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# Association between fetal exposure to phthalate endocrine disruptor and genome-wide DNA methylation at birth

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ABSTRACT

*Background:* Phthalic acid esters are ubiquitous and antiandrogenic, and may cause systemic effects in humans, particularly with *in utero* exposure. Epigenetic modification, such as DNA methylation, has been hypothesized to be an important mechanism that mediates certain biological processes and pathogenic effects of *in utero* phthalate exposure.

*Objective:* The aim of this study was to examine the association between genome-wide DNA methylation at birth and prenatal exposure to phthalate.

*Methods:* We studied 64 infant–mother pairs included in TMICS (Taiwan Maternal and Infant Cohort Study), a long-term follow-up birth cohort from the general population. DNA methylation levels at more than 450,000 CpG sites were measured in cord blood samples using Illumina Infinium HumanMethylation450 BeadChips. The concentrations of three metabolites of di-(2-ethylhexyl) phthalate (DEHP) were measured using liquid chromatography tandem–mass spectrometry (LC–MS/MS) in urine samples collected from the pregnant women during 28–36 weeks gestation.

*Results:* We identified 25 CpG sites whose methylation levels in cord blood were significantly correlated with prenatal DEHP exposure using a false discovery rate (FDR) of 5% ($q$-value < 0.05). Via gene-set enrichment analysis (GSEA), we also found that there was significant enrichment of genes involved in the androgen response, estrogen response, and spermatogenesis within those genes showing DNA methylation changes in response to exposure. Specifically, *PA2G4*, *HMGCR*, and *XRCC6* genes were involved in genes in response to androgen.

*Conclusions:* Phthalate exposure *in utero* may cause significant alterations in the DNA methylation in cord blood. These changes in DNA methylation might serve as biomarkers of maternal exposure to phthalate in infancy and potential candidates for studying mechanisms via which phthalate may impact on health in later life. Future investigations are warranted.

## 1. Introduction

Phthalic acid esters, or phthalates, are a group of synthetic compounds widely used as plasticizers (Koch et al., 2013), with di-(2-ethylhexyl) phthalate (DEHP) being the most frequently used for items including containers, food packaging, vinyl flooring, furniture, and medical devices (Cirillo et al., 2013). Low-molecular-weight phthalates such as di-n-butyl phthalate (DnBP) are frequently used as colorants, lubricants, adhesives, detergents, or deodorants because they are easily mixed with water, oil, and alcohol, are odorless, and exist in liquid form at room temperature. Therefore, phthalates are also commonly present in personal care products such as hair sprays, shampoos, and cosmetics (Buckley et al., 2012). Phthalates do not bond covalently to these products and therefore tend to disperse into food, drink, or the environment. Humans are ubiquitously exposed to phthalates through digestion, inhalation, or dermal absorption (Araki et al., 2014; Dewalque et al., 2014).

Phthalates have strong antiandrogenic (Doyle et al., 2013; Martino-

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Andrade et al., 2016) and weak estrogenic (Huang et al., 2014; Lee et al., 2012) effects. Previous studies have shown that phthalate exposure is associated with an increased risk of allergic diseases (Jaakkola and Knight, 2008), preterm birth (Ferguson et al., 2016), behavioral problems (Engel et al., 2010), and reduced anogenital distance (Swan et al., 2015).

Our long-term follow-up birth cohort study (Lin et al., 2011a, 2011b) demonstrated that in Taiwan, prenatal exposure to phthalates, particularly DEHP, was associated with postnatal health effects, including decreased sex hormone levels (Lin et al., 2011a, 2011b), reduced reproductive organ growth (Su et al., 2015), increased behavioral problems (Lien et al., 2015), and allergic symptoms (Ku et al., 2015) in children, indicating that systemic endocrine, neurobehavioral, and immune effects of *in utero* phthalate exposure are consistently observed, even years after exposure.

Epigenetic modification, such as DNA methylation, was hypothesized to play a critical role in the biological mechanism of the postnatal effects of prenatal phthalate exposure. Animal studies have suggested that phthalate exposure may be associated with altered DNA methylation (Abdel-Maksoud et al., 2015; Rajesh and Balasubramanian, 2015; Wu et al., 2010). However, human studies are scarce; a few studies have reported that phthalate exposure was inversely associated with DNA methylation on selected candidate genes using placenta (LaRocca et al., 2014; Zhao et al., 2016) or blood samples (Wang et al., 2015). One study demonstrated an inverse association between prenatal levels of monoethyl phthalate, a metabolite of low-molecular-weight DEP, and cord blood methylation of Alu repeats. A similar but weaker negative association was also found in relation to long interspersed nuclear element-1 (*LINE-1*) methylation (Huen et al., 2016b). The maternal urinary level of phthalates in the third trimester was found to be associated with decreased placental *LINE-1* methylation and reduced birth weight (Zhao et al., 2015). In the present study, we examine the association of genome-wide DNA methylation at birth with maternal exposure to the most frequently used phthalate, DEHP (Solomon et al., 2017).

## 2. Methods

### 2.1. Subject recruitment

We established a birth cohort study in central Taiwan in 2001. A total of 610 pregnant women without gestational complications were all invited to participate in this environmental health study of the endocrine disruptor between December 1, 2000 and November 30, 2001 (Fig. 1). Among these, 430 pregnant women gave informed consent and completed the structured questionnaire with the assistance of the researchers during their third trimester. A total of 384 newborns from 391 pregnant women who provided urine samples were recruited, their cord blood samples were collected, and a newborn questionnaire and birth record were completed. Among these, 307 newborns had DNA samples extracted from cord blood. Because cigarette smoking is a strong modifier of DNA methylation (Joubert et al., 2014), newborns whose mothers smoked before pregnancy (n = 31) and/or had environmental tobacco smoke (ETS) exposure for more than 4 h per day during pregnancy (n = 21) were excluded. We also excluded newborns whose DNA samples were of insufficient quantity (n = 46) or quality (n = 145) for DNA methylation assays. Finally, 64 newborns were selected for measurement of DNA methylation using Illumina Infinium HumanMethylation450 (HM450k) BeadChips. The study protocol was approved by the Institutional Review Board of the National Health Research Institutes, Taiwan.

### 2.2. Measurement of phthalate exposure

DEHP is rapidly metabolized to its monoester, mono (2-ehtylhexyl) phthalate (MEHP), and MEHP can be further metabolized to oxidative products such as mono (2-ethyl-5-hydroxyhexyl) phthalate (MEHHP) and metabolized into mono (2-ethyl-5-oxohexyl) phthalate (MEOHP). The concentrations of these three phthalate metabolites, MEHP, MEHHP, and MEOHP, were measured in urine samples collected from the 64 pregnant women during the third trimester, at 28–36 weeks. Urine samples were collected and stored in brown glass bottles (National Scientific Supply Company, Claremont, CA, USA) and the concentrations of these phthalate metabolites in spot urine were measured by high-performance liquid chromatography–tandem mass spectrometry (HPLC–MS/MS). Urinary creatinine levels were measured at Union Clinical Laboratory (UCL; Taipei, Taiwan) using an ADVIA 1800 Clinical Chemistry System (Siemens, Erlangen, Germany), as previously described (Lin et al., 2011a, 2011b; Wen et al., 2017). Creatinine-adjusted concentrations of three phthalate metabolites were calculated by dividing unadjusted values by their urinary creatinine levels. Distributions of the unadjusted and creatinine-adjusted concentrations of three phthalate metabolites are provided in Table 1. Because the concentrations of these three phthalate metabolites are highly intercorrelated (r = 0.81–0.95; see Supplementary Table S1), we considered that the total concentration of phthalate metabolites, ΣMEHP, defined as MEHP + MEHHP + MEOHP, represented the potency of prenatal phthalate exposure and used this as the exposure variable in the subsequent epigenome-wide association study (EWAS).

### 2.3. Data preprocessing and quality control

Genomic DNA was extracted from cord blood samples using the DNeasy Blood and Tissue Kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer's instructions. Bisulfite conversion of genomic DNA was performed using the EZ DNA Methylation Kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's instructions. Bisulfite-converted genomic DNA (500 ng) from each sample was subjected to DNA methylation analysis using the HM450k BeadChip according to the manufacturer's instructions.

This assay measures DNA methylation levels at 482,421 CpG sites. We applied data preprocessing in a fashion similar to that described by Shi and colleagues (Shi et al., 2014) with some modifications. This data preprocessing procedure was also suggested by Liu and Siegmund (Liu and Siegmund, 2016). Specifically, we used the *methylumi* package in Bioconductor to import the raw image (.IDAT) files, and raw methylated and unmethylated intensities were background corrected and dye-bias corrected to reduce technical variation between arrays (Triche et al., 2013). To adjust for the difference between methylation distributions of type I design probes and type II design probes, we used a Beta-MIxture Quantile normalization method (BMIQ) implemented in the *BMIQ* function in the *wateRmelon* package (Teschendorff et al., 2013). The DNA methylation level measured by each probe was defined as the DNA methylation percentage at the corresponding CpG site (β-value), estimated as the fraction of the methylated intensity over the total intensity. We excluded unreliable probes from the HM450k BeadChip using the following criteria: (i) probes with detection *P*-value > 0.05 for any sample, meaning that the total intensities of the probes did not differ significantly from background noise; (ii) probes located on the X and Y chromosomes; and (iii) probes whose sequences contained SNPs or copy number variants within the population as defined by the SNPs collected from CHB and CHD in HapMap Phase III (release 3, Human Genome build 36, hg18) and the copy number variants provided by the ASN population in 1000 Genome Project (version 20100804) (Altshuler et al., 2010; Altshuler et al., 2010, 2015). These exclusions resulted in 414,995 CpG sites in autosomes being available for subsequent analyses. We found that differences between experimental batches were not associated with the first and second principal components of the methylation data of the 64 newborns at the 414,995 CpG sites in autosomes (see Supplementary Fig. S1). Note that the gender revealed by the first principal component of the methylation data of the 64 newborns in the X chromosome was consistent with the