



Title	Effect of prenatal exposure to phthalates on epigenome-wide DNA methylations in cord blood and implications for fetal growth : The Hokkaido Study on Environment and Children's Health
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1 **Effect of prenatal exposure to phthalates on epigenome-wide DNA**
2 **methylations in cord blood and implications for fetal growth: The**
3 **Hokkaido Study on Environment and Children's Health**

4

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18 **ABSTRACT**

19 Prenatal exposure to phthalates negatively affects the offspring's health. In particular,
20 epigenetic alterations, such as DNA methylation, may connect phthalate exposure with health
21 outcomes. Here, we evaluated the association of di-2-ethylhexyl phthalate (DEHP) exposure
22 *in utero* with cord blood epigenome-wide DNA methylation in 203 mother-child pairs
23 enrolled in the Hokkaido Study on Environment and Children's Health, using the Illumina
24 HumanMethylation450 BeadChip. Epigenome-wide association analysis demonstrated the
25 predominant positive associations between the levels of the primary metabolite of DEHP,
26 mono(2-ethylhexyl) phthalate (MEHP), in maternal blood and DNA methylation levels in
27 cord blood. The genes annotated to the CpGs positively associated with MEHP levels were
28 enriched for pathways related to metabolism, the endocrine system, and signal transduction.
29 Among them, methylation levels of CpGs involved in metabolism were inversely associated
30 with the offspring's ponderal index (PI). Further, clustering and mediation analyses suggested
31 that multiple increased methylation changes may jointly mediate the association of DEHP
32 exposure *in utero* with the offspring's PI at birth. Although further studies are required to
33 assess the impact of these changes, this study suggests that differential DNA methylation
34 may link phthalate exposure *in utero* to fetal growth and further imply that DNA methylation
35 has predictive value for the offspring's obesity.

36

37 **Keywords:** EWAS, DEHP, MEHP, increased methylation, ponderal index

Abbreviations: EDC, Endocrine-disrupting chemicals; EWAS, Epigenome-wide association studies; DMR, Differentially methylated regions; DEHP, di-2-ethylhexyl phthalate; CpG, cytosine-guanine dinucleotide; PI, Ponderal index; MEHP, mono(2-ethylhexyl) phthalate; BMI, Body mass index; FDR, False discovery rate; DMCpG, differentially methylated CpG, DRHM-CpGs, DEHP-related higher methylated CpGs; KEGG, Kyoto Encyclopedia Genes and Genomes; SD, Standard deviation; TSS200, 200 bases from the transcription start site; IGR, Intergenic region; GO, Gene Ontology; MAPK, Mitogen-activated protein kinase.

38 **1. Introduction**

39 Phthalates are widely used plasticizers (Koch et al. 2013) included in the composition
40 of consumer products, such as food packages, toys, and personal care products, which can
41 lead to chemical exposure through ingestion, inhalation, and skin adsorption (Ait Bamai et al.
42 2015; Jensen et al. 2015). They are potential endocrine-disrupting chemicals (EDCs) and
43 have been found to exert various adverse effects that negatively impact an individual's
44 health. In particular, phthalate exposure *in utero* has been linked to adverse birth outcomes,
45 such as decreased birth size (Minatoya et al. 2017; Song et al. 2018; Whyatt et al. 2009)
46 preterm birth (Ferguson et al. 2017; Huang et al. 2014), pregnancy loss (Gao et al. 2017), and
47 reduced anogenital distance in infants (Swan et al. 2015). Prenatal exposure to phthalates can
48 also affect childhood health outcomes, such as behavioral problems (Engel et al. 2010; Engel
49 et al. 2009; Minatoya et al. 2018b; Tellez-Rojo et al. 2013), obesity (Buckley et al. 2016;
50 Kim and Park 2014), and allergic diseases (Ait Bamai et al. 2018; Jaakkola and Knight 2008;
51 Whyatt et al. 2014). Based on these, although phthalates are rapidly metabolized and
52 excreted, early life exposure to phthalates may contribute to long-term health outcomes
53 (Koch et al. 2013). However, the potential mechanisms underlying their long-lasting effects
54 have not been fully elucidated. Epigenetic modifications, e.g., DNA methylation, may
55 represent potential mechanisms by which phthalate exposure *in utero* exerts long-term
56 effects. Several studies have indicated that epigenetic changes may connect EDC exposure
57 in the developmental stage with long-term adverse health outcomes (Barouki et al. 2018; Ho
58 et al. 2017; McLachlan 2016; Tapia-Orozco et al. 2017). In addition, animal studies have
59 demonstrated that developmental phthalate exposure was associated with DNA methylation
60 changes in the offspring (Abdel-Maksoud et al. 2015; Manikkam et al. 2013; Martinez-
61 Arguelles and Papadopoulos 2015; Rajesh and Balasubramanian 2015; Sekaran and
62 Jagadeesan 2015; Wu et al. 2010). Several human cohort studies have also shown that

63 prenatal phthalate exposure correlates with DNA methylation changes in selected candidate
64 genes, using placenta (LaRocca et al. 2014; Zhao et al. 2016; Zhao et al. 2015) or cord blood
65 samples (Huang et al. 2018; Huen et al. 2016; Montrose et al. 2018; Tindula et al. 2018).
66 Recently, a few epigenome-wide association studies (EWASs) were published, allowing a
67 unbiased assessment of epigenetic modifications associated with environmental factors
68 (Christensen and Marsit 2011). Among them, one study reported that phthalate exposure
69 altered the placental methylome and DNA methylation modification on the epidermal growth
70 factor receptor significantly mediated the associated effects from phthalates exposure on
71 early placental function (Grindler et al. 2018). Moreover, several differentially methylated
72 regions (DMRs) in cord blood associated with prenatal phthalate exposure have been
73 identified (Solomon et al. 2017). Genes with these regions are implicated in the inflammation
74 reaction, cancer, endocrine function, and male fertility. Another study also investigated
75 genome-wide DNA methylation changes in cord blood associated with prenatal exposure to
76 the most common phthalate, di-2-ethylhexyl phthalate (DEHP), and suggested that DNA
77 methylation in genes involved in the androgen response, spermatogenesis, and cancer-related
78 pathways may be affected by prenatal exposure to this chemical (Chen et al. 2018). Although
79 existing evidence supports the role of prenatal phthalate exposure in modifying DNA
80 methylation, few studies have focused on the potential effects of phthalate exposure-
81 associated methylation changes on the developing fetus and later in life.

82 Here, using an epigenome-wide approach, we aimed to elucidate the relation between
83 prenatal DEHP exposure and cord blood DNA methylation from participants of the Hokkaido
84 Study. Furthermore, we explored whether DNA methylation at the identified loci mediated
85 the effect of prenatal DEHP exposure on the ponderal index (PI) at birth as an indicator of
86 fetal growth.

87

88 **2. Materials and Methods**

89 ***2.1 Study population***

90 Details of participants enrolled in the Sapporo cohort of the Hokkaido Study on
91 Environment and Children's Health were previously described (Kishi et al. 2017; Kishi et al.
92 2013; Kishi et al. 2011).

93

94 ***2.2 Measurement of the primary metabolite of DEHP; mono(2-ethylhexyl) phthalate*** 95 ***(MEHP)***

96 Maternal blood samples were obtained during the hospital examination of participants
97 and stored at -80°C . Concentrations of MEHP in maternal blood, as an indicator of DEHP
98 exposure, were measured via gas chromatography mass spectrometry at Nagoya University,
99 as described (Araki et al. 2017; Araki et al. 2014; Jia et al. 2015). The detection limit was
100 0.28 ng/mL.

101

102 ***2.3 450K DNA methylation analysis***

103 Umbilical cord bloods were collected immediately after birth and then stored at -80°C .
104 Cord blood DNA methylation levels at 485,577 CpGs was measured using the Infinium
105 HumanMethylation450 BeadChip (Illumina Inc., San Diego, CA, USA) by G&G Science
106 Co., Ltd. (Fukushima, Japan). Details of the 450K methylation analysis have been described
107 previously (Miura et al. 2019; Miura et al. 2018). After quality control (Aryee et al. 2014),
108 functional normalization (Fortin et al. 2014) and reducing the batch effects (Leek et al. 2012),
109 β -values, ranging from 0-1 for 0% to 100% methylated, at 426,413 CpG probes were
110 obtained.

111

112 ***2.4 Data analysis***

113 Among the 514 participants, 203 mother-infant pairs had detectable MEHP levels in
114 maternal blood and cord blood DNA methylation data. Data analyses methods were
115 previously described (Miura et al. 2019; Miura et al. 2018). Briefly, the associations of the β -
116 values with MEHP natural log (ln)-transformed concentrations were determined using robust
117 linear regression analysis (Fox and Weisberg 2011) with the *limma* package in the
118 R/Bioconductor, which was adjusted for maternal age, level of education, pre-pregnancy
119 body mass index (BMI), smoking status during pregnancy, blood sampling periods,
120 gestational age, infant sex, and estimates of cord blood cell counts for CD4⁺ T cells, CD8⁺ T
121 cells, monocytes, granulocytes, B cells, and nucleated red blood cells. The proportion of cord
122 blood cells was estimated using the *minfi* package in the R (ver.3.3.2)/Bioconductor (ver.
123 3.3). We selected covariates previously reported to be associated with exposure or cord blood
124 DNA methylation. For multiple comparisons, *p*-values were adjusted using a false discovery
125 rate (FDR) to obtain *q*-values. Since we obtained a reduced number of FDR-significant
126 findings, we evaluated the differentially methylated CpGs (DMCpGs) with an uncorrected *p*-
127 value < 2.5E-04. We also assessed DEHP-related higher methylated CpGs (DRHM-CpGs)
128 for functional enrichment with Kyoto Encyclopedia Genes and Genomes (KEGG) pathways
129 (Kanehisa et al. 2002) via the *gometh* function of the *missMethyl* package in R/Bioconductor
130 (Phipson et al. 2016).

131 To ascertain whether MEHP levels were associated with the characteristics of
132 participants, we utilized the Spearman's correlation test, Mann–Whitney *U* test, and Kruskal–
133 Wallis test.

134 Moreover, we examined associations between methylation levels (β -values) at DRHM-
135 CpGs and the PI at birth using a multivariate regression model adjusted for maternal age,
136 level of education, parity, pre-pregnancy BMI, smoking status during pregnancy, gestational

137 age, and infant sex, with JMP Pro 14 (SAS Institute Inc., Cary, NC, USA). The PI was
138 calculated as follows: $PI (kg/m^3) = \text{birth weight (kg)} / (\text{birth length (m)})^3$.

139 After identification of CpGs related to the PI, we tested the methylation patterns of
140 these CpGs for mediation in the association between maternal MEHP levels and the PI, using
141 a structural equation model from *lavaan* in R ver. 3.6.3. CpGs inversely associated with the
142 PI and with p -value < 0.1 were selected, and z-scores for methylation levels were calculated.
143 To determine inter-individual patterns in DNA methylation, we performed hierarchical
144 clustering with Euclidean distance and the Ward D2 agglomeration method (Clifford et al.
145 2011) in R and stratified participants by methylation profile. In the mediation analysis,
146 methylation levels (β) or the methylation cluster was used as a mediator, and models were
147 adjusted for $\ln(\text{MEHP})$, maternal age, gestational age, and infant sex in the association
148 between the methylation ~~cluster~~ and the PI, and for maternal age, smoking during pregnancy,
149 and blood sampling periods in the association between $\ln(\text{MEHP})$ and the methylation. These
150 factors were associated with the PI and methylation, respectively, with $p < 0.1$ in the
151 regression analysis. The clustering approach enables us to clarify whether the methylation in
152 each identified CpGs had occurred simultaneously or independently. In addition, they allow
153 to adequately incorporate the mediators into the model considering the inter-individual
154 patterns in DNA methylation.

155 The flow for the analyses is represented in Supplementary Figure S1.

156

157 **2.5 Ethics**

158 This study was conducted with written informed consent from all subjects. The study
159 protocol was approved by the institutional Ethical Board for Human Gene and Genome
160 Studies at the Hokkaido University Graduate School of Medicine and the Hokkaido

161 University Center for Environmental and Health Science. All experiments were performed in
162 accordance with the relevant guidelines and regulations.

163

164 **3. Results**

165 **3.1 Study population**

166 The characteristics of the subjects are shown in Table 1. The median MEHP
167 concentration in maternal blood was 10.3 ng/mL (interquartile range: 5.8–15.3 ng/mL), with
168 a 100% detection rate. The average \pm standard deviation (SD) of the mothers' age was 29.8 \pm
169 4.9 years. Maternal blood sampling periods were significantly associated with MEHP levels
170 (p -value < 0.01). Of the 203 newborns, 94 (46.3%) were male. The mean gestational age,
171 birth weight, and birth length were 39.9 weeks, 3137.5 g, and 48.5 cm, respectively. The
172 MEHP level was negatively correlated with the PI ($\rho = -0.133$, $p = 0.059$).

173

174 **3.2 EWAS of DEHP exposure in utero**

175 In adjusted robust linear regression models, there were two CpGs with significant
176 epigenome-wide methylation alteration (FDR q -value < 0.05): one located at 200 bases from
177 the transcription start site (TSS200) of *ZC3H10* (cg26409978) and another mapped to *SDK1*
178 (cg00564857), as shown in Figure 1A. Maternal MEHP levels showed more positive
179 association with methylation levels than negative association, as seen in the volcano plot. For
180 instance, of 271 DMCpGs with uncorrected p -values $< 2.5E-04$, 253 (93.4%) were positively
181 associated with MEHP levels (Figure 1B). The list of the DMCpGs with p -values $< 2.5E-04$
182 is available in the Supplemental Table S1.

183 We had very few findings with a significant false discovery rate (FDR) to confirm the
184 effect of prenatal DEHP exposure on DNA methylation changes. We examined the location
185 of the DRHM-CpGs with p -value $< 2.5E-04$ in gene features and CpG islands; notably, we

186 found statistically significant differences in the association with MEHP levels considering the
187 expected proportions (for gene features, X^2 p -value = 0.004; for CpG islands, X^2 p -value =
188 0.01; Figure 2). A decrease of methylation level in island and an increase in the intergenic
189 region (IGR) were particularly observed.

190 Next, we compared our results to those of a published study on the association between
191 prenatal phthalate exposures and DNA methylation in cord blood that used Illumina
192 HumanMethylation450 BeadChips (Solomon et al. 2017). In this study, the authors identified
193 seven DMRs associated with MEHP levels in maternal urine at 26 gestational weeks using
194 two different approaches (see Supplementary Table S2). We extracted the results of our
195 EWAS at CpGs in the DMRs identified by (Solomon et al. 2017) (Table 2). Since the CpGs
196 included in each region showed methylation alteration in the same direction, the average the
197 partial regression coefficients were shown in Table 2. Although no CpG was associated with
198 maternal MEHP levels with genome-wide statistical significance in our cohort, six of the
199 seven DMRs showed increased methylation changes. Among them, five DMRs that mapped
200 to *MUC4*, *C5orf63*, *CNPY1*, *SVIL-AS1*, and *FIBIN*, showed the same positive direction as
201 those identified by (Solomon et al. 2017).

202

203 **3.3 Gene Ontology (GO) analysis**

204 To investigate the biological processes influenced by DEHP-associated increased
205 methylation, we tested for KEGG pathway (Kanehisa et al. 2002) enrichment among the 253
206 DRHM-CpGs with $p < 2.5E-04$. We observed 12 enriched pathways with $FDR < 0.05$. GO
207 analyses of the data obtained from EWAS are inclined for cancer-related genes (Harper et al.
208 2013) and relatively healthier children were included in the analysis; therefore, the enriched
209 pathways excluding cancer and human disease pathways are listed in Table 3. The most
210 significant pathway was “metabolic pathway,” with $FDR = 2.4E-08$. We also observed three

211 pathways involved in the endocrine system—GnRH signaling pathway, renin secretion, and
212 cortisol synthesis and secretion—and two pathways involved in signal transduction: the
213 mitogen-activated protein kinase (MAPK) and Notch signaling pathways.

214

215 ***3.4 Methylation for mediation in the association between prenatal DEHP exposure and the*** 216 ***offspring's PI at birth***

217 Initially, we conducted multiple regression analyses to examine the association between
218 the PI and methylation levels at 16 DRHM-CpGs on genes involved in metabolic pathways
219 (Table 3). Of those, methylation levels at 12 DRHM-CpGs were inversely related to the PI
220 (Figure 3). In particular, the methylation levels at cg27433759:*PIK3CG*,
221 cg10548708:*ACAAI*, and cg07002201:*FUT9* were associated with PI with p -value < 0.1 .
222 Although the methylation levels at the three CpGs were positively correlated (Supplementary
223 Table S3), we could not determine whether the methylation in each identified CpGs had
224 occurred simultaneously or independently. To clarify this, we stratified samples based on the
225 methylation levels (z-scores) at those three CpGs using hierarchical clustering. This approach
226 revealed two distinct methylation clusters: the increased methylation cluster (cluster 1, $n =$
227 59) and the decreased methylation cluster (cluster 2, $n = 144$) (Supplementary Figure S1).
228 Cluster 1 exhibited significantly higher methylation levels at all three CpGs than cluster 2.
229 We then examined the differences in MEHP levels and PI between both clusters. Cluster 1
230 showed higher MEHP levels and lower PI than cluster 2 (Supplementary Figure S2). These
231 results demonstrated that the increased methylation in cg27433759:*PIK3CG*,
232 cg10548708:*ACAAI*, and cg07002201:*FUT9* associated with higher MEHP levels and lower
233 PI simultaneously occurred in the current participants. Finally, we tested the methylation
234 cluster for mediation in the association between MEHP levels and the PI (Figure 4). The
235 mediation path through the methylation cluster explained 28.8% (indirect/total) of the effect

236 of MEHP levels on the PI, although methylation levels at each of the three CpGs did not
237 mediate statistically significant effects (Supplementary Table S4). Since the methylation
238 levels at the three CpGs were positively correlated (Supplementary Table S3), we considered
239 total methylation levels at the three CpGs and observed a mediation effect with p -value $<$
240 0.05 considering the methylation cluster as the mediator, which explained 32.7 % of the
241 effect of MEHP levels on PI (Supplementary Table S4).

242

243 **4. Discussion**

244 Here, we assessed the effect of prenatal DEHP exposure on DNA methylation in cord
245 blood and found that maternal MEHP levels were predominantly associated with increased
246 methylation changes. The genes annotated to DRHM-CpGs were enriched for pathways
247 related to metabolism, the endocrine system, and signal transduction. Further, clustering and
248 mediation analyses suggested that the increased methylation changes related to metabolic
249 pathways may link prenatal DEHP exposure to fetal growth (as indicated by the offspring's
250 PI at birth).

251 As we described previously (Araki et al. 2014), maternal MEHP levels from subjects
252 in-between the second and third trimester (median = 10.3 ng/mL) were higher than those at
253 18 weeks of gestation (median = 1.18 ng/mL). Additionally, in most cases, phthalate
254 metabolite levels in blood samples are noticeably higher than in urine samples (Frederiksen et
255 al. 2010).

256 Noteworthy, we found two DMCPGs with $FDR < 0.05$: cg26409978 located in TSS200
257 of zinc finger CCCH-type domain-containing 10 (*ZC3H10*) and cg00564857 mapped to
258 *SDK1* (sidekick cell adhesion molecule 1), both showing increased methylation changes. We
259 also observed a preference for methylation positively associated with MEHP levels with p -
260 values $< 2.5E-04$. In a previous study using the 450K platform, (Solomon et al. 2017)

261 reported seven DMRs associated with MEHP levels in maternal urine at 26 gestational weeks
262 (n = 332, median: 3.63 μ g/g creatinine). Our study differs in sample size, matrices, sampling
263 time, and analysis methods; nonetheless, when we evaluated the direction of methylation
264 changes in these DMRs, increased methylation in five of them was replicated in our data set
265 (Table 2). The observed phthalate-induced increased methylation was also consistent with a
266 previous study that demonstrated a positive association between prenatal levels of high
267 molecular weight phthalate and cord blood methylation region of *MEG3* (Tindula et al.
268 2018). These results suggested that maternal MEHP would predominantly induce higher
269 methylation in the offspring. However, other studies on cord blood methylation alterations
270 have also reported prenatal phthalate-induced decreased methylation. A previous study
271 demonstrated a negative association between maternal levels of monoethyl phthalate, a
272 metabolite of diethyl phthalate, with *Alu* methylation and a similar but weaker association
273 with the methylation of *LINE-1* (Huen et al. 2016). In addition, mono-n-butyl phthalate and
274 monobenzyl phthalate in maternal urine samples were inversely associated with *Alu*
275 methylation (Huang et al. 2018). Another study showed that a negative association of
276 maternal phthalate concentrations with the methylation of the metabolism-related genes *IGF2*
277 and *PPARA* (Montrose et al. 2018), as well as *LINE-1* methylation. The differences in
278 metabolite type, measuring time, and level of phthalates may account for these disparities.

279 We also observed an enrichment of DRHM-CpGs in the IGR, with a decrease within
280 CpG islands (Figure 2). Previous studies showed that disease-associated and environmentally
281 induced DMCpGs, such as those resulting from obesity or exercise intervention, have
282 accumulated in the IGR or open seas (Grundberg et al. 2013; Huang et al. 2015; Ronn et al.
283 2013; Zhu et al. 2018), suggesting that DNA methylation may also be dynamically regulated
284 outside CpG islands. The enrichment of DMCpGs within the IGR may affect the function of
285 gene expression regulators located within the region. A recent study showed that the

286 methylation levels at CpGs in the IGR were anticorrelated to the nearest gene expression
287 (Zhu et al. 2018).

288 Since prenatal DEHP exposure was predominantly associated with increased
289 methylation changes, we conducted GO analysis for 253 DRHM-CpGs with $p < 2.5E-04$ to
290 examine the effects of DEHP-associated increased methylation on the biological processes.
291 The analysis showed the accumulation of CpGs with DEHP-induced higher methylation in
292 metabolic pathways. The effects on these pathways are accordant with those reported in
293 previous epidemiological studies, which have shown that phthalate exposure *in utero* is
294 associated with fetal metabolic outcomes, such as decreased birth size (Minatoya et al. 2017;
295 Watkins et al. 2016; Whyatt et al. 2009) and adipokine levels, i.e., markers of metabolic
296 function in cord blood (Ashley-Martin et al. 2014; Minatoya et al. 2018a; Minatoya et al.
297 2017). It is possible that increased methylation associated with exposure to DEHP in utero
298 may affect metabolic outcomes due to down-regulation of the expression of certain genes
299 involved in metabolic pathways.

300 Given the above, we hypothesized that these methylation changes would disrupt fetal
301 growth. Therefore, we examined the association between methylation levels at 16 DRHM-
302 CpGs in metabolic pathways and the PI at birth, an indicator of fetal growth, and found that
303 methylation levels at 12 CpGs were negatively associated with the PI (Figure 3). We also
304 analyzed the association of two CpGs that survived FDR correction (*ZC3H10*: cg26409978
305 and *SDK1*: cg00564857) with PI and found that both the CpGs were inversely related to PI;
306 however, it was not statistically significant ($\beta = -6.6$, 95% CI: -59.5 to 46.2 for cg26409978,
307 $\beta = -6.9$, 95% CI: -16.9 to 3.2 for cg00564857).

308 Among them, three CpGs, cg27433759:*PIK3CG*, cg10548708:*ACAA1*, and
309 cg07002201:*FUT9*, approached statistical significance (p -value < 0.1). *PIK3CG*
310 (phosphatidylinositol-4,5-bisphosphate 3-kinase) encodes a class I catalytic subunit of

311 phosphoinositide 3-kinase (PI3K), which phosphorylates inositol lipids and is related to the
312 pathway affecting insulin-like growth factor 1 (IGF1)-Akt (Matheny et al. 2017) and
313 erythropoietin-induced JAK-STAT (Cokic et al. 2012) signaling pathways. *ACAA1* (acetyl-
314 CoA acetyltransferase 1) encodes an enzyme operative in the β -oxidation system of the
315 peroxisomes and is involved in fatty acid metabolism (Islam et al. 2019). *FUT9*
316 (fucosyltransferase 9) belongs to the glycosyltransferase family and is involved in
317 glycosphingolipid biosynthesis (Ogasawara et al. 2011). Hierarchical cluster analysis
318 confirmed that the separation of samples at the DNA methylation level positively correlated
319 with MEHP levels (Supplementary Figures S1 and S2), indicating that the inter-individual
320 increased methylation changes could be induced by prenatal DEHP exposure. Furthermore,
321 although each CpG did not show significant mediation in the association between prenatal
322 DEHP exposure and offspring's PI, both the methylation clusters and the total methylation at
323 the three CpGs represented significant mediation effects (p -value < 0.05) and explained
324 28.8% and 32.7 % of the effect of MEHP levels on the PI (Figure 4 and Supplementary Table
325 S4), respectively. In addition, the direct effects are non-significant after adding the both
326 mediators in the models. Since the direct effects are not closer to the zero than the indirect
327 effects, the mediators not completely but quite robustly mediate the association between
328 maternal MEHP levels and offspring's PI. These results suggest that multiple DEHP-induced
329 higher methylation may jointly contribute to the effects of DEHP exposure *in utero* on fetal
330 development.

331 GO analysis also showed that DEHP-induced increased methylation was associated
332 with the MAPK signaling pathway, including nine genes (Table 3). Of those, four genes,
333 namely *MAP2K6*, *CACNA1D*, *CACNA1C*, and *MAP3K3*, were also involved in the endocrine
334 system, as shown in Table 3. Recently, an experimental study showed that MEHP has an
335 impact on MAPK pathways as well as on peroxisome proliferator-activated receptor γ

336 (PPAR γ) transcriptional activity, leading to the disturbance in lipid metabolism and human
337 villous cytotrophoblast differentiation (Shoaito et al. 2019). MAPK signaling modulates a
338 diverse range of cellular functions, cellular functions cell proliferation, differentiation, and
339 migration. In addition to the metabolic pathway, possibly, increased methylation on the genes
340 related to the MAPK signaling pathway may link prenatal phthalate exposure to adverse
341 health outcomes. The effect of methylation changes identified herein, specifically in the
342 MAPK signaling pathway, on long-term health outcomes warrant further longitudinal studies.

343 Nonetheless, there were some limitations in this study. First, MEHP levels were
344 measured only once between the second and third trimesters. Consequently, we need to
345 consider that a single MEHP measurement could represent a long-term prenatal exposure due
346 to the short half-life of MEHP. In addition, among several metabolites of DEHP, only MEHP
347 levels were measured. MEHP is the primary metabolite of DEHP, but other secondary
348 metabolites, such as mono(2-ethyl-5-hydroxyhexypentyl) phthalate and mono(2-ethyl-5-
349 carboxyl) phthalate, have been detected in maternal serum (Hart et al. 2014). Further,
350 although DEHP is the most common phthalate, there are several phthalates coexisted in the
351 environment, such as *di*-butyl phthalate, dimethyl phthalate, and diethyl phthalate. These
352 chemicals, including other secondary metabolites of DEHP, should be considered and fully
353 examined in the future. Second, since urine samples were unavailable in this study, only
354 blood samples were used to measure maternal MEHP levels. Recently, most studies have
355 measured urinary phthalate levels, which keeps the risk of a potential contamination to a
356 minimum. In this study, we cautiously handled all samples to prevent *ex vivo* hydrolysis of
357 DEHP and contamination. In addition, we calculated the background levels of MEHP and
358 confirmed that external contamination was of no consequence. Third, DEHP is known to
359 affect multiple tissues. Notably, whether the association of prenatal DEHP exposure with
360 cord blood DNA methylation that we observed potentially represents methylation changes in

361 other tissues is unknown. Moreover, replication analysis using a different population or gene
362 expression analysis is important to validate the result from epigenome-wide analysis. Without
363 validation analysis is also a limitation of this study. Fourth, this study limited participants to
364 mothers who delivered vaginally, meaning that relatively healthier children were included in
365 the analysis. Therefore, the effects of DEHP exposure on DNA methylation might be
366 underestimated. Fifth, cord blood DNA methylation and the PI at birth were cross-sectional.
367 Subsequently, the cause and effect relation between them was undetermined. Lastly, we
368 analyzed CpGs showing a p -value $< 2.5E-04$ (not epigenome-wide significance), to confirm
369 the effect that prenatal DEHP exposure had on DNA methylation. We cannot exclude the
370 possibility that some results might be false positives.

371

372 **5. Conclusion**

373 Collectively, this EWAS identified increased methylation changes associated with
374 prenatal DEHP exposure. The DEHP-associated increased methylation changes may jointly
375 contribute to the effects of prenatal exposure to this chemical on fetal development.

376 DNA methylation alterations in cord blood may be involved in modulating the
377 postnatal growth trajectory. In addition, recent studies showed the sex-specific effects of
378 phthalate exposure on DNA methylation (Chang et al. 2020; Svobada et al. 2020). Additional
379 studies with larger sample sizes are needed to fully elucidate the influence of prenatal DEHP
380 exposure on cord blood DNA methylation changes and the subsequent effects on infant long-
381 term outcomes, including sex-specific health outcomes.

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384 **CRedit author contribution statement**

385 T.K. and R.K conceived and supervised the study. R.M., A.A., T.I., K.M., C.M.,
386 T.N., K.S., and M.I. contributed to data curation, formal analysis, investigation, and
387 methodology. R.M., A.A., K.M., C.M., K.S., and R.K. contributed to funding acquisition.
388 R.M., A.A., T.I., C.M, and R.K contributed to writing – original draft. All authors
389 contributed to writing – review editing. All authors reviewed the final version of the
390 manuscript.

391

392 **Declaration of competing Interest**

393 The authors declare no competing interests.

394

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Figures

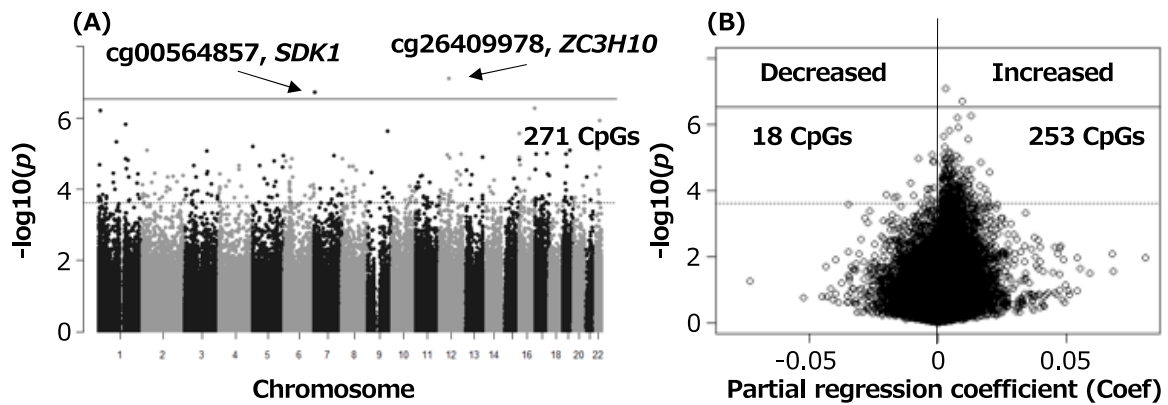


Figure 1. Manhattan (A) and volcano (B) plots of the epigenome-wide DNA methylation associations with prenatal exposure to DEHP.

Adjusted for maternal age, level of educational, pre-pregnancy BMI, smoking status during pregnancy, blood sampling periods, gestational age, infant sex, and estimates of cord blood cell counts. Horizontal solid lines represent the significance threshold of an FDR < 0.05. Horizontal dotted lines represent the threshold of a p -value < 2.5E-04.

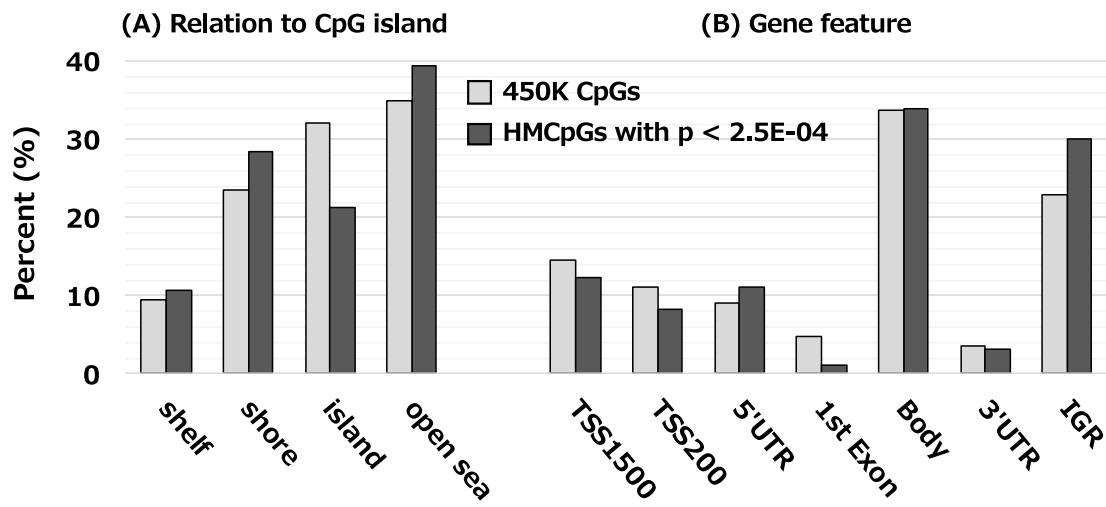


Figure 2. Location of DRHM-CpGs with $p < 2.5E-04$ (253 CpGs) compared to that of all CpGs in the methylation array.

χ^2 test: (A) $p = 0.004$, (B) $p = 0.01$.

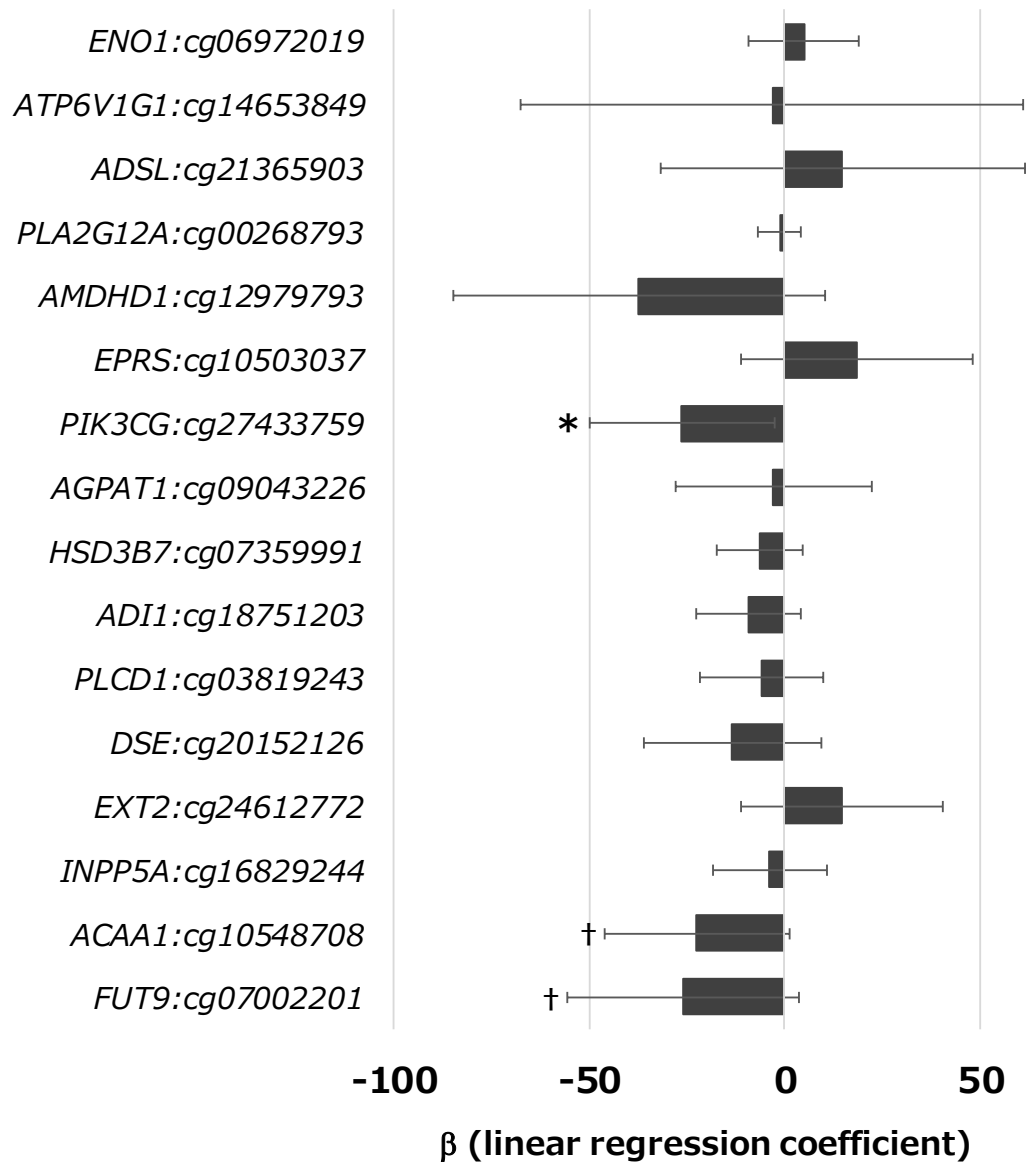


Figure 3. Linear regression coefficients (β) of the PI at birth in relation to the methylation levels, ranging from 0–1 for 0% to 100% methylated, at CpGs positively associated with MEHP with p -value $< 2.5E-04$, mapped to the genes involved in metabolic pathways ($n = 203$).

Linear regression coefficients (β) indicates PI changes with one unit increase in methylation levels.

Error bars indicate a 95% confidential interval. Adjusted for maternal age, level of educational, parity, pre-pregnancy BMI, smoking status during pregnancy, gestational age, and infant sex.

† $p < 0.1$, * $p < 0.05$.

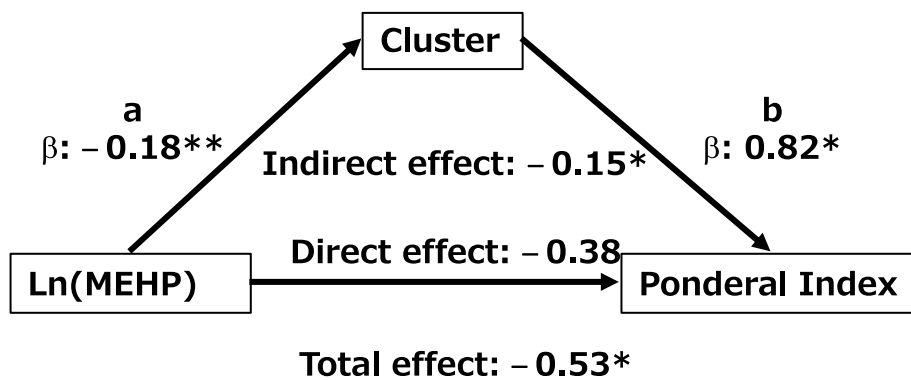


Figure 4. Mediator model for the association of prenatal MEHP exposure, methylation cluster for cg27433759, cg10548708, cg7002201, and PI at birth ($n = 203$).

Models were adjusted for maternal age and smoking status during pregnancy in path “a” and for ln(MEHP), maternal age, parity, gestational age, and infant sex in path “b.”

Effect sizes with * $p < 0.05$ and ** $p < 0.01$ are shown.

Table 1. Characteristics of the study population and their relationships with maternal serum MEHP concentrations (n = 203).

	Mean \pm SD/ N (%)	MEHP (ng/mL)			<i>p</i> -value	
		ρ / Median	25th	75th		
Maternal characteristics						
Maternal age (year) ^a	29.8 \pm 4.9	ρ = 0.038			0.594	
Prenatal BMI (kg/m ²) ^a	21.2 \pm 3.0	ρ = 0.049			0.485	
Parity ^b	0	110 (54.2)	10.00	5.65	15.20	0.644
	\geq 1	93 (45.8)	10.37	6.00	15.65	
Educational level (year) ^b	\leq 12	93 (45.8)	10.37	5.92	14.66	0.831
	> 12	112 (54.2)	9.92	5.65	15.42	
	Annual household income (million yen) ^c	< 3	39 (19.4)	11.53	6.03	
	3–5	103 (51.2)	8.65	5.57	14.92	
	5–7	43 (21.4)	11.41	6.90	16.80	
	> 7	16 (8.0)	9.83	5.42	13.48	
Smoking during pregnancy ^b	No	167 (82.3)	10.41	5.92	15.55	0.424
	Yes	36 (17.7)	7.80	5.23	14.11	
Alcohol consumption during pregnancy ^b	No	132 (65.5)	10.37	5.96	15.72	0.638
	Yes	70 (34.5)	10.22	5.40	15.09	
Caffeine intake during pregnancy (mg/day) ^a	143.0 \pm 125.8	ρ = 0.064				0.374
Blood sampling period (week) ^c	< 32	77 (37.9)	11.41	6.64	15.28	0.009
	32–35	48 (23.6)	12.40	6.64	17.32	
	\geq 35	78 (38.4)	7.08	5.00	13.80	
	Infant characteristics					
Gestational age (week) ^a	39.9 \pm 1.0	ρ = 0.000				0.998
Sex ^b	Male	94 (46.3)	9.86	6.32	14.42	0.673
	Female	109 (53.7)	10.41	5.63	16.31	
Birth weight (g) ^a	3137.5 \pm 333.3	ρ = -0.066				0.352
Birth length (cm) ^a	48.5 \pm 1.5	ρ = 0.057				0.416

PI (kg/m ³) ^a	27.4 ± 2.2	$\rho = -0.133$	0.059
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^aSpearman's correlation test (ρ)
^bMann–Whitney *U* test
^cKruskal–Wallis test

Table 2. Direction of cord blood DNA methylation changes associated with maternal MEHP levels at DMRs identified by Solomon et al. (2017) in the present study.

Gene	Chr	Start	End	Sapporo cohort				Solomon et al. 2017	
				Number of probes	Average Coef ^a	Min <i>p</i> -value ^b	Direction ^c	Max bFC ^d	Direction ^c
<i>MUC4</i>	3	195489306	195490169	8	0.018	0.223	+	0.297	+
<i>C5orf63/FLJ44606</i>	5	126408756	126409553	13	0.017	0.002	+	0.250	+
<i>VTRNA2-1</i>	5	135414858	135416613	16	-0.007	0.320	-	-0.895	-
<i>RNF39</i>	6	30038254	30039801	37	0.005	0.367	+	-0.833	-
<i>CNPY1</i>	7	155283233	155284759	10	0.004	0.082	+	0.171	+
<i>SVIL-ASI</i>	10	29698152	29698685	8	0.002	0.119	+	0.390	+
<i>FIBIN</i>	11	27015519	27016671	8	0.003	0.166	+	0.231	+

^aAverage partial regression coefficient at CpG sites in the region.

^bMinimum *p*-value within the region.

^cDirection of methylation change: +, increase; -, decrease.

^dFold change in the DNA methylation *M*-value per log₁₀ unit increase in phthalate metabolite concentration.

Abbreviations: Chr, chromosome.

Table 3. Significantly enriched pathways (FDR < 0.05) for the gene targets of 253 DRHM-CpGs associated with MEHP levels ($p < 2.5E-04$).

KEGG orthology	KEGG pathway	Genes*	<i>p</i> -value
Metabolism	Metabolic pathways	<i>ENO1; ATP6V1G1; ADSL; PLA2G12A; AMDHD1; EPRS; PIK3CG; AGPAT1; HSD3B7; ADII; PLCD1; DSE; EXT2; INPP5A; FUT9; ACAA1</i>	7.3E-11
Signal transduction	MAPK signaling pathway	<i>MAP2K6; EFNA3; CACNA1D; DAXX; FGF9; DUSP4; PPM1A; DUSP10; CACNA1C; MAP3K3</i>	3.0E-07
	Notch signaling pathway	<i>NUMBL; NCOR2; RFNG; CTBP1; NOTCH1</i>	6.4E-07
Endocrine system	GnRH signaling pathway	<i>MAP2K6; CACNA1D; ITPR2; CACNA1C; MAP3K3</i>	1.3E-04
	Renin secretion	<i>CACNA1D; ITPR2; CACNA1C</i>	6.9E-04
	Cortisol synthesis and secretion	<i>CACNA1D; ITPR2; CACNA1C</i>	1.2E-03
Circulatory system	Vascular smooth muscle contraction	<i>CACNA1D; PLA2G12A; CALD1; ITPR2; CACNA1C</i>	4.0E-04
Nervous system	Dopaminergic synapse	<i>CACNA1D; TH; ITPR2; CACNA1C</i>	7.4E-04

*Genes annotated to the DRHM-CpGs with $p < 2.5E-04$.