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Probing prenatal bisphenol exposures and tissue-specific DNA methylation responses in cord blood, cord tissue, and placenta

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ABSTRACT

The early-gestational fetal epigenome establishes the landscape for fetal development and is susceptible to disruption via environmental stressors including chemical exposures. Research has explored how cell- and tissue-type-specific epigenomic signatures contribute to human disease, but how the epigenome in each tissue comparatively responds to environmental exposures is largely unknown. This pilot study compared DNA methylation in four previously identified genes across matched cord blood (CB), cord tissue (CT), and placental (PL) samples from 28 mother-infant pairs in tthe Michigan Mother Infant Pairs study; evaluated association between prenatal exposure to bisphenols (BPA, BPF, and BPS) and DNA methylation (DNAm) by tissue type; compared epigenome-wide DNAm of CB and PL; and explored associations between prenatal bisphenol exposures and epigenome-wide DNAm in PL. Bisphenol concentrations were quantified in first-trimester maternal urine. DNAm was assessed at four genes via pyrosequencing in three tissues; epigenome-wide DNAm analysis via Infinium MethylationEPIC array was completed on CB and PL. Candidate gene analysis revealed tissue-specific differences across all genes. In adjusted linear regression, BPA and BPF were associated with DNAm across candidate genes in PL but not CB and CT. Epigenome-wide comparison of matched CB and PL DNAm revealed tissue-specific differences at most CpG sites and modest associations between maternal first-trimester bisphenol exposures and PL but not CB DNAm. These data endorse inclusion of a variety of tissues in prenatal exposure studies. Overlapping and divergent responses in CB, CT, and PL demonstrate their utility in combination to capture a fuller picture of the epigenetic effect of developmental exposures.

1. Introduction

Increasing evidence demonstrates that prenatal developmental exposure is associated with the disruption of many biological systems [1]. Just as the organs and tissues of the body operate in separate but synchronous ways to respond to and manage 'typical' functions or insults, so too do these organs and tissues respond in a multi-dimensional way to environmental toxicant exposure [2]. Therefore, organ- or tissue-specific biological effects after fetal exposure must be characterized to enhance understanding of the broader processes that are implicated in exposure studies. One fundamental feature of tissue-specific regulatory systems is the epigenome. This work defines the epigenome as consisting of chemical modifications (e.g. DNA methylation and histone modification) that are mitotically heritable and regulate gene expression but are not the result of a change in the DNA sequence [3]. The epigenome is known to differ across tissues [4] and assist in establishing tissue- or cell-specific gene expression and functions [5]. Infant cord blood (CB) is a commonly used tissue to evaluate DNA methylation in response to prenatal environmental exposures. The use of CB as a surrogate tissue; whereby changes in CB DNA methylation are evaluated as a proxy for target tissues like the brain or liver [6–8], is an acceptable practice both because of the clear, ethical restrictions of

Abbreviations: CB, Cord Blood; CT, Cord Tissue; PL, Placenta; BPA, bisphenol A; BPS, bisphenol S; BPF, bisphenol F; MMIP, Michigan Mother Infant Pairs.

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utilizing target tissues and its correlation with disease biomarkers in epigenome-wide association studies (EWAS). However, there is a growing consensus within the field of environmental epigenetics for the quantitative and comprehensive interrogation of the association between early-life exposures and alterations in epigenome-wide DNA methylation in *multiple* neonatal or infant tissues, beyond and in addition to CB. The epigenome differs in every tissue and even every cell type; a biological feature that assists in establishing tissue- or cell-specific gene expression and functions [5]; and although advances have been made in elucidating how cell- and tissue-type specific epigenomic signatures contribute to human disease, additional studies are needed that address this concept [9].

Parallel epigenetic analyses of CB, cord tissue (CT), and placenta (PL) within a single pregnancy cohort provides an opportunity to elucidate how different tissues respond to prenatal exposures. Importantly, both CT and PL are feasible to collect alongside CB, and their separate embryonic origins offer the potential to gather information about an exposure's impact on DNA methylation in separate developmental lineages. DNA methylation profiling of CB and CT samples from 295 neonates with the Illumina Infinium 450 K array followed by hierarchical clustering analysis with 25 primary tissues and cells confirmed CB and CT tissues and cells clustered in accordance with their germinal origin [10]. Specifically, CB clustered with hematopoietic stem cell (HSC)-derived mesodermic tissue (e.g. blood), while CT clustered with mesenchymal stem cell (MSC)-derived mesodermic tissue (e.g., muscle, heart, kidney) [10]. Placenta, unlike CB and CT, is not composed solely of fetal cells, but rather it contains cells of both fetal and maternal origin. Both CT and PL are key tissues in fetal growth and development. These features of CB, CT, and PL establish a compelling incentive to include all three for epigenetic analysis in DOHaD studies to compare their associations with exposures or outcomes of interest.

The exposure of interest to the current investigation are bisphenols. Bisphenol-A (BPA) is a chemical commonly used in receipts, plastics, and food packaging with striking evidence demonstrating its role as an endocrine disruptor [11]. Human exposure to this toxicant is considered 'ubiquitous,' principally because of its wide-spread use as components in every-day products. Exposure to BPA and two of its commonly used replacement analogues, bisphenol-F (BPF) and bisphenol-S (BPS), are readily detectable in U.S. populations [12–15]. A recent systematic review compared the endocrine and physiological effects of BPA, BPF, and BPS and demonstrated that BPF and BPS have similar in vitro metabolism, potencies, and mechanisms of action to that of BPA and additional toxicity in separate hormonal actions [16]. Multiple studies have shown relationships between prenatal exposures to bisphenols and altered CB DNA methylation [17–20]. However, only a limited number of studies have examined the associations between bisphenol exposures and PL or CT DNA methylation [21,22]. There is a critical gap in the literature of studies that compare CB, CT, and PL tissue DNA methylation both generally and in association with prenatal toxicant exposures.

In this proof-of-concept study we aimed to (1) compare average percent DNA methylation in candidate genes selected for their previously detected association with differential DNA methylation in CB in response to maternal prenatal BPA exposure [17] (*FN1*, *SNAP25*, *HOXA-AS3*, and *PRSS22*) across three tissues - CB, CT, and PL; (2) evaluate the association between prenatal exposure to the bisphenols BPA, BPF, and BPS and DNA methylation by tissue type; (3) move beyond candidate genes evaluated in (1) and compare epigenome-wide DNA methylation patterns of CB and PL; and (4) explore epigenome-wide DNA methylation in PL in association with prenatal bisphenol exposure. We predicted that DNA methylation at *FN1, SNAP25, HOXA-AS3*, and *PRSS22* would display tissue-specific differences and associations with prenatal bisphenol exposure; that we would detect differential, function-specific methylation between CB and PL; and that PL EWAS would reveal additional loci associated with bisphenol exposures.

2. Methods

2.1. Study population

All study procedures were performed under the approval of the University of Michigan (UM) Medical School Institutional Review Board with written informed consent obtained from all the participants. The samples used in this study were derived from the Michigan Mother-Infant Pairs human birth cohort (MMIP), which was initiated in 2010. The details of participant recruitment, inclusion criteria, and study timeline have previously been reported [17]. The study presented here utilized maternal urine collected at the first trimester visit and PL, CT, and CB samples collected at the time of delivery from a subset of families. N = 28 subjects had all three tissues (PL, CT, and CB) available—they are denoted as 'tissue triads.' N = 23 maternal-infant pairs had maternal urinary bisphenol measures in addition to the tissue triads. The maternal and infant samples included in this study were collected between the years 2011–2017. Demographic and anthropometry data collected from participants and extracted from the electronics health record included: maternal age, number of days to delivery, maternal height, maternal weight pre- and post-pregnancy, infant sex, and infant birth weight.

3. DNA extraction and quantification of tissue triads

3.1. DNA extraction

Infant CB samples (N = 28) were collected into PaxGene Blood DNA tubes (PreAnalytix) with the use of butterfly needles at the time of birth and stored at -80 until processing. Total DNA was extracted with the PaxGene Blood DNA kit. PL samples were collected shortly after birth; PL segments were dissected and stored at −80 until processing. Total DNA was extracted with the AllPrep DNA/RNA/miRNA Universal Kit (Qiagen). CT samples were collected by dissecting a portion of the umbilical cord that was closest to the infant side. Samples were stored at −80 until processing. Total DNA was extracted with the DNeasy Blood & Tissue Kit (Qiagen). For all samples, DNA quality and concentration were assessed via Qubit at the University of Michigan Advanced Genomics Core.

3.2. Bisulfite conversion

DNA for each tissue was bisulfite converted using the EZ-96 DNA Methylation Kit (Zymo), wherein approximately 500 ng of input DNA was used. The kit utilized sodium bisulfite to convert unmethylated cytosines to uracil and ultimately thymine, while methylated cytosines were protected [23].

3.3. DNA methylation measurement – candidate gene analysis of cord blood, umbilical cord, and placenta

Pyrosequencing [24] was used to measure DNA methylation levels at CpG sites in the genes *FN1*, *SNAP25*, *HOXA-AS3*, and *PRSS22* in CB, CT, and PL. These genes were selected because they were previously associated with differential DNA methylation in CB in response to maternal prenatal BPA exposure [17]. PCR amplification was performed on the bisulfite converted DNA. Primers utilized for PCR amplification were designed using the PyroMark Assay Design Software 2.0 (Supplementary file 1). DNA methylation levels were then quantified using the PyroMark Q96 ID (Qiagen). Matched sample triads were run on the same plate for PCR amplification and pyrosequencing. All pyrosequencing plates included a 0 % and 100 % human bisulfite converted control, no-template controls, and 10 % of samples run in duplicate.

3.4. DNA methylation measure - epigenome-wide analysis of cord blood and placenta and cord blood

Following bisulfite treatment, DNA methylation at >850,000 CpG sites was evaluated using the Illumina Infinium MethylationEPIC BeadChip ('EPIC') at the University of Michigan Advanced Genomics Core according to standard protocols. CB samples were run on three separate days, while PL were run on two separate days; these experimental batches were considered in statistical models. Epigenome-wide analysis on CT was not included in this study due to limited funds at the time of analysis.

3.5. Maternal bisphenol measurement

Urine samples were collected from mothers during their first trimester visit at the University of Michigan Hospital, which took place between weeks 8–14 of pregnancy. Quantification of bisphenol concentrations and specific gravity (SG) were completed by NSF International (Ann Arbor, MI) and reported earlier for the larger cohort [25]. Urinary bisphenol values below the limit of detection (LOD, 0.2 ng/mL) were replaced with LOD/$\sqrt{2}$ (0.141 ng/mL).

3.6. Statistical analyses

All statistical analyses were performed in R version 3.6.0 (Platform: x86_64-apple-darwin15.6.0 (64-bit) & Running under: macOS Mojave 10.14.6).

3.7. Cross-tissue comparison – candidate genes

We first evaluated cross-tissue differences in average percent DNA methylation at each gene. For a given sample, mean percent methylation was calculated using all CpG sites assessed for each gene. We visualized DNA methylation region averages for each subject through a spaghetti plot. Correlations across loci and tissues were assessed using Spearman's correlation. Finally, paired t-tests were run between tissue pairs to determine whether mean percent methylation at a gene was different across CB, CT, and PL.

3.8. Bisphenol exposure and candidate gene DNA methylation

Additional univariate analyses were completed on those families for whom maternal bisphenol measures were available. Bivariate analyses were completed to classify the correlation between bisphenol exposures and candidate gene methylation, in addition to covariates of interest, utilizing Spearman correlation. Bisphenol measures were first adjusted for specific gravity and natural log (ln) transformed. Mixed effects regression was utilized to assess associations between first trimester bisphenol exposures and DNA methylation in the tissue triads. BPA, BPF, or BPS were regressed on repeat measures of DNA methylation for each gene at the three tissues. Models included a random intercept representing each subject and adjusted for sex and tissue type. An interaction term between tissue type and exposure was included to determine whether any tissue(s) relationship with the exposure differed from the rest. Given that this is a proof-of-concept study with a small sample-size, results with a p-value < 0.20 were discussed.

Since several genes had evidence for differential response across tissues (interaction term with $p < 0.20$) by at least one bisphenol, the mixed effect regression was followed by linear regression for each gene in each separate tissue type to estimate the tissue-specific association between exposure and DNA methylation. These models adjusted for infant sex, and when available, estimated cell type proportions of nucleated red blood cells (nRBCs) and B cells for CB and two surrogate cell type variables for placenta (see next section).

3.9. Processing and quality control of infinium methylation EPIC data

Arrays were assessed for quality of samples and probes using a standard pipeline. Briefly, the pipeline utilized the minfi package [26] (R Project for Statistical Computing) to read in raw data image files. Quality control of samples was assessed by comparing estimated sex (from methylation values on the X and Y chromosomes) with known infant sex, detection p-values of probes, and intensity signals. Probes with poor detection (positions that failed detection in more than 10 % of samples N = 1475), cross-reactive probes, and probes that target polymorphic CpG sites were dropped. [27] The Functional Normalization [28] R package was used to correct for background and perform dye-bias normalization. These preprocessing steps resulted in 822,020 retained probes from N = 28 CB and N = 28 PL samples that passed all quality control measures. Finally, M-values, defined as the log2 ratio of intensities of methylated probe versus unmethylated probes, were generated for each sample at these CpG sites and were used in downstream statistical analyses unless otherwise noted. M-values were selected for statistical analysis given their advantages, which include meeting the assumption of homoscedasticity and superior performance in Detection Rate (DR) and True Positive Rate (TPR), especially for highly methylated and unmethylated sites [29,30].

Using estimateCellCounts in R, the relative proportion of B cells, CD4 + T cells, CD8 + T cells, granulocytes, monocytes, neutrophils, and nucleated red blood cells (nRBCs) were estimated for each cord blood sample using an established algorithm based on DNA methylation profiles of sorted major cord blood cell types. [31] Using RefFreeEWAS, the relative proportion of putative cell types were estimated for PL with a reference-free deconvolution algorithm. This method utilized the participants' PL methylation data to estimate constituent cell types [32]. After these steps, two principal components representing cell type composition were retained.

3.10. Epigenome-wide comparison of CB and PL DNA methylation

Mixed effects regression was utilized to identify loci that were differentially methylated between matched tissue samples, with CB as the reference category. Tissue type was regressed on repeat measures of DNA methylation for each probe in CB and PL. Models included a random intercept representing each subject and adjusted for infant sex. P-value correction by the Benjamini-Hochberg false discovery rate (FDR) method was used, and a 5 % FDR (e.g., $q<0.05$) was considered significant.

3.11. Gene ontology enrichment analysis for tissue comparison

Gene ontology was assessed on the 10,000 most significantly differentially methylated CpG sites between CB and PL with the gometh function in the missMethyl package[33]. Gometh performs gene set enrichment testing on both GO and KEGG pathways for the significant sites.

3.12. Bisphenols and epigenome-wide placenta DNA methylation

Using EPIC array data from PL, linear regression was used to identify differentially methylated CpG sites (using M-values) by each bisphenol exposure, adjusting for covariates infant sex and two surrogate variables for placental cell type. Ln-adjusted bisphenol measures were used in statistical models. An empirical Bayes method in the limma [34] R package was then used to shrink probe-wise variances towards a pooled estimate and calculate a moderated t-statistic. Due to the small sample size and exploratory nature of this part of our study, we used the p-value cut-off of $p < 0.0001$.

3.13. Differentially methylated regions (DMRs)

We utilized dmrcate [35] to test for DMRs by bisphenol exposure. A DMR had to consist of at least 2 consecutive probes. Probes that were two nucleotides or closer to a single-nucleotide polymorphism (SNP) that had minor allele frequency greater than 0.05 were filtered out first. The model was adjusted for placental cell type and infant sex. A significance cut-off of $p < 0.0001$ was used.

3.14. Pathway analysis

LRpath [36] was utilized to perform gene-set enrichment across all probes annotated to genes (using Entrez Gene IDs) using concepts from KEGG and GO (Biological Process, Molecular Function, and Cellular Component). Raw P-values generated from the linear model for the association between each bisphenol and PL DNA methylation at sites within genes were used. Concepts from both GO and KEGG databases were selected, and only gene-sets with a minimum of 10 and a maximum of 250 genes were used; a directional test was included. LRPath tests the odds that the genes in a concept have higher significance values (e.g., lower p-values from the differential methylation analysis) than expected at random. Significance for gene-sets was considered at $q < 0.05$.

4. Results

4.1. Candidate gene analysis: DNA methylation varies across tissue type

Demographics of the study participants are displayed in Table 1. Average percent DNA methylation varied in candidate genes across tissue (Fig. 1). Paired t-tests demonstrated that average percent methylation was significantly different between at least one tissue pair per candidate gene. For example, in both *FN1* and *HOXA-AS3*, CT had the highest average DNA methylation percentage, while PL had the lowest (Fig. 2). Lastly, *SNAP25* and *PRSS22* had two tissue pairs that were not significantly different upon comparison. Correlations between tissues were also assessed. CT and PL average percent methylation were negatively correlated ($p < 0.05$) in *FN1* and *HOXA-AS3,* while CB and PL average percent methylation were positively correlated ($p < 0.05$) in *SNAP25* and *PRSS22*.

4.2. Candidate gene analysis: environmental exposure response across tissue type

We assessed whether DNA methylation at each tissue type had similar or different associations with environmental exposure to bisphenols. 95 % of samples (22/23) had BPA levels above the LOD and 83 % of samples (19/23) had BPF and BPS levels above the LOD.

Table 1
Descriptive statistics [median (25th, 75th percentiles) or n (%)] for N = 28 mother-infant pairs in the MMIP cohort included in this study.

Maternal age (years)	33 (31, 35)
Number of days to delivery (days)	275 (274, 278)
Maternal Race/Ethnicity: White	28 (100%)
Cell type variable 1*	0.502 (0.193, 0.827)
Cell type variable 2*	0.248 (0.0425, 0.385)
Maternal urinary BPA (ng/mL)	1.01 (0.424, 1.30)
Maternal urinary BPF (ng/mL)	1.87 (0.411, 2.05)
Maternal urinary BPS (ng/mL)	0.331 (0.156, 0.375)
Infant Sex	
Female	14 (50 %)
Male	14 (50 %)
Infant birth weight (gms)	3452 (3195, 3698)

*Cell type variables were derived using RefFreeEWAS, the relative proportion of putative cell types were estimated for PL with a reference-free deconvolution algorithm.
Limit of detection (LOD) < 0.2 ng/mL. Urinary bisphenol measures adjusted for specific gravity.

First, mixed effects regression was performed to evaluate whether associations between BPA, BPF, or BPS with DNA methylation were similar or different across tissues (Fig. 3). Significant interactions were detected between BPF and tissue type for both *FN1* ($p = 0.010$) and *HOXA-AS3* ($p = 0.009$), suggesting tissue-specific associations with respect to biomarkers of *in utero* BPF exposure.

We performed linear regression of each separate tissue to estimate the magnitude of associations between exposure and DNA methylation. While none were significant at $p < 0.05$, we discuss results with $p < 0.20$ to inform future research. BPA exposure was associated with PL DNA methylation in *FN1*, *SNAP25*, and *PRSS22* (Fig. 3). For example, every ln-transformed-unit increase in BPA exposure was associated with a decrease of average PL DNA methylation at *FN1* by 5.45 % ($p = 0.094$) (Fig. 3). BPA exposure was only associated with CB DNA methylation in *SNAP25* ($p = 0.169$) and did not display an association with CT DNA methylation. BPF exposure was associated with PL DNA methylation in *FN1* ($p = 0.15$) and *HOXA-AS3* ($p = 0.098$). BPF exposure was also associated with CB DNA methylation in *HOXA-AS3* and CT DNA methylation in *PRSS22*. Lastly, BPS exposure was associated with CT DNA methylation in *FN1* ($p = 0.131$) and CB DNA methylation in *PRSS22* ($p = 0.108$).

4.3. Epigenome-wide analysis: DNA methylation between CB and PL

Mixed effects regression detected over 600,000 differentially methylated sites (DMS) by tissue type, of which >300,000 displayed differences of at least 10 % between tissues. For the purposes of mapping gene ontology, we chose to evaluate the top 10,000 DMS, which corresponded to 3987 genes. Gene ontology analysis revealed 10 gene-sets enriched for GO pathways and five gene-sets enriched for KEGG pathways (Table 2). The most significantly enriched gene-sets included lamellipodium (GO, cellular component, q=0.0144) and Yersinia infection (KEGG, q=0.0014).

4.4. Epigenome-wide analysis: bisphenols and DNA methylation in placenta

We assessed PL DNA methylation at 822,020 CpG sites via EPIC and report associations with bisphenols at $p < 0.0001$ in this exploratory analysis. Maternal first-trimester urinary BPA exposure was associated with 63 DMS at $p < 0.0001$. The genomic inflation factor (lambda) for the analysis was 1.20. Increasing BPA concentrations were associated with increasing DNA methylation at 83 % of these CpG sites (Supplemental Table 2). BPF exposure was associated with 29 sites at $p < 0.0001$ (lambda=1.03) and increasing BPF concentrations were associated with decreasing DNA methylation at 97 % of these CpG sites (Supplemental Table 3). Similarly, BPS exposure was associated with 32 sites at $p < 0.0001$ (lambda=0.797). Increasing BPS concentrations were associated with decreasing DNA methylation at 90 % of these CpG sites (Supplemental Table 4).

4.5. Differentially methylated regions in placenta by bisphenols

Six DMRs were detected in placenta in association with maternal first-trimester urinary BPA exposure at $p < 0.0001$ (Table 3). Each region contained at least five sites, and four of the six regions displayed a decrease in DNA methylation with increasing BPA levels. Sixteen DMRs were detected in placenta in association with maternal first-trimester urinary BPF exposure at $p < 0.0001$ (Table 3). Each region contained at least two sites, and 13/16 (81 %) of sites displayed a decrease in DNA methylation with increasing BPF levels. Two genes, *RPS6KA2* and *CBFA2T3*, contained two separate DMRs in association with BPF exposure. Thirty-seven DMRs were detected in placenta in association with maternal first-trimester urinary BPS exposure at $p < 0.0001$ (Table 3). Each region contained at least three sites, and 23/37 (62 %) of sites

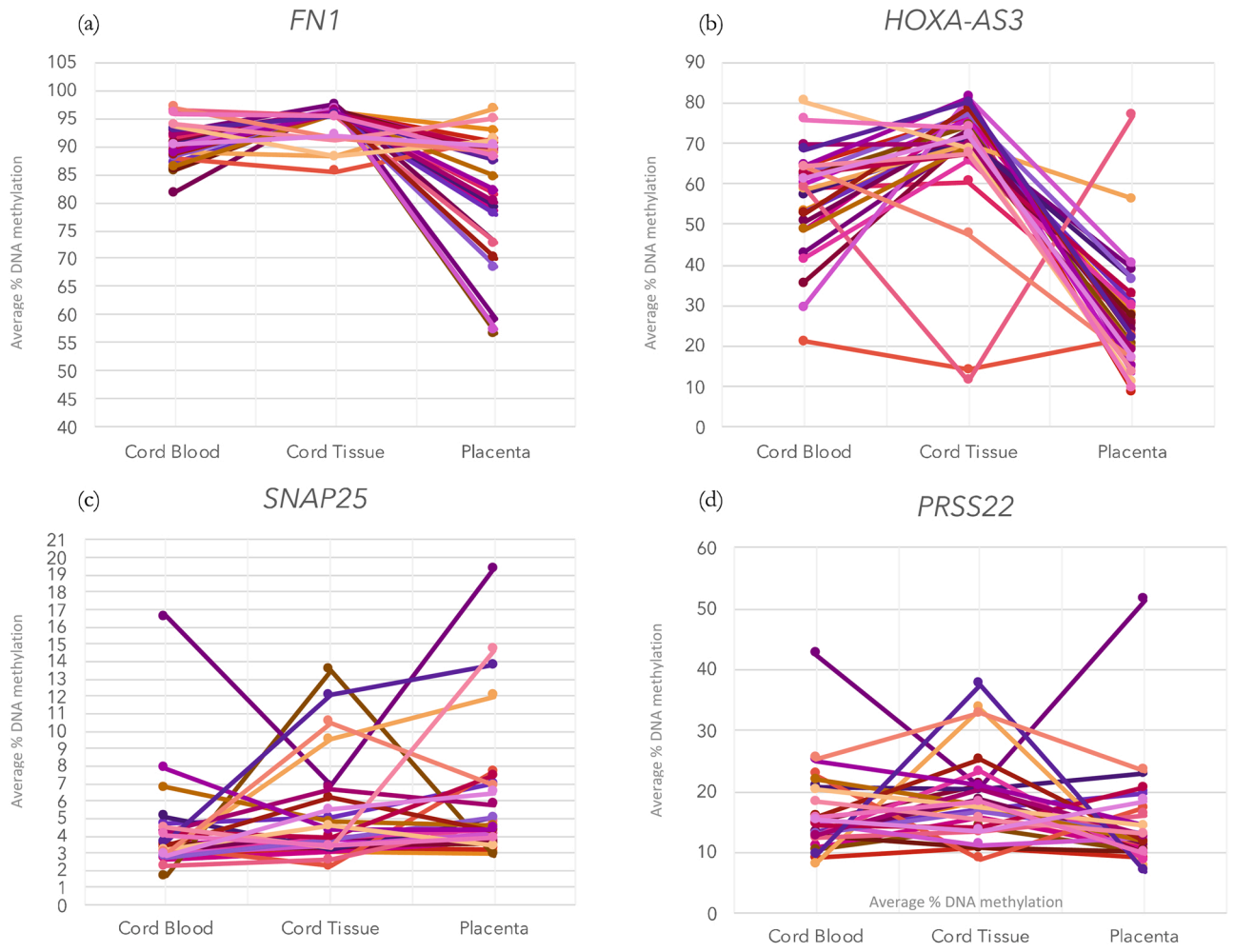
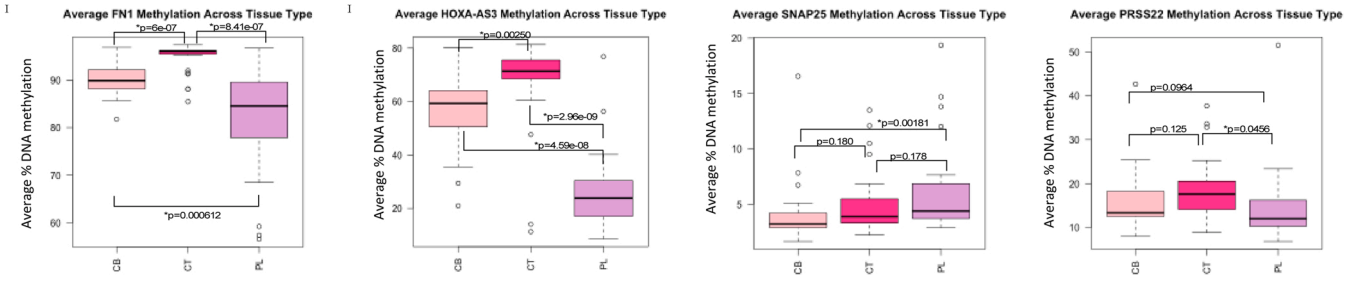


Fig. 1. Average percent DNA methylation across tissue type for four genes in matched samples, Legend: Each graph represents DNA methylation of one candidate gene (averaged across all CpG sites assayed for that gene) in CT, CB, and PL, plotted for each subject (e.g., a subject is represented by a circle, and a subjects' measurements are connected with a line across tissues) in each gene. (a) *FNI* (b) *HOXA-AS3* (c) *SNAP-25* (d) *PRSS22*. DNA methylation was measured via pyrosequencing.



II

	Cord Tissue	Placenta
Cord Blood	-0.253	0.213
Cord Tissue		**-0.533**

II

	Cord Tissue	Placenta
Cord Blood	0.261	-0.268
Cord Tissue		**-0.418**

	Cord Tissue	Placenta
Cord Blood	0.0263	**0.608**
Cord Tissue		0.344

	Cord Tissue	Placenta
Cord Blood	0.0348	**0.840**
Cord Tissue		-0.0315

Fig. 2. DNA methylation comparisons across CB, CT, and PL.

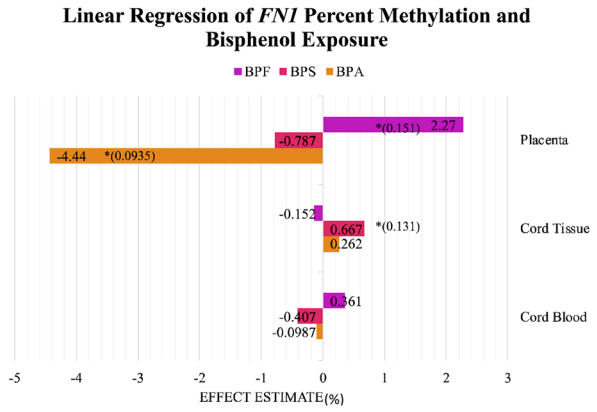
displayed a decreased in DNA methylation with increasing BPS levels. *ADAMTS17* contained three DMRs while *LMF1* contained two DMRs.

4.6. Pathway enrichment analysis of differentially methylated sites by bisphenols

BPA exposure-associated DNA methylation sites were enriched for three gene-sets significant at FDR < 0.05. Higher BPA exposure was associated with decreased methylation for genes in two out of three

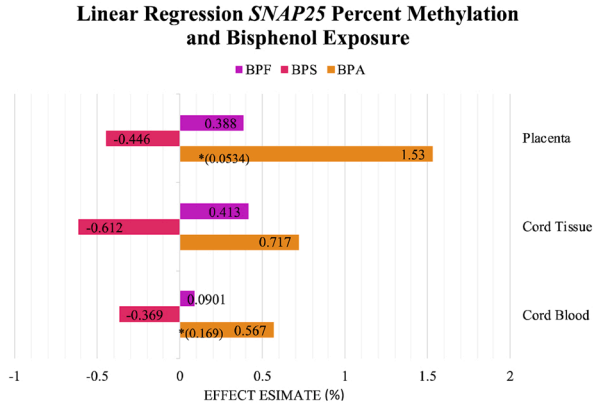
Mixed Effects FN1

	Estimate (se)	p-value
lnSGAdjBPA	-0.213 (1.33)	0.874
lnSGAdjBPA*Sample	-1.17 (1.09)	0.287
lnSGAdjBPF	-0.556 (0.786)	0.483
lnSGAdjBPF*Sample	**1.60 (0.601)**	**0.0102**
lnSGAdjBPS	-0.615 (1.54)	0.690
lnSGAdjBPS*Sample	0.781 (1.20)	0.518



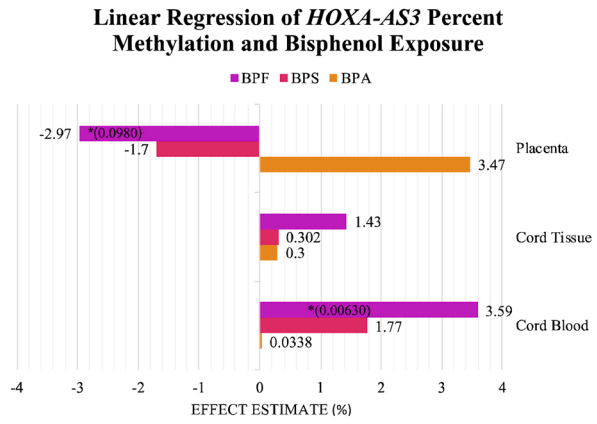
Mixed Effects SNAP25

	Estimate (se)	p-value
lnSGAdjBPA	-0.225 (0.465)	0.631
lnSGAdjBPA*Sample	0.408 (0.316)	0.205
lnSGAdjBPF	-0.0360 (0.280)	0.898
lnSGAdjBPF*Sample	0.0998 (0.193)	0.608
lnSGAdjBPS	-0.427 (0.510)	0.407
lnSGAdjBPS*Sample	0.109 (0.358)	0.763



Mixed Effects HOXA-AS3

	Estimate (se)	p-value
lnSGAdjBPA	-0.376 (2.41)	0.877
lnSGAdjBPA*Sample	2.22 (1.95)	0.260
lnSGAdjBPF	**3.74 (1.41)**	**0.0102**
lnSGAdjBPF*Sample	**-2.96 (1.09)**	**0.00882**
lnSGAdjBPS	0.783 (2.71)	0.773
lnSGAdjBPS*Sample	-1.50 (2.15)	0.488



Mixed Effects PRSS22

	Estimate (se)	p-value
lnSGAdjBPA	-0.415 (1.05)	0.694
lnSGAdjBPA*Sample	0.341 (0.778)	0.663
lnSGAdjBPF	-0.171 (0.560)	0.761
lnSGAdjBPF*Sample	0.0518 (0.424)	0.903
lnSGAdjBPS	-0.722 (1.05)	0.492
lnSGAdjBPS*Sample	0.485 (0.798)	0.546

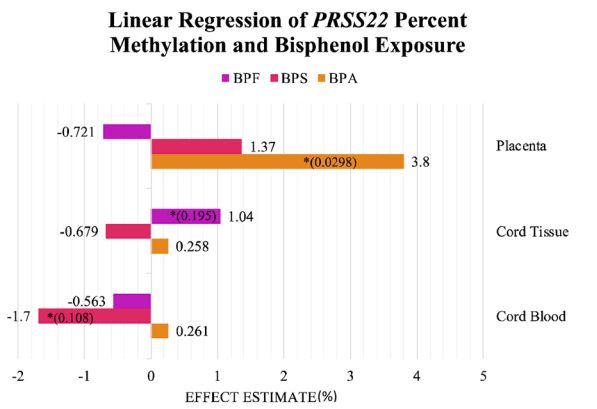


Fig. 3. Mixed-effects and linear regression analysis of candidate gene DNA methylation and bisphenol exposure across CB, CT, and PL. "Sample" represents subject, whereby a single subject has three tissues. Linear regression was performed after mixed-effects regression to determine which tissue type(s) was/were driving the association detected.

Table 2
Enriched Gene Ontology terms among the top 10,000 differentially methylated genes between CB and PL.

Gene ontology from GO Database					
	Description	**Ontology**	**N**	**P-value**	**Q-value**
GO:0030027	lamellipodium	CC	175	2.07E-06	0.0144
GO:0043312	neutrophil degranulation	BP	481	2.40E-06	0.0144
GO:0035579	specific granule membrane	CC	91	2.49E-06	0.0144
GO:0070062	extracellular exosome	CC	2162	7.39E-06	0.0284
GO:0007229	integrin-mediated signaling pathway	BP	83	8.21E-06	0.0284
GO:0005886	plasma membrane	CC	4271	1.05E-05	0.0302
GO:0001772	immunological synapse	CC	36	1.35E-05	0.0333
GO:0006955	immune response	BP	311	1.74E-05	0.0376
GO:0002250	adaptive immune response	BP	279	2.38E-05	0.0457
GO:0030667	secretory granule membrane	CC	92	2.79E-05	0.0482
Gene ontology from KEGG Database					
	Description		**N**	**P-value**	**Q-value**
hsa05135	Yersinia infection		137	3.97E-06	0.0014
hsa04611	Platelet activation		124	1.98E-04	0.0219
hsa04660	T cell receptor signaling pathway		104	2.25E-04	0.0219
hsa04062	Chemokine signaling pathway		192	2.56E-04	0.0219
hsa04060	Cytokine-cytokine receptor interaction		295	5.97E-04	0.0408

Note: Gene ontology was assessed on the 10,000 most significantly differentially methylated CpG sites between CB and PL by utilizing *gometh* in missMethyl. N represents the number of genes in the pathway. Q-value is the FDR adjusted p-value.

enriched pathways (Table 4). The pathway or concept with the greatest odds of enrichment for differential methylation was RIG-I-like receptor signaling pathway. In general, the enriched pathways were associated with immune sensing and inflammation/inflammatory host defenses. BPF exposure-associated DNA methylation sites were enriched for 13 pathways significant at FDR < 0.05. Higher BPA exposure was associated with decreased methylation for genes in 12/13 pathways (Table 4). The pathway or concept with the greatest odds of enrichment for differential methylation was ascorbate and aldarate metabolism. In general, the enriched pathways were associated with metabolism and drug metabolism. No pathways were significant for BPS exposure at FDR < 0.05.

5. Discussion

Although advances have been made in elucidating how epigenomic signatures associated with cell types and tissues contribute to human disease [9], the extent to which tissues respond to environmental exposures in a similar or different manner is limited. This has only recently become an active area of research in the basic sciences [37] and epidemiology [9]. In this study, we found tissue-specific differences in DNA methylation using both candidate gene and EWAS approaches in addition to associations with prenatal bisphenol exposures. PL displayed the greatest number of associations with bisphenol exposures across candidate genes, and the epigenome-wide analysis of DNA methylation in the PL further emphasized these modest associations.

The inclusion of matched CB, CT, and PL in this pilot study was an important first step in deconstructing tissue-specific response to prenatal bisphenol exposure. Studies are needed that not only compare tissue-specific DNA methylation in multiple tissue types, but that also evaluate such differences in the context of toxicological exposure. Our previous investigation of maternal exposure to BPA, BPF, and BPS in association with epigenome-wide DNA methylation in infant CB revealed differential methylation in both single sites and gene regions [17]. This study expanded upon these findings to reveal modest associations between maternal bisphenol and exposure and differential methylation in PL. These findings suggest that relying solely on one embryonic tissue to explain the epigenetic impact and consequence of maternal prenatal exposures in human pregnancy cohorts must be reconsidered. The importance of including CT and PL in early-life exposure studies is derived both from their separate embryonic origins from that of CB and their differential physiological responses to environmental toxicants. For example, CT has an increased potential to accumulate lipophilic toxicants like BPA during gestation as compared to CB and may be a better tissue in which to assess fetal toxicant exposure [38]. Despite this, currently no published studies have evaluated storage of bisphenols in CT. In a similar fashion, PL possesses mechanisms to protect the fetus from potentially toxic or harmful substances in maternal circulation [39] likely increasing its toxicant burden and harboring consequences for its nascent physiological functions. Birth cohort studies have also reported associations between DNA methylation in PL and prenatal exposures including arsenic [40], cadmium [41], copper [42], and maternal smoking [43]. Furthermore, recent studies have begun to identify and document the differential DNA methylation patterns across CB and PL epigenomes [44,45]. Tissue-specific differentially methylated regions in CT and PL (cord white blood cells as a reference) are enriched for genes that function in embryogenesis, vascular development, and the regulation of gene expression [46].

Our comparison of DNA methylation at four candidate genes revealed differences across CB, PL, and CT. In completing an epigenome-wide comparison of CB and PL methylation data, we detected significant differences—whereby $>$ 300,000 DMS displayed differences of at least 10 % between tissues. This large number of differences aligns with other comparisons of PL and CB. Groleau et al., performed a genome-wide comparison of DNAm in 444 PL and CB samples, determining that large portions of the genome are differentially methylated between these two tissues [44]. While some evidence indicates modest correlations between CpG sites in CB and PL, an increasing number of studies demonstrate CB and PL methylation patterns are distinct with limited cross-tissue predictability [47,48]. Our study did not have EWAS data of UC. However, Sakurai et al., reported that UC exhibits hypomethylation across the genome as compared to CB, with location-specific hypomethylation around transcription start sites [49]. They also noted that the UC methylome possesses enrichment of DMRs associated with HOX gene clusters and genes related to developmental body patterning and lineage-specific cellular differentiation [49]. The addition of UC to prenatal studies has revealed additional and novel epigenetic associations and biological pathways with prenatal exposures and disease. Wu et al., in their study of preterm birth, illustrated the complementary benefit of including CT methylome in their analyses; of the 994 CpGs associated with preterm birth, only 10 were detected in CB [50]. Moreover, Herzog et al. detected tissue-specific differentially methylated regions (tDMRs) associated with human umbilical vein endothelial cells and with PL [46]. These tissues should all be examined with respect to their responses to exposures and contribution to pregnancy and child health outcomes.

The results from the candidate gene analysis suggest tissue-specific associations with prenatal exposure to bisphenols. As of this writing, no published studies have evaluated prenatal bisphenol exposure and CT DNA methylation. Similarly, only three human epidemiological studies are currently published that assessed placental DNA methylation in association with bisphenol exposure. Nahar et al. demonstrated the tissue-

Table 3
Differentially methylated regions in placenta in association with maternal first-trimester urinary bisphenol exposure.

A.

Location	Gene Name	Number of CpG Sites	P-value[a]	Max Beta Change per ln-ng/mL BPA increase[b]
Chr7: 100424355–100425827	*EPHB4*	12	1.24E-12	-0.0620
Chr1: 55246867–55247408	*TTC22*	5	1.14E-08	0.0779
Chr1: 234367145–234367586	*SLC35F3*	5	6.36E-08	0.0642
Chr11: 46367725–46368295	*DGKZ*	5	9.81E-08	-0.0517
Chr2: 202125088–202125310	*CASP8*	5	1.22E-07	-0.0506
Chr11: 67170528–67171585	*PPP1CA*	7	5.15E-10	-0.0335

B.

Location	Gene Name	Number of CpG Sites	P-value[a]	Max Beta Change per ln-ng/mL BPF increase[b]
chr6:167275395–167276650	*RPS6KA2*	16	1.87E-09	-0.0512
chr17:79098772–79099882	*AATK*	9	2.09E-08	-0.0250
chr16:88948617–88950197	*CBFA2T3*	9	7.01E-07	-0.0435
chr6:167190034–167190226	*RPS6KA2*	3	9.82E-07	-0.0318
chr2:219135802–219135936	*PNKD*	2	4.04E-06	-0.0041
chr6:31829644–31829960	*NEU1*	9	7.48E-06	-0.0297
chr8:91013575–91014327	*DECR1*	6	1.97E-05	0.0329
chr5:150618948–150619039	*GM2A*	2	2.10E-05	0.1078
chr1:76080294–76080727	*SLC44A5*	5	2.10E-05	0.0597
chr13:114111864–114112218	*DCUN1D2*	6	2.36E-05	-0.0407
chr4:124232–124622	*ZNF718*	6	2.98E-05	-0.0183
chr9:116225793–116225992	*RGS3*	4	3.11E-05	-0.0478
chr16:88942335–88942358	*CBFA2T3*	2	4.88E-05	-0.0316
chr11:3187511–3187939	*OSBPL5*	15	5.25E-05	-0.0165
chr6:31478822–31478830	*MICB*	2	5.34E-05	-0.0445
chr11:16761290–16761533	*C11orf58*	4	6.91E-05	-0.0273

C.

Location	Gene Name	Number of CpG Sites	P-value[a]	Max Beta Change per ln-ng/mL BPS increase[b]
chr15:92398726–92399195	*SLCO3A1*	2	2.34E-06	-0.0519
chr16:983381–983870	*LMF1*	2	5.78E-05	-0.0506
chr17:77245306–77245327	*RBFOX3*	2	2.34E-06	-0.0656
chr6:29690766–29692183	*HLA-F*	26	7.23E-16	-0.0967
chr6:28983835–28985069		23	3.63E-13	0.0637
chr6:29648161–29649084	*ZFP57*	22	2.87E-16	-0.1067
chr10:102278918–102280155	*SEC31B*	15	4.31E-11	0.0859
chr2:70994758–70995607	*ADD2*	15	6.45E-11	0.1172
chr12:96183791–96185064	*NTN4*	12	2.72E-13	0.0830
chr9:33473445–33474350	*NOL6*	12	4.27E-08	0.0331
chr12:81331012–81331863	*LIN7A*	11	4.25E-07	0.0797
chr5:170735973–170736572	*TLX3*	11	1.73E-05	0.0323
chr6:32116653–32116963	*PRRT1*	10	5.78E-05	0.0619
chr3:185000208–185001026	*MAP3K13*	9	2.35E-09	-0.0740
chr6:32022929–32023409	*TNXB*	9	1.10E-05	0.0364
chr2:177042493–177043501	*HOXD-AS1*	8	4.10E-11	-0.1317
chrX:154842296–154842856	*TMLHE*	8	3.43E-05	0.0738
chr1:68517177–68517462	*GNG12-AS1*	7	1.76E-06	0.0353
chr1:161228203–161228877	*PCP4L1*	6	5.06E-07	-0.0603
chr16:1251787–1252484	*CACNA1H*	6	6.77E-06	-0.0663
chr17:28088578–28088749	*SSH2*	6	6.58E-05	0.0897
chr2:131792521–131793064	*ARHGEF4*	6	1.38E-05	-0.0862
chr15:100532781–100533336	*ADAMTS17*	5	1.56E-05	-0.0693
chr8:1814096–1814957	*ARHGEF10*	5	5.30E-06	-0.0357
chr8:2003810–2004488	*MYOM2*	5	1.96E-06	-0.0814
chr8:20831094–20831500		5	5.94E-07	-0.0427
chr15:100537304–100537761	*ADAMTS17*	4	4.11E-05	-0.0659
chr4:19756214–19756485	*RP11–608O21.1*	4	5.32E-06	-0.0798
chr6:13873924–13874251		4	5.73E-06	0.0500
chr1:158900384–158901032	*PYHIN1*	3	3.46E-07	-0.0915
chr10:131686425–131686574	*EBF3*	3	4.95E-05	-0.0537
chr15:72565016–72565039	*CELF6*	3	6.63E-05	-0.0170
chr15:99408804–99409194	*IGF1R*	3	3.55E-05	0.0507
chr15:100666162–100666305	*ADAMTS17*	3	3.27E-06	-0.0663
chr16:971556–971820	*LMF1*	3	1.28E-05	-0.0458
chr17:77657538–77657578		3	2.57E-07	-0.0535
chr8:13372453–13372491	*DLC1*	3	6.73E-05	-0.0672

A. BPA B. BPF C. BPS

[a] Minimum FDR p-value for the region

[b] For interpretability, changes across the DMR are reported as proportion methylated (beta), though models used logit-transformed beta values (M-values). Models adjusted for infant sex and two surrogate variable cell types. Significance considered at $p < 0.0001$

specific effects of prenatal BPA exposure on DNA methylation and found that repetitive element (LINE-1) percent DNA methylation in PL but not in fetal liver or kidney was significantly positively associated with total and free BPA concentrations [51]. Song et al., similarly, combined an epigenome-wide analysis with pyrosequencing analysis of prenatal BPA exposure in PL and identified over 200 differentially methylated genes and a gene-specific associations with exposure level [21]. Alternatively, Jedynak et al., evaluated PL DNA methylation in 201 mother-son pairs in association with prenatal synthetic phenol exposure but did not detect differential DNA methylation related to prenatal BPA exposure [22].

Table 4
Gene-sets enriched for differentially methylated genes in placenta by maternal first-trimester urinary bisphenol exposures using LRPath.

A.					
BPA					
Pathway ID	**Pathway Name**	**Database with Concept**	**No. of Genes in Concept**	**Q-value**	**Direction**[a]
hsa04622	RIG-I-like receptor signaling pathway	KEGG	67	0.00576	down
hsa00650	Butanoate metabolism	KEGG	29	0.00576	up
hsa04060	Cytokine-cytokine receptor interaction	KEGG	249	0.0259	down
B.					
BPF					
Pathway ID	**Pathway Name**	**Database with Concept**	**No. of Genes in Concept**	**Q-value**	**Direction**[a]
hsa00053	Ascorbate and aldarate metabolism	KEGG	22	1.04E-10	down
hsa00040	Pentose and glucuronate interconversions	KEGG	27	2.83E-09	down
hsa00982	Drug metabolism - cytochrome P450	KEGG	67	2.50E-08	down
hsa00983	Drug metabolism - other enzymes	KEGG	46	4.81E-08	down
hsa00140	Steroid hormone biosynthesis	KEGG	52	4.81E-08	down
hsa00830	Retinol metabolism	KEGG	60	4.81E-08	down
hsa00860	Porphyrin and chlorophyll metabolism	KEGG	38	4.81E-08	down
hsa00980	Metabolism of xenobiotics by cytochrome P450	KEGG	66	4.81E-08	down
hsa00514	Other types of O-glycan biosynthesis	KEGG	42	1.37E-07	down
hsa00500	Starch and sucrose metabolism	KEGG	46	1.39E-07	down
hsa04742	Taste transduction	KEGG	50	0.0301	down
hsa04950	Maturity onset diabetes of the young	KEGG	24	0.0349	down
hsa04144	Endocytosis	KEGG	197	0.0491	up

[a] Direction indicates whether most enriched genes in the pathways had reduced methylation ('down') or increased methylation ('up') in association with increasing bisphenol levels.

Our analysis of epigenome-wide PL DNA methylation using the Illumina Infinium EPIC array revealed modest associations with maternal first-trimester BPA, BPF, and BPS exposure. Given the small sample size, we reported associations at $p < 0.0001$ for each bisphenol; model results revealed that each exposure was associated with about the same number of differentially methylated sites. The gene family *DNAH* (dynein axonemal heavy chain) appeared in association with both BPA and BPS exposure, while the gene family *TRP* (transient receptor potential cation channel) appeared in association with both BPA and BPF exposure. However, no CpG sites were statistically significant when considering multiple comparisons via the false discovery rate approach ($q<0.05$) [52].

We detected DMRs and biological pathways significantly associated with differential methylation in the PL in response to maternal exposure to BPA, BPF, and BPS revealing a consistent pattern of association with key developmental processes, like vascular development of the PL and disorders like preeclampsia and intrauterine growth restriction (IUGR) [53–59]. Gene-set analysis further emphasizes these associations and possibly delineates the way in which these bisphenols may differ overall. The three significant gene-sets associated with BPA exposure are implicated in immune-sensing, the microbiome and inflammation, inflammatory host defenses [60,61], and epigenetic dysregulation of placental gene expression in preeclampsia [62]. This correlates to evidence that BPA exposure is associated with dysregulated placentation and elicits 'preeclampsia-like' features in mice [63]. BPF-associated gene-sets include steroid and drug metabolism, a feature that is commonly linked to BPA exposure with increasing evidence also implicating BPF [64]. Taken together, the DMR and gene-set analyses suggest a connection between prenatal exposure to bisphenols and increased inflammation, impaired vascularization, and endocrine disruption in the placenta. Although these births were uncomplicated and no pathology of the PL was detected, it is relevant to consider whether an increased sample size and a broader range of BPA exposure might validate these associations and increase our ability to detect complications. While there are studies that have evaluated BPA exposure and its association with changes in epigenetic marks like DNA methylation [65], histone modifications and chromatin remodeling [66], the specific mechanism of how bisphenol exposure elicits those changes is unclear. The similar, although at times divergent responses in CB and CT, and the unique PL epigenetic response, demonstrates their utility in combination—as a means to capture a fuller picture of the developmental effect of an exposure through epigenetic programming. Future work is needed to characterize whether DNA methylation in these surrogate tissues correlates with health outcomes and of which health outcomes CT and CB are the best predictors. Including overlapping tissues in EWAS expands our ability to detect associations between exposure and biological pathways—associations which can then be translated into detangling human disease.

5.1. Limitations

Despite the limited availability of families with all three tissues, we consider it a notable strength of this study that we compared DNA methylation at four candidate genes in matched tissue samples of CB, CT, and PL. However, the small sample size across our analyses limits the reliability and generalizability of our results. Reported results should be considered trends to explore in future studies as we lacked statistical power to detect all true associations with typical significance cut-offs. This study does not include epigenome-wide DNA methylation in CT. Available funds prevented the inclusion of CT tissue in our analysis and limits the comparison of epigenome-wide DNA methylation in the tissue triads. Furthermore, bisphenols exhibit non-monotonic dose responses and evaluating families with exposure level extremes would add key information about the risks posed for that portion of the population. It is also a limitation that we only evaluated four candidate genes and regions in all three tissues.

6. Conclusions

This study is a proof-of-concept demonstrating similarities and differences in DNA methylation across CB, CT, and PL, at four genes and in their associations with prenatal bisphenol exposures. The inclusion of matched CB, CT, and PL in this pilot study was an important first step in deconstructing tissue-specific response to prenatal bisphenol exposure. These findings underscore the importance of reconsidering the use of only one embryonic tissue to explain the epigenetic impact and consequence of maternal prenatal exposures in human pregnancy cohorts. CT and PL are important surrogate tissues because of their separate embryonic origins from that of CB and their differential physiological responses to environmental toxicants. Our exploratory analysis of epigenome-wide DNA methylation revealed broad differences between CB and PL throughout the epigenome and modest associations between prenatal bisphenol exposures with PL DNA methylation. We recommend that future investigations consider the addition of CT and/or PL as

surrogate tissues in conjunction with CB to broaden the ability to decipher biological pathways associated with developmental exposures to environmental toxicants.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.reprotox.2022.11.005.

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