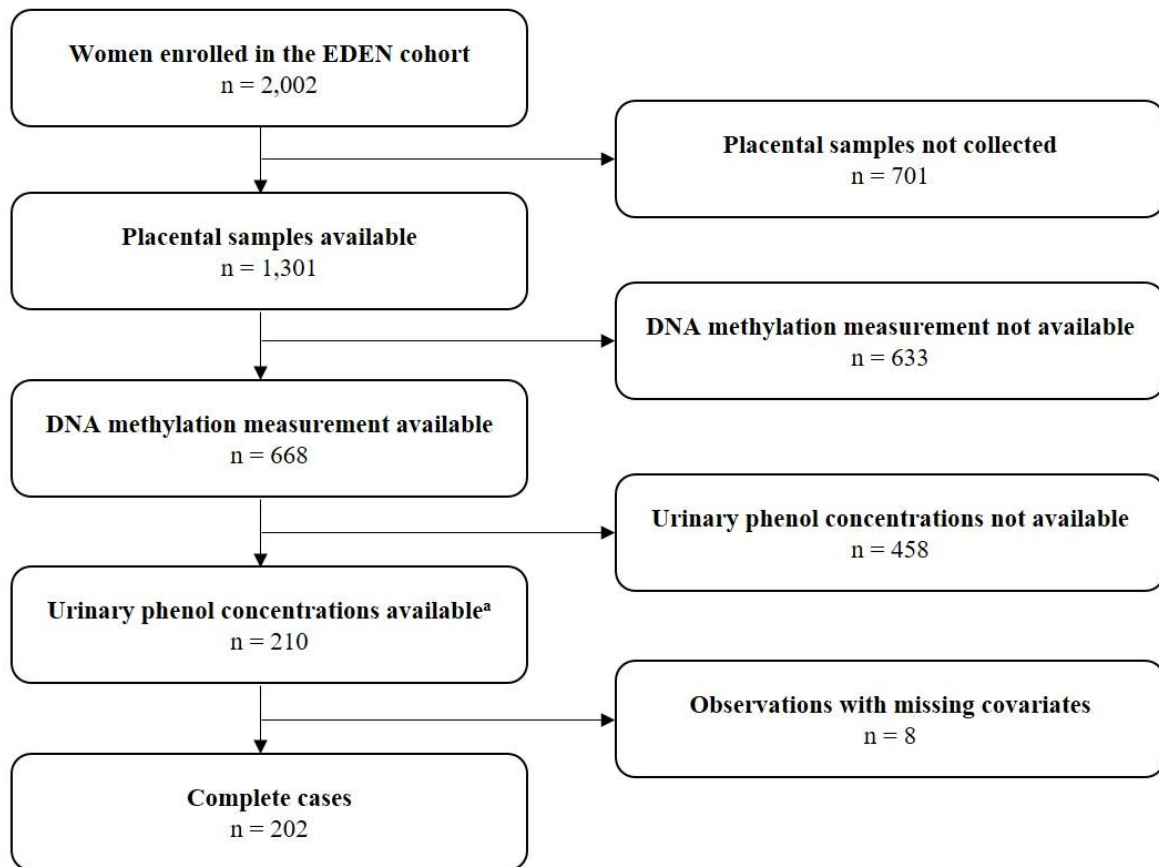
187  
 188 GAMP models were adjusted for recruitment center, maternal active smoking in the three months  
 189 preceding pregnancy and during pregnancy, maternal age, parity, maternal education level, season of  
 190 conception, batch, plate, and chip.

191 <sup>a</sup> Tests the association of the CDF of the observed methylation distributions for each individual with  
 192 each exposure variable.

193 <sup>b</sup> Tests whether the densities of the observed methylation distributions for each individual are  
 194 associated with an exposure variable.

195 Abbreviations: CDF = cumulative distribution function. GAMP = global analysis of methylation  
 196 profiles.

197 **Appendix Figures**



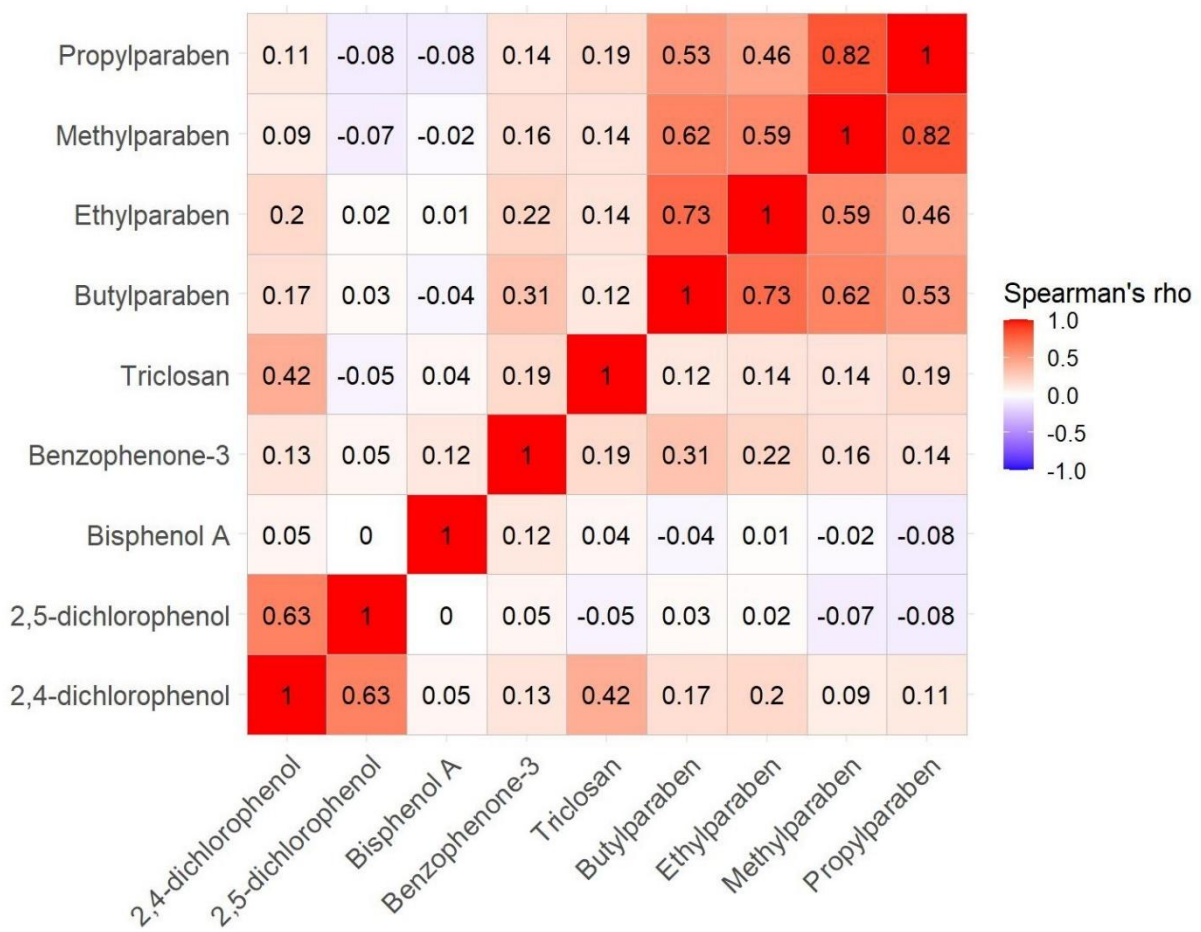
198

199 **Appendix Figure 1:** Study flow chart.

200 <sup>a</sup> Phenol concentrations were assessed in the framework of a previous study restricted to boys and with  
201 at least one maternal urine sample available for phenol measurements and complete data on prenatal  
202 and postnatal growth (Philippat et al. 2014).

203

204

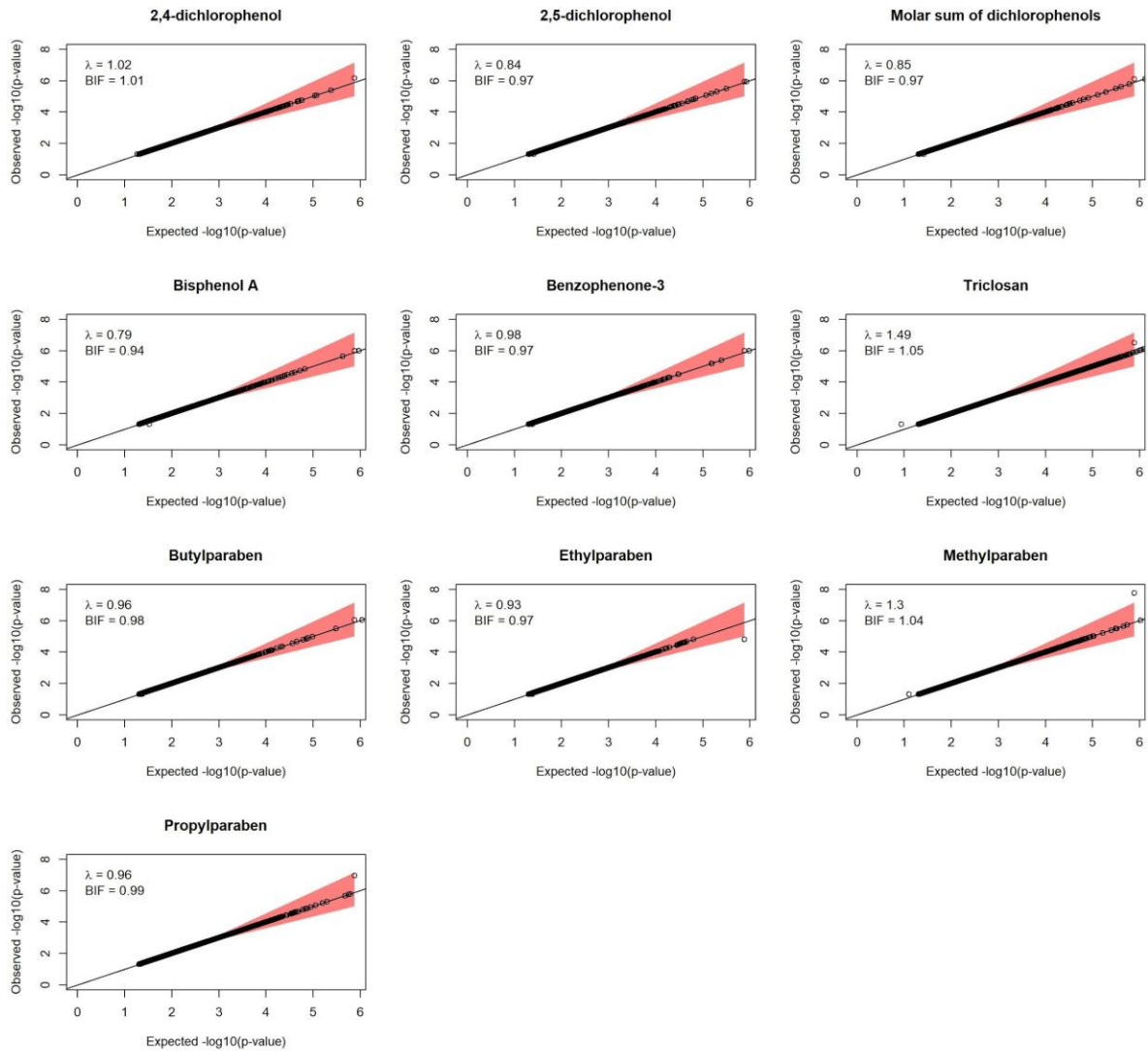


205

206 **Appendix Figure 2:** Spearman's correlation coefficients (rho) between standardized urinary phenol  
207 concentrations (n = 202).

208

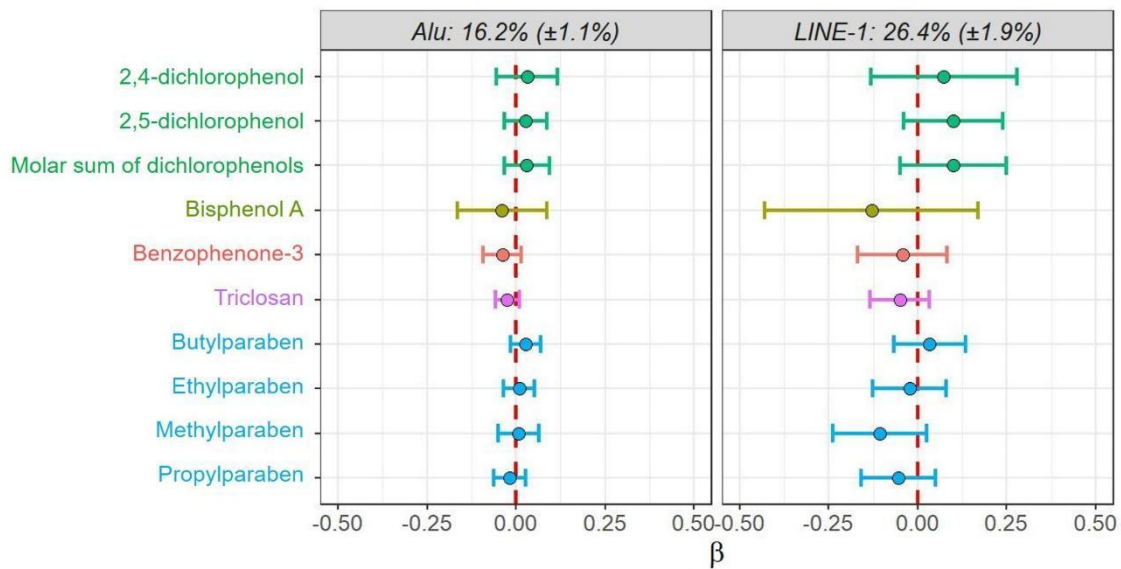
209



210

211 **Appendix Figure 3:** Q-Q plots with genomic inflation factor ( $\lambda$ ) and Bayesian inflation factor (BIF)  
 212 for the association between phenol concentrations and DNA methylation sites in the EWAS ( $n = 202,$   
 213  $379,904$  CpGs). Red area marks the confidence interval of the fit. Regression models were adjusted  
 214 for recruitment center, maternal active smoking in the three months preceding pregnancy and during  
 215 pregnancy, maternal age, parity, maternal education level, season of conception, batch, plate, and  
 216 chip.

217



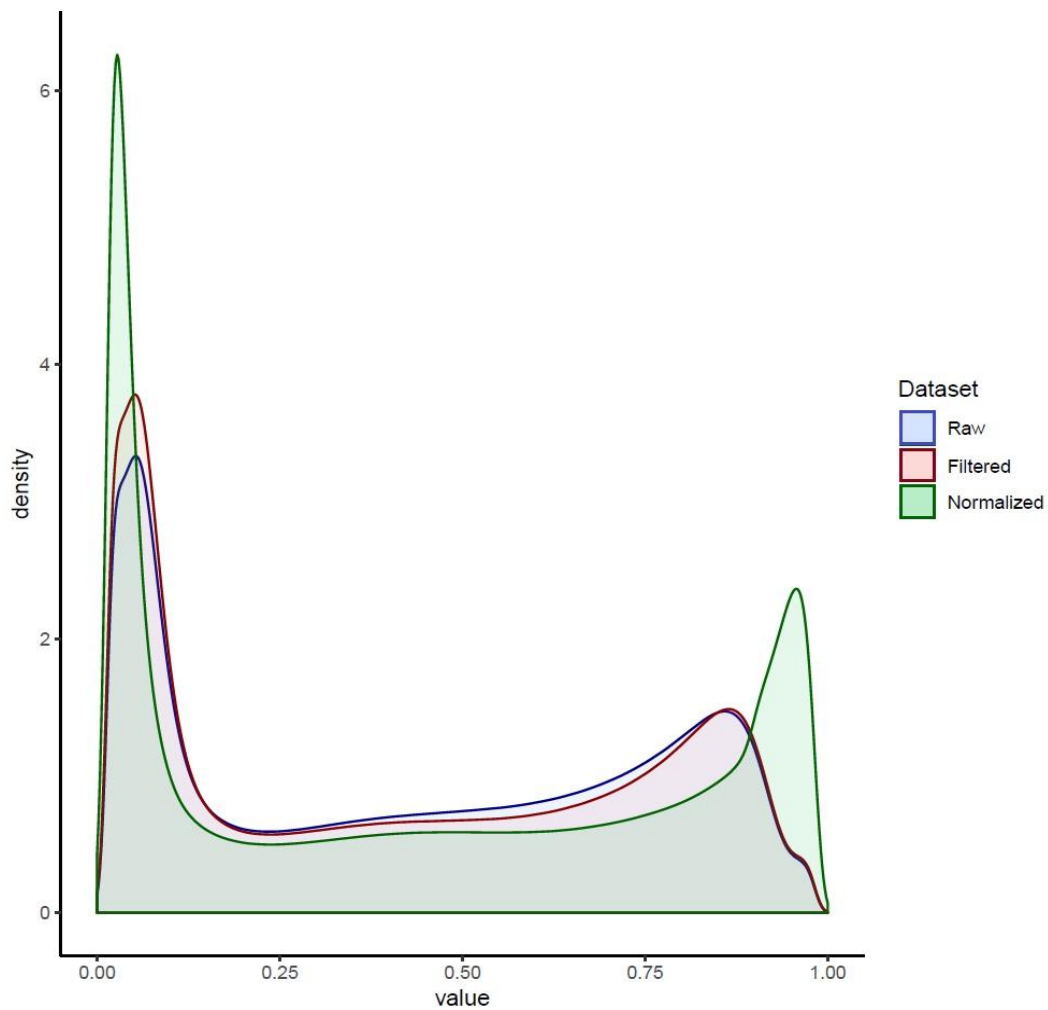
218

219 **Appendix Figure 4:** Adjusted associations between pregnancy concentrations of phenols and  
 220 methylation levels of the repetitive elements *Alu* and *LINE-1* (n = 201). Circles represent  $\beta$  regression  
 221 coefficient estimates reported with 95% CIs and correspond to a change in the global DNA  
 222 methylation level for doubling of the urinary phenol concentration. Same color represents phenols  
 223 belonging to the same family. Regression models were adjusted for recruitment center, maternal  
 224 active smoking in the three months preceding pregnancy and during pregnancy, maternal age, parity,  
 225 maternal education level, season of conception, batch, and plate.

226 Abbreviations: CI = confidence interval.

227





228

229 **Appendix Figure 5:** Impact of data processing and normalization on the overall DNA methylation  
 230 profile of placental samples ( $n = 767, 668$  samples and replicates) using the ChAMP. Blue color  
 231 represents raw data as extracted from the .idat files; red color represents filtered data (removal of  
 232 probes with detection p-values  $>0.01$ , with a beadcount  $<3$  in at least 5% of samples, probes not  
 233 targeting CG, associated with SNPs or unspecific probes); green color represents BMIQ normalized  
 234 data.

235 Abbreviations: BMIQ = Beta Mixture Quantile. ChAMP = Chip Analysis Methylation Pipeline.  
 236 SNP = single nucleotide polymorphism.

237

238

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