

Sex-Specific Programming of Cardiac DNA Methylation by Developmental Phthalate Exposure

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ABSTRACT: Phthalate plasticizers are ubiquitous chemicals linked to several cardiovascular diseases in animal models and humans. Despite this, the mechanisms by which phthalate exposures cause adverse cardiac health outcomes are unclear. In particular, whether phthalate exposures during pregnancy interfere with normal developmental programming of the cardiovascular system, and the resulting implications this may have for long-term disease risk, are unknown. Recent studies suggest that the effects of phthalates on metabolic and neurobehavioral outcomes are sex-specific. However, the influence of sex on cardiac susceptibility to phthalate exposures has not been investigated. One mechanism by which developmental exposures may influence long-term health is through altered programming of DNA methylation. In this work, we utilized an established mouse model of human-relevant perinatal exposure and enhanced reduced representation bisulfite sequencing to investigate the long-term effects of diethylhexyl phthalate (DEHP) exposure on DNA methylation in the hearts of adult male and female offspring at 5 months of age (n=5–7 mice per sex and exposure). Perinatal DEHP exposure led to hundreds of sex-specific, differentially methylated cytosines (DMCs) and differentially methylated regions (DMRs) in the heart. Pathway analysis of DMCs revealed enrichment for several pathways in females, including insulin signaling, regulation of histone methylation, and tyrosine phosphatase activity. In males, DMCs were enriched for glucose transport, energy generation, and developmental programs. Notably, many sex-specific genes differentially methylated with DEHP exposure in our mouse model were also differentially methylated in published data of heart tissues collected from human heart failure patients. Together, these data highlight the potential role for DNA methylation in DEHP-induced cardiac effects and emphasize the importance of sex as a biological variable in environmental health studies.

KEYWORDS: Developmental origins of health and disease (DOHaD), DNA methylation, phthalate, heart, sex differences, toxicopigenetics

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Introduction

Cardiovascular diseases (CVD) are a major cause of morbidity and mortality worldwide, and significant sex differences exist in their incidence, pathogenesis, and prognosis.¹ It is increasingly evident that environmental exposures play an important role in CVD etiology, but how sex and environment interact to influence CVD risk and prognosis are unclear. Phthalates are industrial chemicals that are widely used in processed food packaging, medical devices, and building materials.^{2,3} The major exposure route for phthalates in the United States is the consumption of contaminated food. Other sources of exposure include dermal (through the use of cosmetics), inhalation of indoor air, and household dust.⁴ Patients receiving medical procedures (dialysis, blood transfusion, EMCO) are also exposed to high levels of phthalates through tubing and medical devices.⁴ Given the ubiquitous human exposure to these chemicals, and evidence of harm in human and animal studies, exposure to phthalates represents a significant public health concern.^{3,5} In rodent models, phthalate exposure during gestation or the early postnatal period is associated with metabolic

diseases and reproductive abnormalities.^{6–9} Recent animal studies have also linked gestational phthalate exposures to altered heart development and function.^{10–12} Likewise, human studies demonstrate an association between both pediatric and adult phthalate exposure and adverse cardiovascular outcomes.^{13–16} However, few studies in humans have investigated the effects of *perinatal* exposures on cardiovascular health.^{17,18} The molecular mechanisms linking phthalate exposures to deleterious cardiovascular effects are poorly understood, although *in vitro* studies suggest that phthalate exposures may disrupt cardiomyocyte differentiation.^{19,20} Importantly, the majority of studies investigating the effects of gestational phthalate exposures on cardiovascular outcomes have focused only on males.^{11,12,21} Although work from our lab and others demonstrate sexually dimorphic effects of endocrine-disrupting chemicals, including phthalates, in humans and animal models,^{6,22–24} little is known about the sex-specific effects of gestational exposure to phthalates on the heart.

One important mechanism by which early-life exposures influence long-term disease risk is by disrupting the epigenetic



programs governing normal development. DNA methylation (DNAm) is an epigenetic mark that is critical for the regulation of stem cell differentiation and the establishment of tissue and cell-type-specific gene expression patterns.^{25,26} Because DNAm is relatively stable, mitotically heritable, and undergoes rapid reprogramming during early development, environmental insults during this period may lead to changes in DNAm that persist later in life.²⁷ Indeed, it is increasingly clear that disruption of normal DNAm patterning during early cardiovascular development may have long-term, adverse health consequences.^{28,29} Recent studies demonstrate that DNAm in the heart is altered in disease states,^{28,29} after obesogenic diet exposure,²⁸ and with toxicant exposures,^{30,31} suggesting that this modification may play a key role in CVD pathogenesis. Despite this, the effects of early-life phthalate exposure on cardiac DNAm have not been investigated. Early-life exposure to diethylhexyl phthalate (DEHP), one of the most widely used phthalates, reprograms DNAm in other tissues.^{32,33} The effects of DEHP exposure on DNAm in developing heart, however, are unknown. We have also previously shown that the effects of perinatal DEHP exposure DNAm are sex-specific.⁶ Based on this evidence, we hypothesized that perinatal exposure to DEHP would lead to sex-specific alterations in the programming of cardiac DNAm that would be present in adulthood. To test this hypothesis, we utilized an established mouse model of human physiologically relevant perinatal DEHP exposure and measured DNAm in adult male and female mouse hearts. These studies were conducted as part of the National Institute of Environmental Health Sciences (NIEHS) Toxicant Exposures and Responses by Genomic and Epigenomic Regulators of Transcription II (TaRGET II) Consortium, which aims to determine how the environment affects disease susceptibility across the life course through changes to the epigenome.³⁴

Materials and Methods

Animal exposure paradigm

Mouse studies were conducted according to procedures established by the NIEHS TaRGET II Consortium.^{34,35} The work outlined in this manuscript is part of a larger study looking at the effects of perinatal lead and DEHP exposures on multiple tissues and time points. This work is focused on DEHP and the 5-month time point. The mice for this experiment were wild-type *a/a* non-agouti mice derived from a colony of the viable yellow agouti (*A^{vy}*) strain maintained for more than 230 generations. This results in forced heterozygosity on an invariant genetic background, which is approximately 93% identical to the C57BL/6J strain.^{36,37} Virgin *a/a* females (6–8 weeks old) were mated with virgin *a/a* males (7–9 weeks old) and randomly assigned to receive control or DEHP through consumption of chow. Control animals received standard chow (AIN-93G, TD.95092, ENVIGO, Madison, WI) with 7% corn oil. Treated animals received DEHP (Sigma) dissolved in 7% corn oil (25 mg DEHP per kg of chow). Dams began

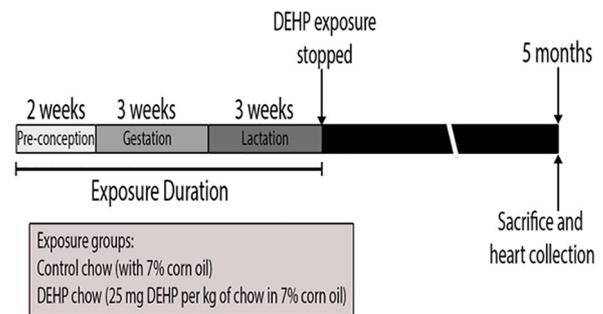


Figure 1. Experimental paradigm depicting timing of DEHP exposure and animal sacrifice/heart collection. Dams were exposed to control chow containing 7% corn oil alone or 25 mg DEHP per kg chow in 7% corn oil. Exposures started 2 weeks prior to mating and continued through gestation, until offspring were weaned on postnatal day 21. At this point, DEHP exposure ceased and all offspring were administered control chow. At 5 months of age, hearts were collected for DNA methylation analysis. DEHP indicates diethylhexyl phthalate.

consumption of control or DEHP diets 2 weeks before mating, and exposure was continued during gestation and lactation. The DEHP exposure level was selected based on a target maternal dose of 5 mg/kg-day, assuming that pregnant and nursing female mice weigh approximately 25 g and eat approximately 5 g of chow per day. This target dose was selected based on literature demonstrating increased body weight and other metabolic effects in offspring that were developmentally exposed to DEHP in this dose range.^{38,39} The 5 mg/kg-day dose used in this study falls within or below the range of no-observed-adverse-effect level (NOAELs) for oral DEHP exposure established by US and European agencies^{4,40} and is estimated to result in an amniotic fluid concentration within the ranges observed in human amniotic fluid.⁶

After weaning at postnatal day 21, pups were weighed, and all animals received DEHP-free 7% corn oil control chow for the remainder of the study (Figure 1). Approximately 1 to 2 male and 1 to 2 female offspring per litter were followed until 5 months of age ($n=6$ control animals per sex, $n=7$ DEHP males, $n=5$ DEHP females). All animals had access to food and drinking water *ad libitum* throughout the experiment, remained on a 12-hour light/dark cycle, and were housed in polycarbonate-free cages. Health checks were carried out daily by lab personnel and the University of Michigan Unit for Laboratory Animal Medicine (ULAM). This study protocol was approved by the University of Michigan Institutional Animal Care and Use Committee (IACUC).

Euthanasia and tissue collection

Each mouse was weighed weekly (Mettler Toledo). Upon euthanasia at 5 months of age, heart samples were collected following protocols established by the TaRGET II Consortium (Figure 1).³⁴ Briefly, before euthanasia, mice were fasted for 6 hours. Euthanasia was carried out via CO₂ asphyxiation and bilateral pneumothorax. Blood was removed by cardiac puncture, followed by whole-body perfusion with cell culture grade

0.9% saline solution (Sigma Life Sciences) to remove any residual blood that could confound the heart-specific epigenomic analysis. Next, whole hearts were collected and weighed. Relative heart weights were expressed as a percent of total body weight. Heart samples were immediately snap-frozen in liquid nitrogen and stored at -80°C until DNA extraction.

Statistical analysis of litter parameters

Differences in mortality rates, sex ratio, and litter size between control and DEHP-exposed animals were analyzed using unpaired *t*-test. For body and heart weights, animals were stratified by sex, and linear mixed-effects regression was carried out using the *lmer4* and *lmerTest* packages in R version 3.6.1 (www.r-project.org). Litter-specific random effects were included to account for within-litter correlation.

DNA extraction and enhanced reduced representation bisulfite sequencing

In all, 1 to 2 male and 1 to 2 female mice per litter were included in each condition. Heart tissue was cryo-pulverized, and DNA extraction was performed using the AllPrep DNA/RNA/miRNA Universal Kit (Qiagen #80224) according to the manufacturer's instructions. Sample concentration and quality were assessed using the Qubit (ThermoFisher) and 2200TapeStation system (Agilent), respectively, and all samples met the quality standard for next-generation sequencing library preparation (Supplementary Table 1). Enhanced reduced representation bisulfate sequencing (ERRBS) was performed at the University of Michigan Epigenomics and Advanced Genomics Cores as described previously.^{35,41,42} Bisulfite conversion efficiencies for all samples exceeded 99.8% (Supplementary Table 1). Single-end, 50 nucleotide sequencing was performed on a HiSeq4000 platform (Illumina). Libraries were multiplexed and sequenced over 2 lanes. Library sizes (with adapters) ranged from 200 to 400 bp, and the average sequencing depth was >118 million reads. On average, the percentage of genomic CpGs captured using this method was 4.8%.

Bioinformatics pipeline, quality control, and differential methylation analysis

For analysis of DNAm data, quality control, trimming, alignment, and methylation calling were conducted as outlined previously.³⁵ CpGs with read coverage >1000 were removed because they were likely the result of PCR amplification; CpGs with read coverage <10 were removed due to decreased power to detect differential methylation. Opposite strand CpGs at the same position were combined via destranding. Sex chromosomes were included in the analysis. We performed differential methylation testing on individual CpG sites (DMCs), requiring sufficient sequencing coverage for a minimum of 4 samples from the DEHP group and 4 samples from the control group for a site to be tested. Differentially methylated regions (DMRs)

were identified in 1000bp tiles using the same process. To identify differentially methylated CpGs and regions, we utilized the *methylSig* R package⁴³ (v0.5.0). Differential methylation for each comparison (control male, control female, DEHP male, DEHP female) was tested using *methylSigDSS()*, which tests for differential methylation under general experimental design using a beta-binomial approach with the 'arcsine' link function.⁴⁴ To control for batch effects, run was included as a covariate in the model. After obtaining *P* values, we adjusted for multiple testing using the false discovery rate (FDR) approach. Sites and regions with $\text{FDR} < 0.05$ and an absolute difference in methylation of >10% were considered significant. To determine where differentially methylated sites were distributed across the genome, we used the *annotatR* R Bioconductor package (v1.5.9) to annotate the CpGs to the mouse mm10 genome.⁴⁵ The *annotate_regions* function was used to generate genomic annotations, including CpG annotations (CpG islands [CGI], shores, shelves, open sea [InterCGI]), genic annotations (exon, intron, promoter, 5'-UTR, 3'-UTR), and gene IDs. To determine whether the proportion of DMCs falling into each annotation was significantly different from the total regions tested, we conducted a Chi-Square test, comparing the number of CpGs falling in each annotation relative to all of the regions tested to the number of hypermethylated or hypomethylated DMCs falling within each category relative to the total number of hypermethylated or hypomethylated DMCs identified.

Pathway analysis of DMCs

PolyEnrich⁴⁶ was used to assess biological pathways enriched among the DMCs. Analyses were stratified by sex and direction of differential methylation. Only sites within 1 kb from transcription start sites were included in the analysis. Biological Process, Cellular Component, and Molecular Function Gene Ontology pathways were used in the analysis. Pathways with $\text{FDR} < 0.05$ were considered statistically significant. For GREAT⁴⁷ analysis, BED files of DMCs for each sex and direction of methylation change (4 separate datasets total) were uploaded to the GREAT web interface: <http://great.stanford.edu/public/html/>, using the mouse mm10 species assembly. Association rule setting "basal plus extension" was utilized with the following parameters: Proximal: 5 kb upstream, 1 kb downstream, plus Distal: up to 1000 kb.

Overlap in DMCs and DMC-associated genes between sexes and human heart failure

Annotated lists of DMCs and DMRs were compared between males and females to identify chromosomal locations that directly overlapped between sexes. In a further analysis, the full lists of DMC- and DMR-associated genes in males were compared to those in females to identify a list of genes in common between sexes. To determine relevance to human disease, DMC- and DMR-associated genes for each sex were

Table 1. Litter Parameters.

EXPOSURE GROUP	NO. OF LITTERS	NO. OF PUPS BORN	NO. OF PUPS DIED	MEAN PUPS PER LITTER (\pm SD) ^a	PUP MORTALITY RATE (%) ^b	% FEMALES ^c
Control	6	53	3	7.57 \pm 3.36	5.7	46
DEHP	6	41	1	6.83 \pm 1.94	2.4	43

Abbreviation: DEHP, diethylhexyl phthalate.

Statistical analyses (DEHP vs Control) were conducted using Fisher Exact Test.

^{a,b}P = .63.

^cP = .29.

compared to genes differentially methylated in heart tissue collected from human heart failure patients.⁴⁸ In this study, whole-genome bisulfite sequencing was conducted to compare DNA methylation between non-failing donor heart tissue and biopsy samples from heart failure patients undergoing left ventricular assist device implantation.⁴⁸ The overlap between gene lists was determined and diagrammed using Venny 2.1 (J.C. Oliveros, <http://bioinfogp.cnb.csic.es/tools/venny/index.html>). Statistical significance of overlap was determined using a hypergeometric test.

Results

Litter parameters and phenotype

Perinatal phthalate exposure had no significant effect on mortality rates ($P = .63$), sex ratio ($P = .29$), or litter size ($P = .63$, unpaired t -test; Table 1). Bodyweight and relative heart weights were not significantly different between control and DEHP-exposed animals (Figure 2).

Differential DNA methylation after perinatal DEHP exposure

To determine the effect of perinatal DEHP exposure on cardiac DNA methylation in offspring mice, we performed enhanced reduced representation bisulfate sequencing on DNA from hearts of control and DEHP-exposed offspring at 5 months of age ($n = 6$ control animals per sex, $n = 7$ DEHP males, $n = 5$ DEHP females). Although exposure to DEHP stopped at 3 weeks of age, we observed hundreds of differentially methylated cytosines (DMCs) and regions (DMRs) with DEHP exposure in both sexes (Table 2 and Supplementary Table 2). In females, 44% of DMCs were hypermethylated and 56% were hypomethylated, while in males, 64% of DMCs were hypermethylated and 36% were hypomethylated (Table 2). As expected, we observed similar results with analysis of DMRs (Supplementary Table 2). In females and males, we observed maximum DEHP-induced methylation changes of 67% and 55%, respectively (Figure 3). The magnitude of methylation changes was similar for DMRs (maximum 63% and 64% in females and males, respectively; Supplementary Figure 1). The locations and annotations of the top 10 hypomethylated and hypermethylated cytosines (DMCs) and regions (DMRs) in

each sex are shown in Tables 3 and 4. Compared to all of the CpGs tested, DMCs were significantly more enriched in open sea and intronic regions compared to promoters (Figure 4).

Pathway analysis of hypomethylated and hypermethylated DMCs

We next performed pathway analysis to identify biological pathways enriched among DMCs. First, we utilized PolyEnrich and focused our analysis only on promoter regions (within 1000 bp of a transcription start site). We stratified the data based on sex and direction of methylation change. This analysis yielded a small number of significantly enriched pathways. In females, among hypermethylated DMCs, we observed significant enrichment in pathways associated with receptor binding, neurotransmitter transport, smooth muscle differentiation, histone demethylation, insulin signaling, and meiosis (Supplementary Table 3). In males, hypomethylated DMCs were significantly enriched for glucose transport (Supplementary Table 4). To further investigate the biological significance of the DMCs, we used a parallel approach for pathway analysis, the Genomic Regions Enrichment of Annotations Tool (GREAT).⁴⁷ GREAT incorporates distal methylation changes in non-coding regions of the genome.⁴⁷ Again, we stratified the data based on sex and direction of methylation change (Figure 5). This analysis revealed sex-specific enrichment for several pathways relevant to heart development and function, including tyrosine phosphorylation, phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) activity, embryonic heart tube development, and regulation of precursor metabolites and energy (Figure 5A-D, respectively).

Sex specificity of differential DNA methylation

We next investigated whether the DEHP-induced changes in methylation were sex-specific. Consistent with our hypothesis, only 2 DMCs and 3 DMRs directly overlapped between males and females (Supplementary Tables 5 and 6). Both overlapping DMCs were located in intergenic regions and did not map to genes. One of the 2 sites exhibited a change in DNAm that was in the same direction for both males and females (Supplementary Table 5). One of the 3 DMRs mapped to a gene (*Slc5a4a*), and all 3 regions were hypomethylated with DEHP exposure in

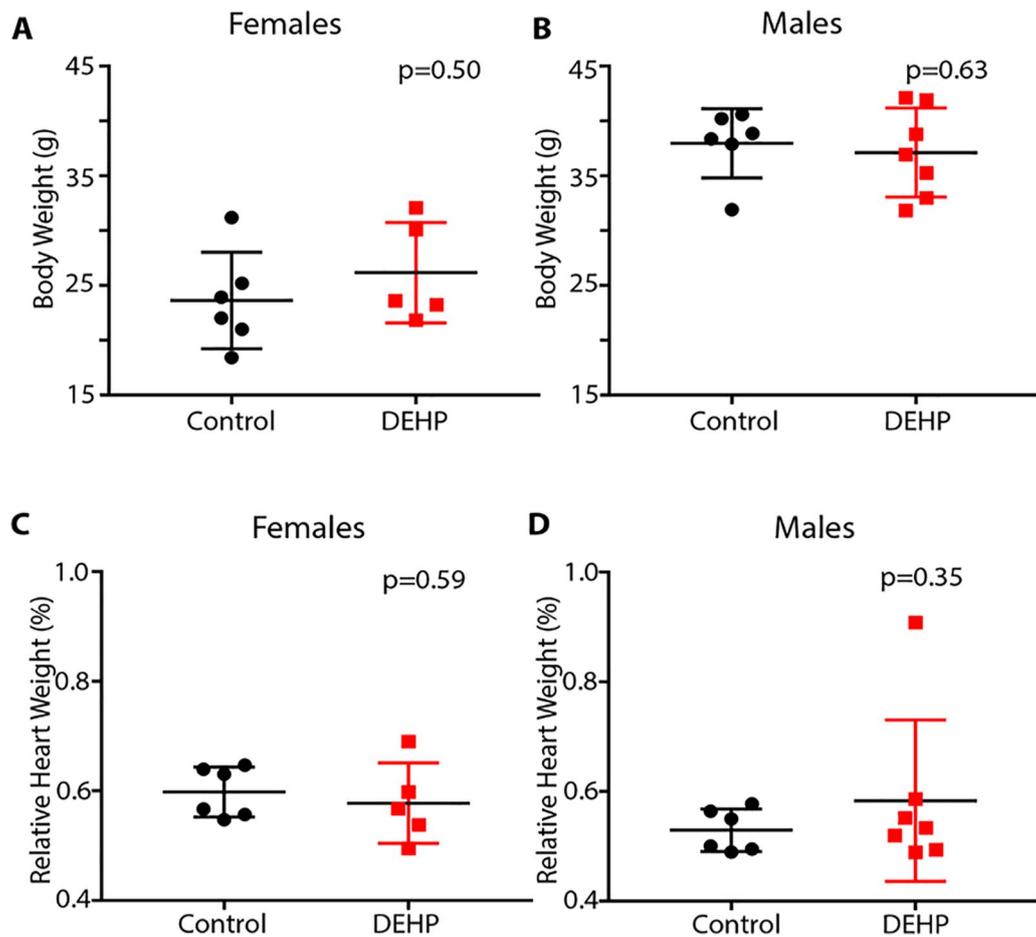


Figure 2. Box plots depicting relative heart weights of control vs DEHP-treated (A) female and (B) male offspring at 5 months of age (n=6 controls for each sex, n=5 DEHP-exposed females, n=7 DEHP-exposed males). Linear mixed effects regression with litter-specific random effects to account for within-litter correlation was used to evaluate statistical significance. Differences between control and DEHP-exposed animals were not statistically significant for either sex. DEHP indicates diethylhexyl phthalate.

Table 2. Differentially methylated cytosines (DMCs) in 5-month offspring mouse hearts.

CONDITION	TOTAL	NO. HYPERMETHYLATED (% TOTAL)	NO. HYPOMETHYLATED (% TOTAL)	TOTAL TESTED
Female DEHP	1654	736 (44%)	918 (56%)	1 170 283
Male DEHP	1187	764 (64%)	423 (36%)	1 250 997

both males and females (Supplementary Table 6). We next determined whether the genes that mapped to the DMCs and DMRs were sex specific. Overall, DMCs in females mapped to 1055 genes and DMCs in males mapped to 794 genes (Supplementary Tables 7 and 8). The majority of DMC-associated genes were highly sex-specific, with only 106 genes overlapping between sexes (Figure 6A and Supplementary Table 9). Differentially methylated regions (DMRs) mapped to 288 genes in females and 173 in males (Supplementary Tables 10 and 11). Only 6 DMR-associated genes overlapped between sexes (Figure 6B and Supplementary Table 12). Among DMC-associated genes that overlapped, we determined whether the changes in methylation at these loci were significantly correlated. To do this, we identified the CpG in

each gene with the highest methylation difference and compared them between sexes. Methylation changes were not significantly correlated (Figure 6C); 42.5% of genes exhibited methylation changes in the same direction with DEHP exposure between males and females (Supplementary Table 9).

Sex-specific methylation and human heart disease

We next evaluated the relevance of DEHP-induced changes in cardiac methylation to human heart disease. To this end, we determined the overlap between genes differentially methylated with DEHP exposure and those reported to be differentially methylated in the hearts of human heart failure patients.⁴⁸ For DMC-associated genes, among females,

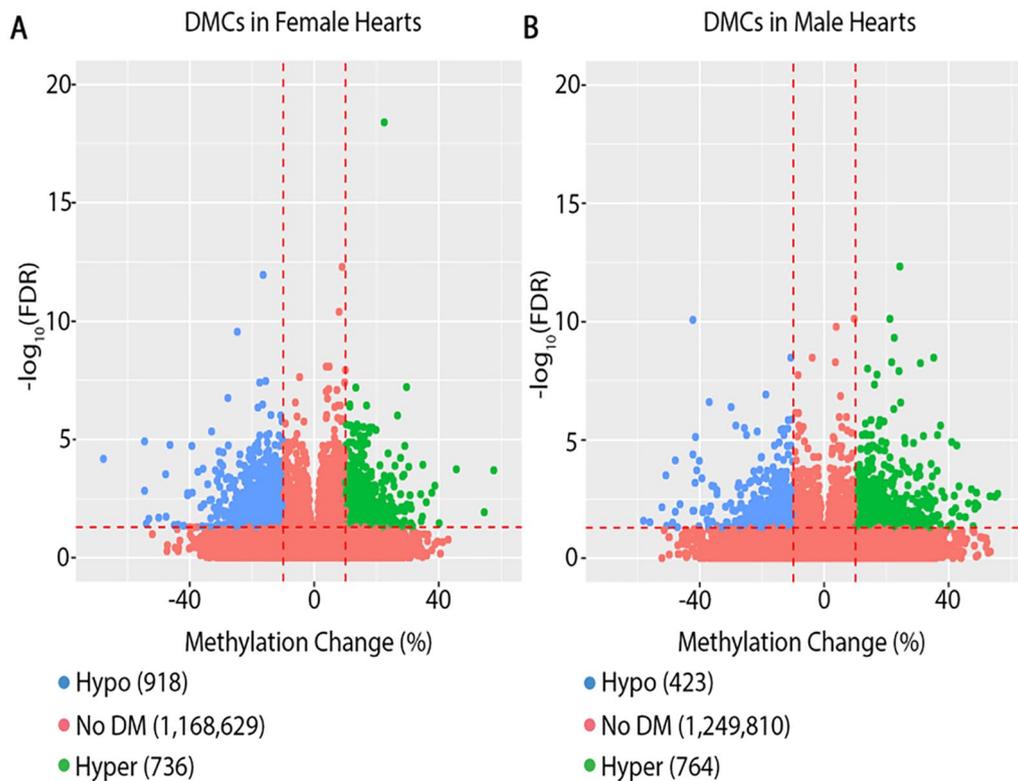


Figure 3. Volcano plots showing differentially methylated cytosines (DMCs) for DEHP vs control in (A) female heart and (B) male heart. Green: regions significantly hypermethylated with DEHP exposure. Blue: regions significantly hypomethylated with DEHP exposure.

Table 3. Top 10 differentially hypomethylated and hypermethylated cytosines (DMCs) in each sex, ranked by methylation change.

FEMALES					
CHROMOSOME	CHROMOSOMAL COORDINATE	METHYLATION CHANGE	FDR	GENE	GENOMIC ANNOTATION
8	108936845	-67.68	6.83E-5	<i>Mir3108/Zfthx3</i>	Promoter/Intron
12	105514846	-54.53	0.002	<i>AU015791</i>	Exon
10	82847363	-54.53	1.25E-5	<i>Txnrd1/Gm38560</i>	Intron/Intron
2	152358290	-53.16	0.022	<i>Gm14164</i>	Intron
9	39187003	-47.60	0.019	<i>Olfir943</i>	Exon, 3'-UTR
14	67253811	-46.40	1.72E-5	<i>Ebf2</i>	Intron
10	79892045	-45.00	0.041	<i>Cfd</i>	Exon
8	120090741	-43.75	0.041	<i>Zdhhc7/Galn2</i>	1to5 kb, Intron/Intron
8	122501577	-41.93	0.045	<i>Piezo1</i>	1to5 kb, Exon
9	77464154	-40.73	0.019	<i>Lrrc1</i>	Intron
17	23742647	54.48	0.012	<i>9530082P21Rik/Paqr4/Kremen2</i>	Promoter/1to5 kb/Exon
5	20925654	45.59	1.80E-4	<i>Rsbni1</i>	Intron
6	28910055	40.12	0.034	<i>Snd1</i>	Intron
5	135105148	35.95	0.004	<i>Mlxipl</i>	1 to 5 kb, Intron

(Continued)

Table 3. (Continued)

FEMALES					
CHROMOSOME	CHROMOSOMAL COORDINATE	METHYLATION CHANGE	FDR	GENE	GENOMIC ANNOTATION
17	29216167	34.78	1.17E-4	<i>Cpne5</i>	Intron
15	30995593	34.65	0.018	<i>Ctnnd2</i>	Intron
19	6297667	34.17	0.002	<i>Ehd1</i>	Promoter/Exon
3	122044452	31.06	0.008	<i>Abca4</i>	1 to 5kb, 5'-UTR, Exon
18	36022234	31.02	0.035	<i>Nrg2</i>	Intron
6	108593678	30.84	0.006	<i>0610040F04Rik</i>	Intron
Males					
11	68340065	-52.15	0.007	<i>Ntn1</i>	Intron
11	85458213	-50.88	3.19E-4	<i>Bcas3</i>	Intron
16	34946664	-47.81	0.011	<i>Mylk/ E130310I04Rik</i>	Intron/Intron
7	143071074	-47.81	7.32E-5	<i>Tssc4/Trpm5</i>	Exon, 3'-UTR/Intron
18	74959378	-47.14	0.048	<i>Lipg</i>	Intron
11	115488732	-42.27	0.010	<i>Armc7</i>	Exon
11	107305047	-42.19	8.39E-11	<i>Pitpnc1</i>	Intron
4	154425413	-42.18	4.08E-5	<i>Prdm16</i>	Intron
19	46471486	-40.97	1.93E-4	<i>Sufu</i>	Intron
7	100998188	-40.14	7.67E-5	<i>P2ry2</i>	Exon
19	45156160	55.71	0.002	<i>Tlx1</i>	Exon, 3'-UTR
1	36944000	48.94	0.002	<i>Tmem131</i>	Promoter, 1to5kb
5	113144949	48.51	0.004	<i>2900026A02Rik</i>	Intron
14	54595141	48.31	0.008	<i>4931414P19Rik</i>	Exon
3	84478963	47.77	0.044	<i>Fhdc1</i>	Exon, Intron
19	45156143	46.28	0.004	<i>Tlx1</i>	Exon, 3'-UTR
1	58740574	45.37	0.018	<i>Cflar</i>	Intron
6	8728976	43.43	0.017	<i>Ica1</i>	Intron
5	143400871	42.16	0.008	<i>Kdelr2</i>	1to5kb
4	89689601	41.73	0.002	<i>Dmrta1</i>	Intron

Table 4. Top 10 differentially hypo-methylated and hypermethylated regions (DMRs) in each sex, ranked by methylation change.

FEMALES					
CHROMOSOME	CHROMOSOMAL COORDINATE	METHYLATION CHANGE	FDR	GENE	GENOMIC ANNOTATION
15	25789001	-62.92	0.032	<i>Myo10</i>	Intron
12	105514001	-54.53	0.004	<i>AU015791</i>	Exon, Intron

(Continued)

Table 4. (Continued)

FEMALES					
CHROMOSOME	CHROMOSOMAL COORDINATE	METHYLATION CHANGE	FDR	GENE	GENOMIC ANNOTATION
2	152358001	-53.16	0.014	<i>Gm14164</i>	Intron
17	45632001	-49.53	0.001	<i>Capn11</i>	Exon, Intron
10	77881001	-44.21	0.005	<i>Tspear</i>	Exon, Intron
7	96773001	-41.76	0.010	<i>Tenm4</i>	1to5kb, Exon, Intron
17	36272001	-37.98	3.90E-4	<i>Trim39</i>	Promoter, 1to5kb, 5'UTR, Exon
14	63982001	-37.60	0.032	<i>4930578I06Rik</i>	Intron
13	38399001	-36.69	0.030	<i>Bmp6</i>	Intron
2	134787001	-33.40	0.037	<i>Plcb1</i>	Intron
15	30995001	34.65	0.012	<i>Ctnnd2</i>	Intron
19	21804001	29.16	0.002	<i>Cemip2</i>	Intron
7	118547001	28.20	2.12E-4	<i>Tmc7</i>	Exon, Intron
15	73544001	27.76	0.028	<i>Dennd3</i>	Exon, Intron
19	46322001	26.98	0.012	<i>Psd</i>	1to5kb, Exon, Intron
5	135105001	26.30	0.025	<i>Mlxip1</i>	Promoter, 1to5kb, Intron
1	62749001	26.04	7.22E-4	<i>Nrp2</i>	Exon, Intron
6	136807001	25.97	8.82E-4	<i>H2afj/ Hist4 h4</i>	Promoter, 1to5kb/1to5kb
11	116131001	24.44	0.015	<i>Trim65/Trim47/ Mrpl38</i>	Promoter, 5'UTR, Exon/1to5kb/Exon, 3'UTR
19	47258001	24.22	0.002	<i>Neur1a</i>	Exon, 3'UTR
Males					
4	131489001	-64.16	7.49E-5	N/A	N/A
1	118981001	-59.33	4.67E-5	<i>Gli2</i>	Intron
11	85458001	-43.09	0.004	<i>Bcas3</i>	Intron
11	107305001	-42.19	2.68E-11	<i>Pitpnc1</i>	Intron
19	46471001	-40.49	0.021	<i>Sufu</i>	Intron
5	122950001	-35.04	0.042	<i>Kdm2b</i>	1to5kb, Intron
4	137949001	-35.52	0.050	<i>Ece1</i>	Exon, Intron
11	75524001	-25.33	0.003	<i>Scarf1</i>	Exon, Intron, 3'UTR
16	34028001	-25.24	0.035	<i>Kalrn</i>	Exon, Intron
8	83710001	-25.22	0.037	<i>Ddx39</i>	1to5kb
12	111572001	54.38	0.043	<i>Mark3/ 2810029C07Rik</i>	1to5kb/Exon
1	74719000	50.69	1.01E-5	<i>Cyp21A1</i>	Intron
1	36943001	48.93	0.001	<i>Tmem131</i>	Promoter, 1to5kb, 5'UTR, Intron, Exon
2	76105001	41.24	5.05E-18	<i>Pde11a</i>	Intron
14	32644001	39.76	0.032	<i>Prrxl1</i>	Intron

(Continued)

Table 4. (Continued)

FEMALES					
CHROMOSOME	CHROMOSOMAL COORDINATE	METHYLATION CHANGE	FDR	GENE	GENOMIC ANNOTATION
7	143082001	39.68	1.68E-4	<i>Trpm5</i>	Promoter, 1to5kb, Intron, Exon
15	63351001	37.83	0.004	<i>Gm20740</i>	Intron
4	155739001	33.27	0.014	<i>Tmem240</i>	Exon, Intron, 3'UTR
6	55457001	30.86	2.64E-5	<i>Adcyap1r1</i>	1to5kb, Intron
X	7167001	30.24	1.20E-4	<i>Clcn5</i>	Intron

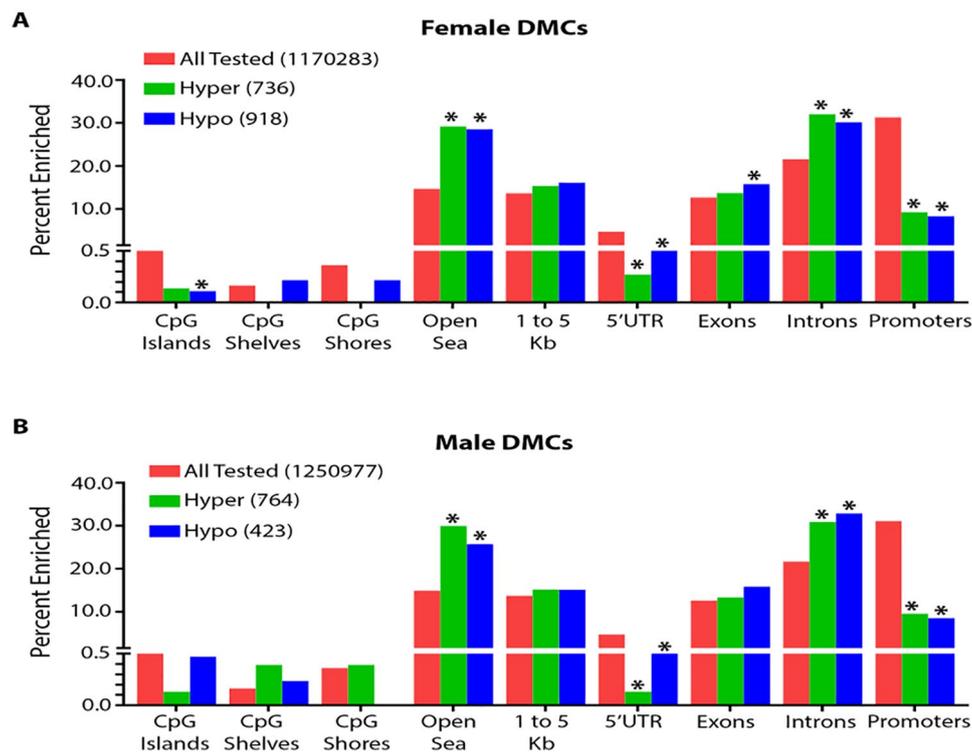


Figure 4. Summary plots depicting the total number of tested CpGs (red), hypermethylated CpGs (green), and hypomethylated CpGs (blue) for each genomic annotation using the R annotatr package. Data are from (A) female and (B) male offspring at 5 months of age. (* $P < .05$ using chi-square test.) DMC indicates differentially methylated cytosine.

111 of the 1346 genes differentially methylated in human heart failure were also differentially methylated with DEHP exposure (Figure 7A and Supplementary Table 13). This overlap was significantly greater than that expected by chance ($P < 3.67 \times 10^{-9}$, hypergeometric test). Among males, 87 genes were shared between the groups (Figure 7B and Supplementary Table 14). Again, the overlap was significant ($P < 3.12 \times 10^{-8}$, hypergeometric test). Only 15 genes were in common between DEHP-exposed males, DEHP-exposed females, and heart failure patients (Supplementary Figure 2A and Supplementary Table 15). To determine whether methylation changes were correlated at overlapping DMC-associated genes between DEHP exposure and heart failure, we compared the CpG with the

highest methylation change for each gene (Figure 7C and D). Overall, changes in methylation were not significantly correlated between heart failure and DEHP exposure in either males or females (Figure 7C and D). However, in DEHP-exposed females, 50.5% of the overlapping genes exhibited changes in methylation in the same direction as in the heart failure patient samples. In DEHP-exposed males, methylation changes in 55.2% of genes were concordant with those in heart failure patient samples. For DMR-associated genes, 32 and 14 genes were in common with human heart failure patients among females and males, respectively (Figure 7C and D and Supplementary Tables 16 and 17), with only 2 genes in common across all groups (Supplementary Figure 2B). Thus, perinatal DEHP

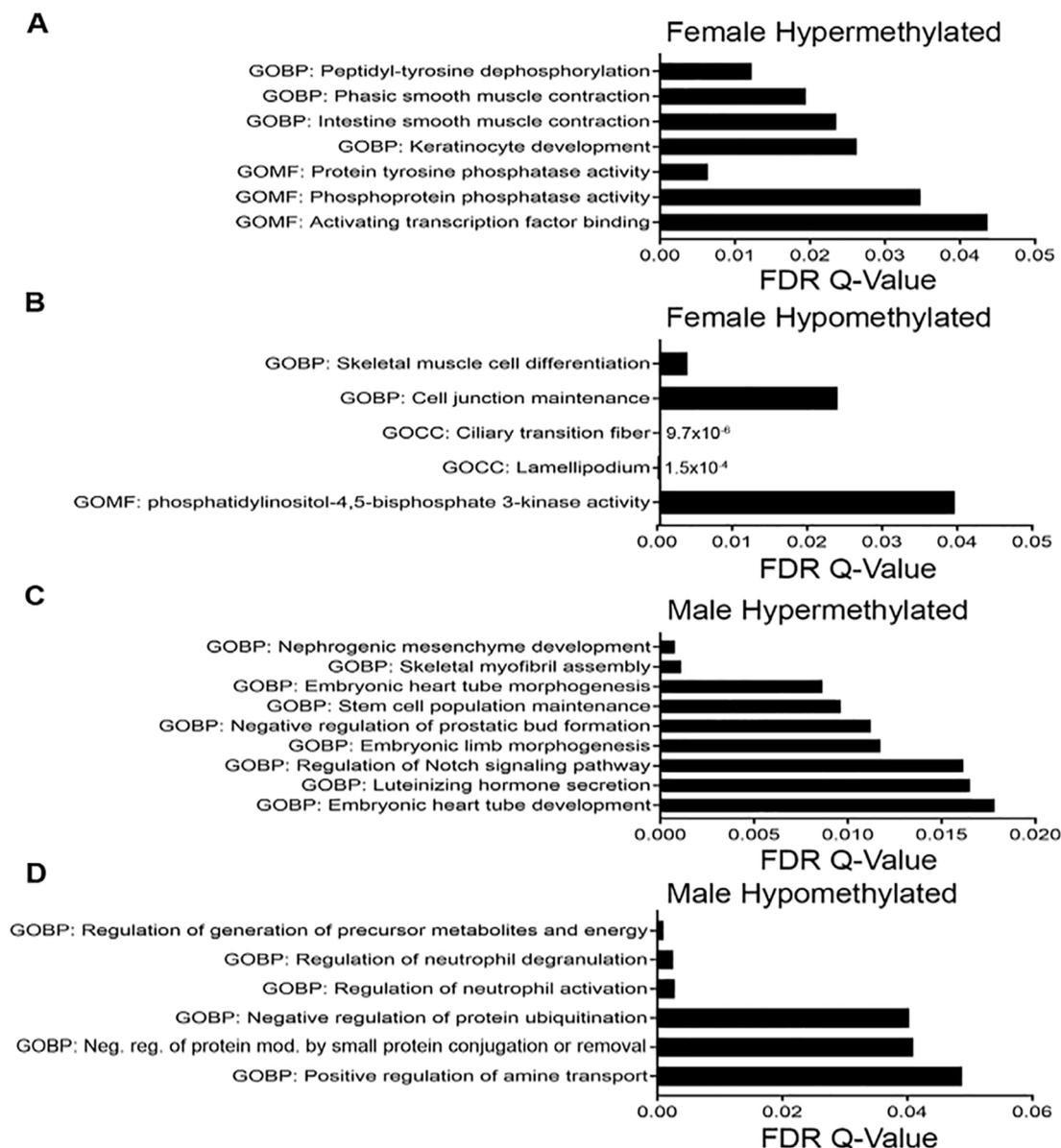


Figure 5. Results of analysis of DMCs with Genomic Regions Enrichment of Annotations Tool. Analyses were stratified by sex and direction of methylation. All significant Gene Ontology terms are shown, with the exception of Male Hypermethylated (Panel C), which depicts the top 10 most significant pathways. DMC indicates differentially methylated cytosine.

exposure resulted in altered methylation at many genes common to human heart failure, with distinct sex differences. In females, these genes included *PRKCE*, *SPRY1*, and *GJA5*, which have all been demonstrated to contribute to sex differences in ischemia-reperfusion injury, cardiac fibrosis, and sudden cardiac death.⁴⁹⁻⁵² In males, common genes included *ECE1*, *SMAD7*, and *DNMT3A*, which play a critical role in cardiac fibrosis and development.⁵³⁻⁵⁵ Notably, changes in methylation at these genes were consistent across individual animals within each treatment group (Supplementary Figure 3).

Discussion

Cardiovascular diseases pose a grave health threat to both males and females. However, the incidence, clinical

presentation, and prognosis of these diseases differ markedly between sexes.¹ Although sex disparities in medical care may underlie some of these differences,⁵⁶ it is clear that genetic, epigenetic, and hormonal factors are important contributors to sex specificity.¹ Indeed, at the cellular level, there are intrinsic sex differences in how cardiomyocytes respond to drug treatments.^{57,58} Expression of ion channels and transporter subunits differ between men and women, and women are more prone to drug-induced arrhythmias.^{59,60} Despite these findings, the sex-specific effects of environmental chemicals on cardiac function, particularly early developmental exposures, are poorly understood. In this work, we demonstrate that perinatal DEHP exposure leads to sex-specific, genome-wide changes in DNAm in the hearts of adult offspring mice. Because exposure to DEHP ceased at 3 weeks of age, these

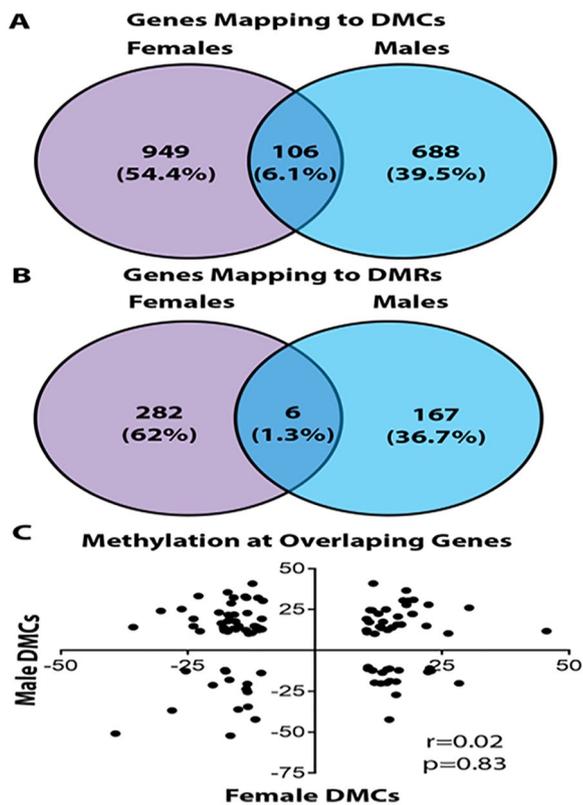


Figure 6. Venn diagrams showing overlap between females and males in genes mapping to (A) differentially methylated cytosines and (B) differentially methylated regions. Panel (C) depicts the correlation between methylation changes occurring in the overlapping genes. The r value represents the Pearson correlation coefficient, and the P value is based on a 2-tailed test. The correlation plot represents the CpG within each overlapping gene that had the largest methylation change after DEHP exposure. DEHP indicates diethylhexyl phthalate; DMC, differentially methylated cytosines.

data suggest that perinatal DEHP exposure may have long-term effects on the epigenome.

The implications the observed changes in methylation have for cardiac function require further investigation. Phthalate-induced epigenetic changes have been identified as potential mediators of altered hormone levels and disease,⁶¹⁻⁶³ suggesting a possible causative role for DNAm in phthalate-induced health effects. In this study, we did not observe significant changes in relative heart weights with DEHP exposure, and we did not determine the effects of DEHP exposure on cardiac function. However, we have previously demonstrated that DEHP exposure in this model results in increased body fat and decreased lean mass in adult females,⁶⁴ suggesting that this chemical has long-term, sex-specific effects on metabolic health. Moreover, adverse effects of perinatal environmental exposures may not manifest until subsequent challenges later in life, such as hormonal stimulation, poor diet, or additional environmental exposures.⁶⁵⁻⁶⁷ Further studies are necessary to determine the consequences of developmental DEHP exposure on cardiac function and the potential role for DNAm in mediating these effects.

Pathways associated with insulin signaling and glucose transport were significantly enriched among DMCs in the hearts of females and males, respectively. Likewise, *Slc5a4a*, which contained a DMR that directly overlapped between males and females, encodes a glucose-sensing sodium channel.⁶⁸ These observations are notable, given the profound and unyielding energetic demands of the heart. Insulin signaling plays a pivotal role in regulating cardiac mitochondrial function, glucose, and fatty acid uptake, as well as promoting hypertrophy in response to exercise.⁶⁹⁻⁷¹ Healthy hearts exhibit metabolic plasticity and are capable of utilizing fatty acids or glucose for generation of ATP.⁷² Both glucose uptake and insulin signaling are impaired in diseases such as coronary heart disease and heart failure.^{71,73} Likewise, additional pathways relevant to cardiac development and function were identified by GREAT analysis, including PI3K pathway function (female, hypomethylated) and embryonic heart tube morphogenesis (male, hypermethylated). The PI3K pathway plays an important role in regulation of cardiac hypertrophy and contractility, and altered function of this pathway is implicated in heart failure and other diseases.⁷⁴

It is further noteworthy that several genes mapping to DMCs and DMRs with DEHP exposure were also differentially methylated in heart tissue from human heart failure patients,⁴⁸ and that the overlapping genes were distinct based on sex. Importantly, in at least 50% of the overlapping genes, methylation changes occurred in the same direction. Notably, *PRKCE* encodes protein kinase C epsilon, which is increased in female hearts compared to males subjected to ischemia-reperfusion injury. Expression of this protein may confer reduced susceptibility to long-term deleterious effects of myocardial infarction. *SMAD7*, which was differentially methylated in DEHP-exposed males and in heart failure samples, plays an important protective role against cardiac fibrosis induced by myocardial infarction or hyperglycemia.^{54,75} Phthalate exposures are linked in human and animal models to several CVDs associated with heart failure, including congenital heart defects, arrhythmias, hypertension, and coronary heart disease.^{10-16,76-78} We are actively pursuing further mechanistic and functional studies to determine the implications of our findings for sex-specific cardiac health.

The mechanisms by which DEHP affects DNAm are currently unclear. Animal studies suggest that perinatal DEHP exposure alters expression of DNA methyltransferases (DNMTs), which catalyze the addition of methyl groups to the 5-position of cytosine bases in DNA.^{79,80} However, because these studies were conducted in males, the sex-specific effects of perinatal phthalate exposures on expression and function of DNMTs are unknown. In this study, we observed differential methylation of *Dnmt3a* in DEHP-exposed males but not females, highlighting a potential sex-specific mechanism of regulation of this gene by DEHP

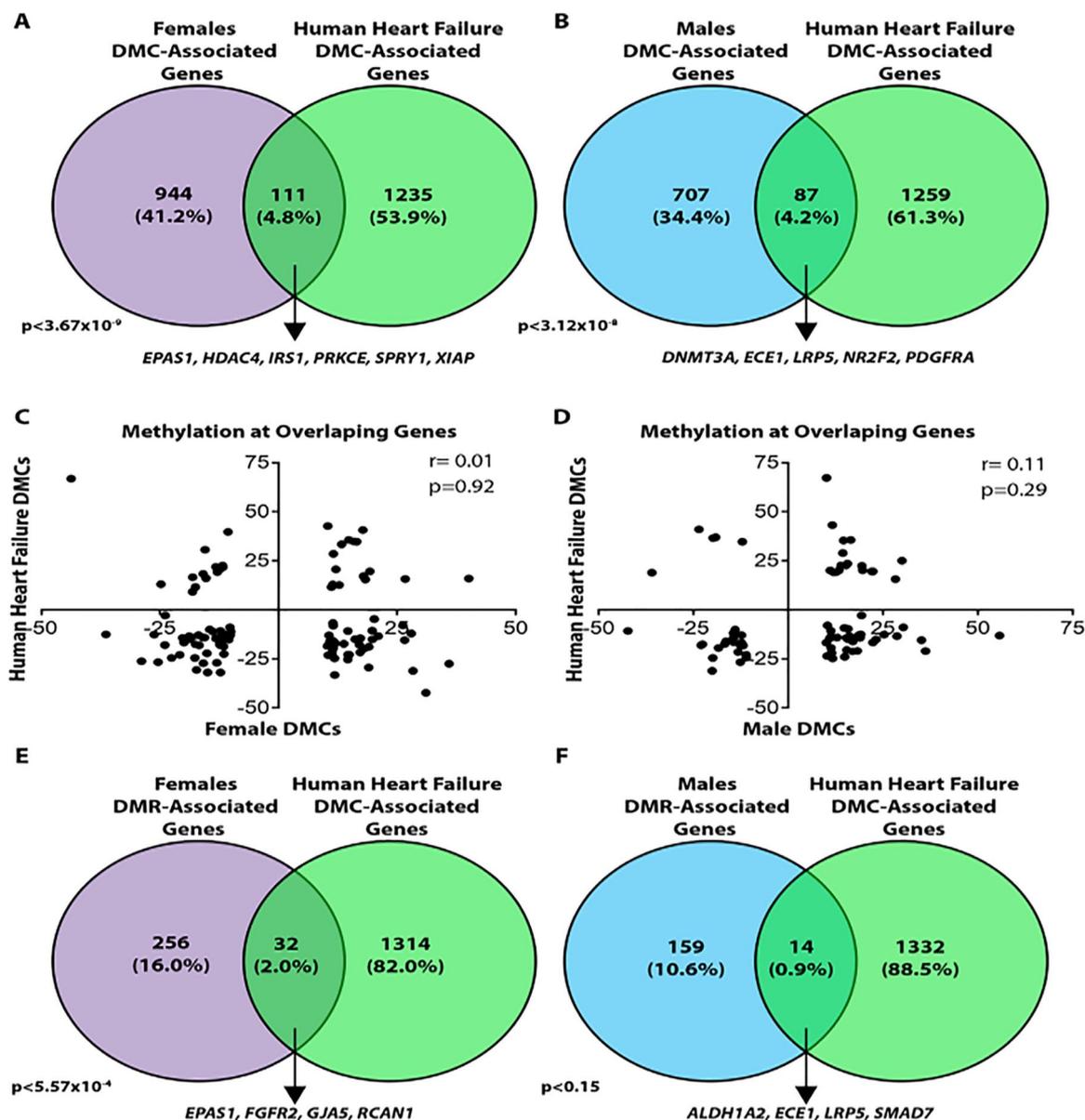


Figure 7. Venn diagrams depicting overlap in genes mapping to DMCs/regions between DEHP-exposed animals and human heart failure samples.⁴⁸ DMC data are depicted in Panels (A) and (B). Panels (C) and (D) depict the correlation between methylation changes occurring in the genes overlapping between human heart failure and DEHP exposed (C) females and (D) males. The r values represent the Pearson correlation coefficient, and the P values are based on a 2-tailed test. The correlation plots represent the CpG within each overlapping gene that had the largest methylation change after DEHP exposure or in heart failure. Panels (E) and (F) depict overlapping genes that mapped to DMRs. Selected overlapping genes strongly linked to cardiovascular development and disease are shown. P values represent the significance of overlap between differentially methylated genes by DEHP exposure and differentially methylated genes in heart failure. DEHP indicates diethylhexyl phthalate; DMC, differentially methylated cytosine; DMR, differentially methylated region.

exposure. The functional effects of this epigenetic programming require further investigation. Importantly, other environmental exposures lead to changes in epigenetic regulators that are highly sex-specific,⁸¹ highlighting a potential mechanism by which toxicants, including DEHP, may induce sex-specific epigenomic programming. Alterations in DNMT function are another potential mechanism by which DEHP may alter DNAm. DNA methyltransferase methylate cytosine bases in DNA using S-adenosylmethionine as a methyl donor, which is supplied by the 1-carbon metabolic

pathway.⁸² Perinatal phthalate exposures lead to perturbation of methyl donor levels and global hypomethylation in rat testis,⁸³ and administration of the methyl donor choline attenuates the teratogenic effects of phthalate exposure in chick embryos,⁸⁴ suggesting that phthalates may interfere with 1-carbon metabolism. Moreover, phthalates have been reported to bind to and modulate the activity of nuclear hormone receptors, including peroxisome proliferator-activated receptors (PPARs),^{85,86} which function in part through regulation of DNAm.^{87,88} Studies to determine the sex-specific

mechanistic basis for DEHP-induced perturbations in cardiac DNAm are currently underway.

Although we provide novel evidence that perinatal phthalate exposure leads to alterations in cardiac DNAm detectable in adulthood, key limitations to this study should be considered. First, to assess changes in DNAm with perinatal DEHP exposure, we utilized ERRBS, which detects DNAm at basepair resolution at CpG rich loci in the genome. This approach captured, on average, 4.8% of the total CpGs in the genome. Thus, other biologically relevant genomic loci may have been missed. ERRBS provides enrichment for CGI, CpG shores, introns, exons, and intergenic regions.^{42,89} Thus, the enrichment of DMCs/DMRs at specific genome-wide regions may have been affected by this bias. In addition, ERRBS employs sodium bisulfite to convert unmethylated cytosines to uracil, which are replaced with thymine in subsequent PCR.⁴² Methylated cytosines are resistant to sodium bisulfite treatment, allowing the quantity of DNAm to be assessed based on C to T transitions. However, oxidized derivatives of 5-methylcytosine, including 5-hydroxymethylcytosine, are also resistant to sodium bisulfite conversion.⁹⁰ Thus, ERRBS and other assays based on traditional sodium bisulfite conversion methods do not discriminate between 5-methylcytosine and 5-hydroxymethylcytosine. Future studies will investigate the effects of perinatal DEHP exposure on 5-hydroxymethylcytosine in several tissues as part of the NIEHS TaRGET II Consortium. A second limitation of this study is that analysis of DNAm was conducted in bulk tissue. We, therefore, cannot determine the effects of DEHP exposure on specific subpopulations of cells. Future studies using single-cell epigenomics and transcriptomics^{91,92} would permit assessment of cell-specific effects of DEHP and other environmental exposures on the heart.

Conclusions

In this work, we describe novel findings related to the effects of perinatal phthalate exposure on the heart. We show, for the first time, that perinatal DEHP exposure leads to altered DNAm in the hearts of offspring mice in adulthood, long after cessation of exposure. Given the reported adverse effects of phthalate exposures on cardiac health, the role for epigenetic programming in phthalate-induced cardiac dysfunction warrants further investigation. We also demonstrate that changes in DNAm are highly sex-specific, a finding that underscores the need for environmental health studies that consider the critical role of sex in toxicant-induced health outcomes.

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Author Contributions

All authors provided critical feedback and helped shape the research, analysis and manuscript. KN performed experiments. LKS, KW and RGC analyzed data. DCD, JAC, and MAS provided advice and guidance on data analysis and interpretation. LKS wrote manuscript.

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Data Availability Statement

Raw and processed ERRBS data are available on GEO, accession number GSE152711.

Supplemental Material

Supplemental material for this article is available online.

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