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Dioxin-like compound exposures and DNA methylation in the Anniston Community Health Survey Phase II

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Abstract

The Anniston Community Health Survey (ACHS-I) was initially conducted from 2005–2007 to assess polychlorinated biphenyl (PCB) exposures in Anniston, Alabama residents. In 2014, a follow-up study (ACHS-II) was conducted to measure the same PCBs as in ACHS-I and additional compounds e.g., polychlorinated dibenzo-p-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), and dioxin-like non-ortho (cPCBs) substituted PCBs. In this epigenome-wide association study (EWAS), we examined the associations between PCDD, PCDF, and PCB exposures and DNA methylation. Whole blood DNA methylation was measured using Illumina EPIC arrays (n=292). We modeled lipid-adjusted toxic equivalencies (TEQs) for: ΣDioxins (sum of 28 PCDDs, PCDFs, cPCBs, and mPCBs), PCDDs, PCDFs, cPCBs, and mPCBs using robust multivariable linear regression adjusting for age, race, sex, smoking, bisulfite conversion batch, and estimated percentages of six blood cell types. Among all exposures we identified 10 genome-wide (Bonferroni p≤6.74E-08) and 116 FDR (p≤5.00E-02) significant associations representing 10 and 113 unique CpGs, respectively. Of the 10 genome-wide associations, seven (70%) occurred in

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Author Credit Statement

GSP: Methodology, Formal data analysis, Visualization, Writing – Original Draft, review and editing
XW: Formal data analysis
MRC: Sample preparation, Investigation
SJC: Sample preparation
JRO: Resources
MP: Conceptualization, Resources, Writing- Review and Editing, Project Administration
LSB: Conceptualization, Resources, Writing – Review and Editing, Funding
DAB: Conceptualization, Supervision, Resources, Writing- Review and Editing, Project Administration

Disclosure Statement

J.R. Olson served as an expert witness for the plaintiffs in legal actions regarding the residents of Anniston, Alabama being exposed to PCBs. The other authors declare that they have no competing interests.

Declaration of interests

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.

the PCDDs and four (40%) of these associations had an absolute differential methylation ≥1.00%, based on the methylation difference between the highest and lowest exposure quartiles. Most of the associations (six, 60%) represented hypomethylation changes. Of the 10 unique CpGs, eight (80%) were in genes shown to be associated with dioxins and/or PCBs based on data from the 2019 Comparative Toxicogenomics Database. In this study, we have identified a set of CpGs in blood DNA that may be particularly susceptible to dioxin, furan, and dioxin-like PCB exposures.

Keywords

Polychlorinated biphenyls (PCBs); mono-ortho PCBs; non-ortho PCBs persistent environmental pollutant (POP); 2,3,7,8-Tetrachlorodibenzodioxin (TCDD/dioxin); Polychlorinated dibenzo-p-dioxins (PCDDs); Polychlorinated dibenzofurans (PCDFs); DNA methylation; Anniston Community Health Survey (ACHS)

1. Introduction

The major US producer of polychlorinated biphenyls (PCBs) operated in Anniston, Alabama from 1929 until 1971. Due to poor environmental controls at the facility, releases of dioxins, furans, and PCBs over time caused wide-spread environmental contamination in the Anniston community. Because these compounds are highly lipophilic and have long half-lives, they bioaccumulate in the food chain and persist in human tissues. The Anniston Community Health Survey (ACHS-I) was established in 2005 to assess the health effects of PCB exposure in the general population of the community (Birnbaum and Staskal-Wikoff, 2010; Pavuk et al., 2014a) and in 2014, a follow-up study was conducted (ACHS-II) (Birnbaum et al., 2016). Prior ACHS-I research has found links between PCB exposures and hypertension (Goncharov et al., 2010), blood pressure (Goncharov et al., 2011), race (Pavuk et al., 2014b), liver disease (Clair et al., 2018), metabolic syndrome (Rosenbaum et al., 2017), serum lipid levels (Aminov et al., 2014), diabetes (Silverstone et al., 2012), and leukocyte telomere length (Callahan et al., 2017).

A number of studies have found associations between altered DNA methylation and dioxin, furan, and PCB exposures in several populations, including Faroe Islanders (Leung et al., 2018), Dutch (van den Dungen et al., 2017), Taiwanese (Su et al., 2019), Greenlandic Inuits (Rusiecki et al., 2008), Koreans (Kim et al., 2010), and Japanese (Kobayashi et al., 2017). We have recently reported on 28 genome-wide significant associations between serum PCB concentrations in ACHS-I and whole blood DNA methylation (Pittman et al. 2019). We carried out a look-up analysis of the 369 FDR significant ACHS-I associations in the ACHS II methylation dataset reported in the present project and determined that the most significantly altered ACHS-I CpG, cg00475490 in the *PRSS23* gene, was still significantly associated with the group, summed tri/tetra-ortho substituted, nondioxin-like PCBs (p=1.33E-04) in ACHS-II.

The current ACHS-II study focuses on dioxins and dioxin-like compounds and their toxicities have generally been attributed to sustained aryl hydrocarbon receptor (AHR) activation as measured in model systems, and this property is used in human risk assessment (Theobald and Peterson 1994). Ligand-mediated AHR activation results in upregulation of

genes in the AHR pathway, including cytochrome P450s (e.g. *CYP1A1* and *CYP1B1*) and the aryl hydrocarbon receptor repressor (*AHRR*), a negative regulator of *AHR* (Hahn et al., 2009). DNA methylation levels at the CpG cg05575921 in *AHRR* are strongly associated with adult and prenatal tobacco smoke exposure (Joehanes et al., 2016; Joubert et al., 2012; Reynolds et al., 2017; Su et al., 2016; Wan et al., 2018), and this could potentially be related to tobacco smoke polyaromatic hydrocarbons (PAHs), which are also AHR ligands. We recently explored the hypothesis that PCB exposures in ACHS might be associated with whole blood DNA methylation levels in *AHRR* or other AHR pathway genes, but found no relationship (Pittman et al., 2019). Dioxins are considered stronger ligands for AHR (Theobald and Peterson 1994), so in this work we also tested if dioxin and dioxin-like exposures might lead to methylation changes in *AHRR* cg05575921. Because dioxin exposures have been linked with altered immune system effects, we used the Houseman model (Houseman et al. 2012; Houseman et al. 2016) to test if exposure was associated with changes in methylation-based estimated cell-type percentages in whole blood. However, the primary objective was to carry out an epigenome-wide association study (EWAS) assessing if exposures to AHR ligands such as PCDDs, PCDFs, cPCBs, and mPCBs were associated with altered whole blood DNA methylation at CpGs across the genome.

2. Methods

2.1 Study population

ACHS-II was conducted in 2014 as a follow-up study to ACHS-I (2005–2007). Both study designs have been previously reported (Birnbaum et al., 2016; Pavuk et al., 2014a). In ACHS-I, two-stage stratified random sampling was used to select households and adults within the household of Anniston, Alabama. We contacted over 1,800 households; individuals living in west Anniston (the area closest to the PCB manufacturing facility) were over-sampled (two-thirds of eligible participants). 1,100 participants were interviewed and 778 volunteered to have their blood samples taken. PCB measurements and covariate data were available for 765 participants. For ACHS-II, 438 of 582 surviving participants from ACHS-I were successfully contacted and 359 eligible individuals were enrolled. While all ACHS-II subjects were recruited from the ACHS-I cohort, due to the lack of available DNA samples, not all ACHS-II subjects overlapped with our previous study (Pittman et al., 2019). We successfully measured DNA methylation in a subset (n=299) of the ACHS-II cohort from frozen (−70°C) whole blood samples. Seven individuals were excluded from analyses as they lacked measures for dioxins, furans and non-ortho substituted PCBs. The Institutional Review Boards at the Centers for Disease Control and the University of Alabama at Birmingham provided clearance for human subjects research. Table 1 contains the ACHS-II study demographics for individuals analyzed here.

2.2 Serum dioxins, furans, and dioxin-like PCBs and lipid measurements

The CDC's National Center for Environmental Health laboratory measured serum level of 28 dioxins, furans, and dioxin-like PCBs (Pavuk et al., 2014a). We measured 1) seven PCDDs (2378-TCDD, 12378-PeCDD, 123678-HxCDD, 123478-HxCDD, 123789-HxCDD, 1234678-HpCDD, and 12346789-OCDD); 2) ten PCDFs (2378-TCDF, 12378-PeCDF, 23478-PeCDF, 123678-HxCDF, 123789-HxCDF, 123478-HxCDF, 234678-HxCDF,

1234678-HpCDF, 1234789-HpCDF, and 12346789-OCDF); 3) three cPCBs (PCB81, 126, and 169); and 4) eight mPCBs (PCB105, 114, 118, 123, 156, 157, 167, and 189). We assigned exposure values below the limit of detection using the limit of detection divided by square root of 2 (Hornung and Reed, 1990). Lipid-substituted toxic equivalencies (TEQs) were calculated using the World Health Organization's 2005 toxic equivalency factors list (TEFs; the compound's potency relative to 2378-TCDD) (Van den Berg et al., 2006). TEQ calculation involved multiplying the lipid-substituted exposure value by the TEF for that compound (Supplemental Table 1). Lipid-substituted TEQs were grouped into five categories: 1) ΣDioxins (the sum of all 28 compounds); 2) PCDDs (2378-TCDD, 12378-PeCDD, 123678-HxCDD, 123478-HxCDD, 123789-HxCDD, 1234678-HpCDD, and 12346789-OCDD); 3) PCDFs (23478-PeCDF, 123678-HxCDF, 123478-HxCDF, 234678-HxCDF, and 1234678-HpCDF); 4) cPCBs (PCB126 and 169); and 5) mPCBs (PCBs 105, 114, 118, 156, 157, 167, and 189) (Table 2). The following compounds from the PCDF and PCB groups were excluded because ≥60% of participants had levels below the limit of detection: 2378-TCDF, 12378-PeCDF, 123789-HxCDF, 1234789-HpCDF, 12346789-OCDF, PCB81, and PCB123. It should be noted that all of the compounds examined in this study were unique to ACHS-II, with the exception of the eight mPCBs. However, in this current study we have used mPCB TEQ values, making these analyses distinct from our previous report (Pittman et al. 2019).

We examined if wet-weight-substituted TEQs would differ substantially from using lipid-substituted TEQs. For the ΣDioxins TEQs, there was a strong correlation between the two exposure measurements, r^2=0.91, p=6.95E-155 (Supplemental Figure 1). Among the other exposure groups there was also good concordance between the two TEQ measures: PCDDs r^2=0.87, p=1.63E-129; PCDFs r^2=0.86, p=9.34E-128; cPCBs r^2=0.98, p=8.21E-235; and mPCBs, r^2=0.96, p=1.03E-209 (Supplemental Figure 2). Based on these data and to be consistent with previous analyses, we used the lipid-substituted TEQs.

Serum lipid levels for total cholesterol, HDL cholesterol, LDL cholesterol, and triglycerides were measured by the University of Washington (Williams et al., 2013), and total lipids were calculated using the Bernert et al. method (Bernert et al., 2007).

2.3 DNA methylation measurement

We isolated genomic DNA from whole blood samples (300 μL per extraction) robotically using the Agencourt Genfind v2 Solid Phase Reversible Immobilization (SPRI) paramagnetic bead-based technology (Beckman Coulter). Prior to bisulfite-conversion, DNA concentrations were measured using a QUBIT dsDNA BR assay kit (Invitrogen). The National Cancer Institute's Cancer Genomics Research Laboratory performed DNA bisulfite conversion using the EZ-96 DNA Methylation MagPrep kit (Zymo Research). Samples were then run on Illumina EPIC methylation arrays (Illumina).

2.4 Statistical analysis

Bioconductor's ChAMP package v2.12.0 (Aryee et al., 2014; Fortin et al., 2017; Morris et al., 2014) was used to normalize methylation data. Methylation data exclusions included: 1) any samples which failed array QC standards; 2) all CpG probes on the X and Y

chromosomes; 3) probes containing a SNP with a minor allele frequency ≥1% at the CpG site; and 4) probes failing QC standards. We also removed an additional 43,254 probes reported to hybridize to one or more non-target sites in the genome (Pidsley et al., 2016). There were 741,471 CpG probes remaining after exclusions.

Our analysis approach is shown in Figure 1. In brief, after running methylation arrays, associations between exposures and methylation were analyzed using robust multivariable linear regression (M-estimation) with the *rlm* function in Modern Applied Statistics with S (MASS v.7.3–51.1) (Ripley, 2002). We $\log_{10}$-transformed TEQ exposure measurements, consistent with previous analyses. All models were adjusted for age, race, sex, smoking status (current/never), bisulfite-conversion batch, and estimated white blood cell percentages. We estimated six white blood cell types: CD4 and CD8 T-cells; B-cells; monocytes; natural killer cells; and granulocytes, using the Houseman method (Houseman et al. 2012) (Houseman et al., 2016) based on the Reinius reference panel (Reinius et al., 2012). We also tested if exposure was associated with estimated cell-type percentages in whole blood. We tested other covariates e.g., body mass index and alcohol use; however, no other covariates substantially changed the estimates, contributed to the amount of variance explained, or improved model fit. We used chi-square tests and two-sided t-tests to examine demographic differences between African-Americans and whites.

For CpGs significant at Bonferroni p≤6.74E-08, we calculated differential methylation (ΔM) between the highest and lowest exposure quartiles and identified Benjamini-Hochberg false discovery rate (FDR) significant CpGs at p≤5.00E-02. For testing CpGs with an absolute ΔM≥1.00% across exposure quartiles, we used ANCOVA (adjusting for age, race and sex) and pair-wise two-sided t-tests. We evaluated potential effect measure modification by race and sex for CpGs at p≤6.74E-08 and with an absolute ΔM≥1.00%. Effect modification was assessed by including an interaction term in our regression models. Interaction terms were considered significant at p≤5.00E-02. We identified differential methylated regions (DMRs) using Bioconductor's DMRcate v.1.18.0 (Peters et al., 2015), comparing the highest versus lowest tertiles for each exposure. We calculated FDR p-values for identified DMRs. Statistical analyses were conducted in R (R Development Core Team, 2018), SAS v9.4, and JMP 13.0.0 (SAS Institute Inc.).

2.5 Functional analysis and Comparative Toxicogenomics Database

Enrichr functional analysis was used to identify functional enrichment of molecular signatures of the group of genes containing FDR significant CpGs among biological processes (BP) or disease (OMIM) (Chen et al., 2013; Kuleshov et al., 2016). In addition, the PCDD-associated FDR-significant CpGs were entered into the eForge web tool hosted at https://eforge.altiusinstitute.org/ and run against the cell type specific epigenomic database (Breeze et al. 2019) and we used the gometh function in the missMethyl package (Phipson et al. 2016) to test enrichment of gene sets defined in Gene Ontology (GO) (Harris et al. 2004) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases (Kanehisa and Sato 2020). We characterized gene associations with dioxin and dioxin-like compounds using curated chemical– gene association data from the 2019 Comparative Toxicogenomics Database (CTD) (Davis et al., 2019), MDI Biological Laboratory, Salisbury Cove, Maine,

and NC State University, Raleigh, North Carolina. World Wide Web (URL: http://ctdbase.org) [August 2019].

3. Results

3.1 Demographics

Table 1 provides the demographics for subjects with available DNA methylation and exposure data (n=292). The cohort was primarily female (74.3%) and had a higher mean BMI (31.6 ± 0.5) relative to that reported (26.0 ± 0.1) in the National Health and Nutrition Examination Survey 2015–2016 (CDC National Center for Health Statistics, 2018). White study participants were older than African-American participants (64.3 vs 60.9 years). African-American participants were more likely to live in west Anniston (93% vs 80%), the section of Anniston where the PCB production facility was located.

3.2 Serum dioxin levels by age, race, and sex

Dioxin levels in ACHS-II were previously reported to be higher than the general population. Table 3 shows the distribution of our five TEQs groups (ΣDioxins, PCDDs, PCDFs, cPCBs, and mPCBs) by age, race, and sex. TEQs were modeled on the log10-scale.

3.3 Correlation among the dioxin, furan and PCBs exposure groups

We examined the correlation among exposure TEQs among the four dioxin exposure groups (PCDDs, PCDFs, cPCBs, and mPCBs). Supplemental File 1, Table S2 shows the coefficient of determination (r^2) for each of the comparisons. TEQs were modeled on the log10-scale and adjusted for age, race, and sex. Correlations between the PCDDs and PCDFs and the PCB groups (cPCBs and mPCBs) were relatively weak (r^2 range, 0.50–0.58). However, PCDDs and PCDFs had a strong correlation (r^2=0.80) as did the cPCBs and mPCBs (r^2=0.83). While the summed TEQs for these structurally related groups were relatively strongly correlated, the differences might be reflected in different biological effects, thus we have assessed all of their associations with DNA methylation independently.

3.4 Smoking and methylation

Numerous studies have demonstrated a strong association between tobacco smoke exposure and methylation at the *AHRR* CpG cg05575921 (Joehanes et al., 2016; Joubert et al., 2012; Monick et al., 2012; Reynolds et al., 2015; Su et al., 2016; Wan et al., 2018). Using robust linear regression, we tested whether this association held for the ACHS-II cohort and found a strong association between current smoking and *AHRR* cg05575921 methylation levels (p=9.63E-207). *AHRR* differential methylation between smokers and nonsmokers was 24.1% (t-test, p=2.73E-24). Among nonsmokers, we found no association between ΣDioxins and *AHRR* cg05575921 (robust linear regression, p=1.71E-01).

3.5 Dioxins, furans, and dioxin-like PCBs, cell-type estimation and DNA methylation

We tested if exposures were associated with methylation-based estimates of cell-type percentages in whole blood and these results are provided in Supplementary Excel File 1, Table S3. We observed nominally significant associations between estimated CD8 T cell

percentages and PCDDs (p = 0.029) and PCDFs (p = 0.041). However, as is standard practice in EWAS stud all methylation analyses are adjusted for estimated cell-type percentages to account for any cell type shifts. We observed 10 genome-wide significant CpGs (representing 10 unique genes) associated with the dioxin exposure groups (Table 4). Four (40%) of these associations had ΔM≥1.00% (based on the methylation difference between the highest and lowest exposure quartiles). Seven of the 10 (70%) associations were most significant in the PCDD exposure group. There were 116 CpG/exposure FDR significant associations, representing 113 unique CpGs (Supplemental Excel File 1 Table S4). Figure 2 illustrates the distribution of these CpGs across the dioxin exposure groups, with the majority of CpGs (n=104, 92%) significant exclusively in the PCDD exposure group. There were no FDR significant associations for the mPCB exposure group.

The CpG with the largest absolute ΔM was associated with the allantoicase gene (*ALLC,* Figure 3). The *ALLC* CpG cg00999904 had differential methylation of −4.64 ± 0.21 (p=1.05E-09) across low and high quartiles of the PCDD exposure group. *ALLC* cg00999904 is located ~1.3 kb upstream of the *ALLC* first exon and is 15 bp downstream of three forkhead-box (FOX) group of transcription factor binding sites (*FOXA1*, *FOXA2*, *FOXP2*) determined by the ENCODE project (Wang et al., 2012).

3.6 Effect modification by race and sex

We examined potential effect modification by race or sex for the four Bonferroni significant CpGs with an absolute ΔM ≥1.00%. We ran multivariable robust linear regression models with an interaction term (sex*exposure or race*exposure). We observed effect modification by race for the PCDD vs cg21401636 (*KIF5C*) association based on an interaction term with p=2.10E-03. The ΔM for African-Americans was −7.02 ± 0.36 (p=4.33E-03) and for whites −5.02 ± 0.26 (p=1.01E-02) (Supplemental Excel File 1 Table S5).

3.7 DMR analysis

We searched for DMRs, groups of CpGs within a genomic region displaying similar directional changes, that were associated with dioxin and dioxin-like exposures, using DMRcate and exposure group tertiles. We identified one DMR/exposure association in the ΣDioxins data at p=2.75E-13 (Figure 4). This DMR was just beyond the 3-prime end of ubiquitin D (*UBD*) and gamma-aminobutyric acid type B receptor subunit 1 (*GABBR1*) at chr6:29521138–29521272 (containing cg02149189, cg13641185, cg00674706, cg27546977, cg21834061, cg10491628, cg15814717, and cg22762215) and was in a CpG island. Also, ENCODE transcription factor binding ChIPseq data indicates this region is potentially transcriptionally active and ENCODE histone modification data from multiple cell types (GM12878, H1-hESC) suggests this region is a potentially repressed enhancer. These DMR CpGs did not overlap with any CpGs in our FDR p≤5.00E-02 list (Supplemental Excel File 1, Table S4).

3.8 Quartile analysis

We examined methylation across quartiles for the four CpGs significant at Bonferroni p≤6.70E-08 and that had an absolute ΔM ≥1.00% (Figure 5) using ANCOVA adjusting for race, age and sex. These CpGs were cg00999904 in *ALCC*; cg06169091 in immunoglobin

superfamily member 21 (*IGSF21*); cg21401636 in kinesin family member 5C (*KIF5C*); and cg03459668 in RAB11 family interacting protein 3 (*RAB11FIP3*). For *ALCC* cg00999904, the methylation in the fourth quartile of PCDDs was significantly different than the first and second quartiles, p=7.42E-03 and p=1.93E-03 respectively (ANCOVA p=9.68E-03). For *IGSF21* cg06169091 there were no significant differences among the quartiles (ANCOVA p=5.25E-01). Fourth quartile methylation in the PCDDs was significantly higher than the first and second quartiles for *KIF5C* cg21401636, p=8.55E-04 and p=1.42E-03 respectively (ANCOVA p=7.70E-41). And for *RAB11FIP3* cg03459668, there were significant differences among all quartiles (ANCOVA p=4.17E-14), except between the first vs. second quartile and the third vs. fourth quartile.

3.9 Functional analysis and Comparative Toxicogenomics Database associations

For the genes with FDR significant CpGs, we used Enrichr functional analysis as well as eForge, missMethyl (GO) and missMethyl (KEGG) (Supplemental Excel File 2). Among the Enrichr, KEGG and GO analyses we observed nominal significance for numerous signatures but all failed to reach FDR significance. The eForge analysis identified that altered CpGs were marginally overrepresented in regions with the repressive histone modification H3K27me3 at FDR q= 0.28 in CD8+ T cells, a cell type that displays nominal-dioxin associated changes in estimated percentage. Using the same gene list, we also used the 2019 Comparative Toxicogenomics Database (CTD) to determine if these genes had reports of interactions with dioxins or dioxin-like exposure in scientific literature. For genome-wide significant CpGs, 8 of the 10 (80%) unique genes (Table 4) and 70 (62%) of the 113 FDR significant unique genes (Supplemental Excel File 1,Table S4) were listed as having interactions with dioxins or dioxin-like exposures.

4.0 Look up analysis

We searched the literature for array-based methylation data and associations with dioxins, furans and dioxin-like PCBs. We identified three studies with 450k methylation array data (Leung et al., 2018; Su et al., 2019; van den Dungen et al., 2017). These studies examined the associations between whole blood DNA methylation and PCBs 105 and 118; 23478-PeCDF, 123478-HxCDF, PCBs 153 and 156; and PCBs 28, 52, 105, 118, 138, 153, 156, 170, 180, and 187, respectively (Supplemental File 1 Table S6). Of the 254 unique CpGs identified among the three studies, there was no overlap among the studies. There was also no overlap between these studies' results and our FDR significant CpGs. Among all CpG look-ups, we observed nominal significance (p<5.0E-02) for 12/254 among PCDF associations and 21/254 mPCB associations; however, none of these reached the calculated Bonferroni-adjusted p-value (p≤2.16E-04) level of significance. We had no data for 22 CpGs probes that failed our quality control screening.

Discussion

In this current study we have examined the associations between dioxin, furan, and dioxin-like PCB exposures and whole blood DNA methylation in the second phase of the Anniston Community Health survey. Among all exposures we identified 10 (Bonferroni p≤6.74E-08) and 116 (FDR p≤5.00E-02) significant associations representing 10 and 113 unique CpGs,

respectively. The PCDDs exposure group had the most Bonferroni and FDR significant CpG associations (seven and 107, respectively), and the majority were in genes shown to be associated with dioxins and/or PCBs, based on data from the 2019 CTD. Of the CTD-based dioxin-associated genes, the most significant CpG was upstream of allantoicase (*ALLC*), a gene in the uric acid degradation pathway. Based on a RNA-seq study, *ALLC* is expressed primarily in the testis, but also has expression in the adrenal gland, adipose tissue, gall bladder, heart, kidney, liver, and ovaries. *ALLC* expression in CD34+ hematopoietic stem and progenitor cells was reported to be increased with TCDD exposure (CTD database). However, it is unclear what the function of *ALLC* is, and it is not considered to be part of the AHR-regulated gene pathway.

Many of the identified CpGs were in gene regulatory regions where numerous transcription factors bind (Figure 4); however, it is not known if changes in methylation in these regions affect transcription factor binding or gene expression. Among FDR-significant CpGs, the B cell lymphoma 3 gene, a proto-oncogene, (*BCL3*, cg23269663), is of interest due to its connection to NF-kappaB regulation (Keutgens et al., 2010) and lymphoma (Viatour et al., 2004). A CpG (cg26686732) in the *FOXA2* gene was in the top 25 FDR-significant CpGs, and it should be noted that several significant CpGs (cg00999904, cg25251562, cg19052272, and cg19825600) in the *ALLC* gene are near three FOX family transcription factor binding sites (FOXP2, FOXA1, and FOXA2). FOXA2 is a hepatocyte transcription factor, which is of interest because a study in ACHS-I found an association between PCB exposure and toxicant-associated steatohepatitis (Clair et al., 2018).

In our previous study of DNA methylation in ACHS-I, we tested the hypothesis that DNA methylation in *AHRR*, a gene in the AHR pathway, might be affected by exposure to PCBs, but observed no association. We tested the same hypothesis regarding dioxin-like exposures (stronger AHR ligands) in the present study. Smoking had an association with *AHRR* cg05575921 methylation levels; however, in nonsmokers we detected no association between dioxin or dioxin-like exposures and changes in *AHRR* methylation.

In dioxins and furans, the two phenyl groups are in a permanent coplanar orientation, making these compounds strong AHR ligands. In non-ortho and mono-ortho substituted PCBs, the phenyl groups are not in a fixed coplanar configuration, but the phenyl groups can assume this configuration. When PCBs are in this coplanar configuration, they become dioxin-like (i.e. become AHR ligands) (Ericksson, 2001). Based on our original hypothesis, we expected to observe the most significant changes in methylation among the most dioxin-like exposures group i.e., the PCDDs. Indeed, we found the most genome-wide significant ($p \leq 6.74E-08$) CpG/exposure associations in the PCDDs, and many of these genes are linked to dioxin-like exposures in model systems (Table 3). However, none of the genes in this group (or FDR list, Supplemental Excel File 1 Table S4) were part of the canonical AHR pathway (e.g. *ARNT, AHRR, CYP1A1, CYP1B1*). These results suggest blood DNA methylation alterations associated with dioxin and dioxin-like exposures are not directly related to AHR pathway activation.

Other studies have reported associations between dioxins and dioxin-like exposures and altered DNA methylation; however, most of these studies examined global methylation or

methylation changes in specific candidate genes. Only a few of these studies had measured TCDD and other dioxins or dibenzofuran congeners or non-ortho PCBs. In the Arctic Monitoring and Assessment Program, it was observed in a cohort of Greenlandic Inuits (n=70) that higher serum levels of 14 PCBs (28, 52, 99, 101, 105, 118, 128, 138, 153, 156, 170, 180, 183, 187) were related to global hypomethylation (Rusiecki et al., 2008); however, only three of these PCBs (105, 118, and 156) have dioxin-like properties. Two studies of TCDD (n=516) (Pilsner et al., 2018) and PCB153 (n=315) (Consales et al., 2016) exposures found an association with sperm DNA hypomethylation. In a cohort of cancer-free Koreans (n=368), researchers reported a non-monotonic association between promoter hypomethylation of *MGMT* and PCBs 105, 138, and 153 (Park et al., 2015). Two compounds (PCB126 and OCDD) were linked to global DNA hypermethylation (Lind et al., 2013) in a cross-sectional cohort of elderly Swedish men and women (n=524).

Other studies have found potential sex-specific effects. Results from a sub-cohort of the Hokkaido Birth Cohort Study on Environment and Children's Health (n=169) showed an association between PCBs 170, 178, 180, and 182 and cord blood *H19* and *LINE1* hypermethylation (Kobayashi et al., 2017). The results also suggested these associations were stronger in females compared to males. A study in a prospective birth cohort of Faroe Islanders with high levels of PCB exposure at birth (n=72) using the 450K array reported an association between PCB105 and sex-specific methylation of a number of CpGs (Leung et al., 2018). Another study in Taiwan used the 450K array and identified 20 CpGs associated with 23478-PeCDF, 123478-HxCDF, PCB153, PCB156, PCB170, and PCB180 exposures in the 2nd generation (n=60) of a cohort whose mothers had been exposed to these compounds due to contaminated cooking oil (Su et al., 2019). However, 13 of the CpG associations reported were in genes that have been associated with smoking exposure in studies by our laboratory (Su et al., 2016) and numerous others (Joehanes et al., 2016; Joubert et al., 2012). The authors of the Taiwanese study reported that their findings could have been confounded by smoke exposure status of the mothers and/or offspring. In in a cohort of Dutch men (n=80), serum levels of dioxin-like PCBs (105, 118, and 156) and the sum of several indicator PCBs (28, 52, 138, 153, 170, 180 and 187) were significantly associated with DMR hypermethylation, with an average 7.4% differential methylation between the subjects with highest and lowest exposure (van den Dungen et al., 2017). From these three array-based studies, we obtained the CpG identifier for the loci associated with dioxin and dioxin-like compounds and compared them to our results. It is of interest to note that the reported CpGs from these studies had no overlap among them; nor was there any overlap with our FDR significant CpGs. The dioxin-like compounds concentrations in these other cohorts were generally lower than in the Anniston population and this may explain the lack of overlap between our findings and these other studies.

Another finding of interest relates to the correlation among PCDDs, PCDFs, cPCBs, and mPCBs. While structurally similar compounds, PCDDs and PCDFs, and the cPCBs and mPCBs were strongly correlated, the correlations among the PCDDs and PCDFs vs the cPCBs and mPCBs were relatively weak. These results are consistent with previously published ACHS-II results (Yang et al., 2018) and suggests that the source of PCDDs/PCDFs exposure may differ from that for cPCBs/mPCBs. Interestingly, significant

methylation effects were observed almost exclusively with exposure to the dioxin group (PCDDs).

Our previous analysis of mono-ortho, di-ortho, and tri/tetra-ortho substituted PCB exposures in ACHS-I identified 369 CpG/exposure associations that were significant at FDR p≤5.00E-02 but only one remained significant in a lookup analysis in ACHS-II (Pittman et al., 2019). We compared the ACHS-I FDR results with our current analysis of dioxin-like compounds in ACHS-II. Again, there was no overlap between our previous PCB results and our genome-wide or FDR significant associations with current dioxin and dioxin-like compounds in this report. This finding is not necessarily conflicting. The most significant associations (80%) in our previous study occurred only with tri/tetra-ortho PCBs, which have no dioxin-like properties. Non-dioxin-like PCBs may perturb DNA methylation through a different mechanism than dioxin-like compounds. The CpGs identified in the present study may represent CpG loci that are most sensitive to dioxin-like exposures given the predominant CpG/exposure associations (92%) were found in the PCDDs, the most dioxin-like compounds. Among our most significant CpGs, we tested for sex and race specific effects and observed effect modification by race for cg21401636 in *KIF5C*. This finding may reflect the significantly (p=6.17E-04) higher PCDD exposure in African-Americans versus whites.

The primary strengths of this current study were the number of dioxin-like compounds measured (n=28) and the use of the Illumina 850K methylation array (the first study we are aware of to examine these exposures using the 850K array). A limitation of our study was the relatively small sample size in ACHS-II (n=292); however, compared to the three referenced 450K assay studies, our sample size is far larger, making our results a significant contribution to the scientific literature. Another limitation was the use of whole blood DNA for methylation analysis. As has been pointed out, cell-type specific effects might be poorly detected in a mixture of cell types because the differential methylation may occur in a relatively rare cell type population or might be in opposite directions in different cell types(Su et al. 2016). In addition, a general issue with the use of genome-wide arrays is the need for multiple-testing correction; therefore, we may have missed true CpG/exposure associations that did not reach FDR significance.

4. Conclusions

This study contributes to the understanding of the effects of dioxin and dioxin-like exposures on whole blood DNA methylation in a human population study. This is the first report to examine dioxin-like exposures and alterations in DNA methylation using the Illumina 850K methylation array. We identified one highly significant DMR and 10 genome-wide significant CpG/exposure associations with 40% having differential methylation ≥1.00%, based on the methylation difference between the highest and lowest exposure quartiles. We observed effect-measure modification by race for cg21401636 (*KIF5C*). Most of the FDR significant CpGs (113 unique CpGs) we found were associated with PCDD exposure, the most dioxin-like of the exposures we measured. These CpGs and the DMR identified may represent a group of CpGs in blood cell types that are particularly susceptible to dioxin exposure. Our current results did not overlap with three previous 450K methylation array

studies; however, all three of those studies had very small sample sizes (60–72 individuals), and only one of those measured dioxins, dibenzofurans and non-ortho PCBs (Su et al., 2019).

In our identification of biological pathway associations, a CpG in *FOXA2* (a hepatocyte transcription factor) was significant at 8.87E-07. Since a previous ACHS-I study found an association between PCB exposure and liver disease (Clair et al. 2018), a future goal will be to explore whether changes in DNA methylation modify this exposure/disease association.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Anniston, Alabama has high levels of environmental dioxin-like compounds.
- Using 850k arrays, we found 10 significant exposure/DNA CpG methylation associations.
- Seven methylation associations were with polychlorinated dibenzo-p-dioxins.
- Four methylation associations had an absolute differential methylation ≥1.00%.
- Eight associations were in genes reported to interact with dioxin exposures.

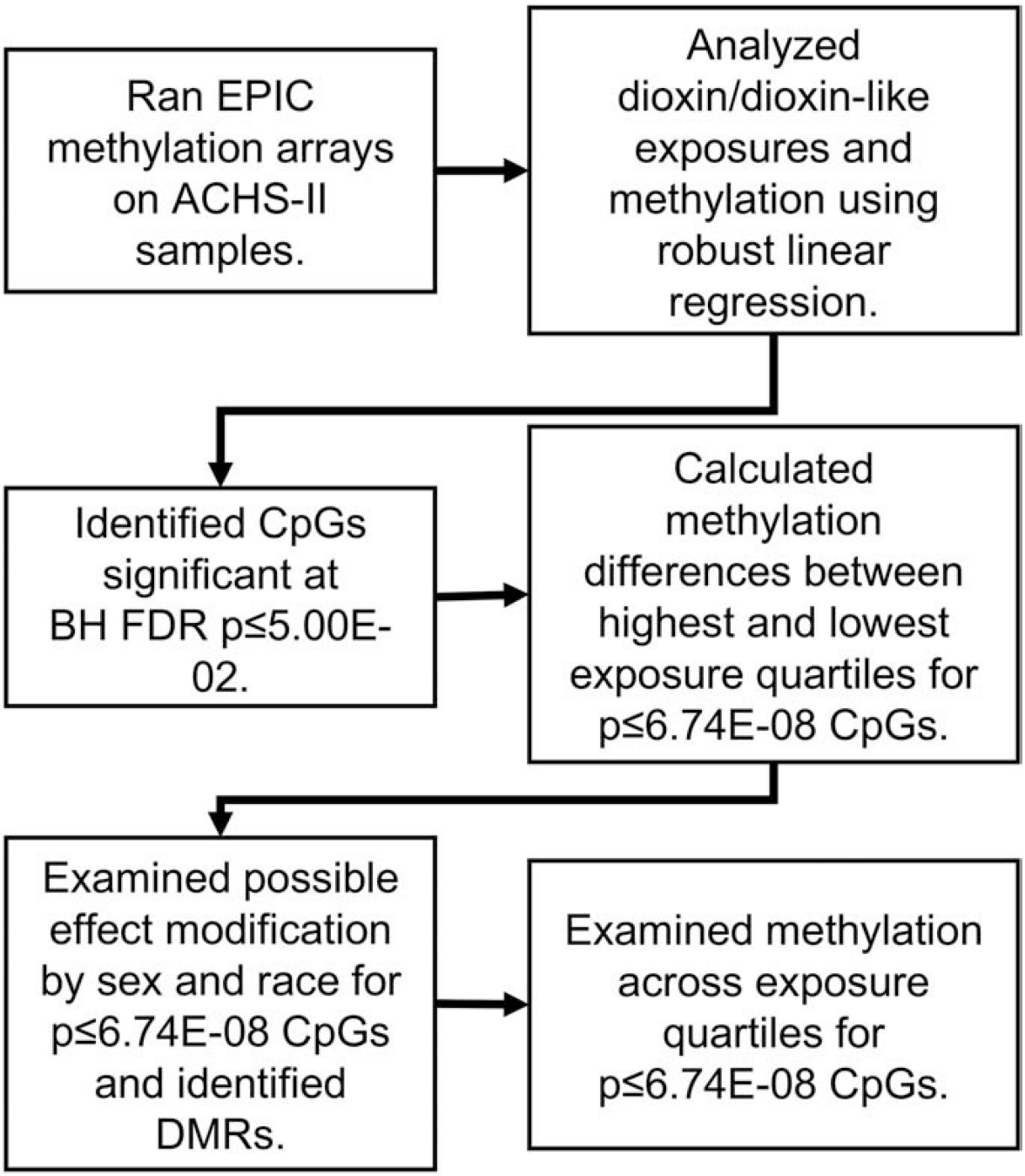

Figure 1.

Overview of data analyses strategy. Robust linear regression models were adjusted for age, race, sex, smoking status, and percentages for six different white blood cell types. Five different exposure groupings were used in regression: the sum of all 28 compounds (ΣDioxins), polychlorinated dibenzodioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), non-ortho substituted PCBs (cPCBs), and mono-ortho substituted PCBs (mPCBs) Exposure measures were lipid-substituted toxic equivalencies (TEQs).

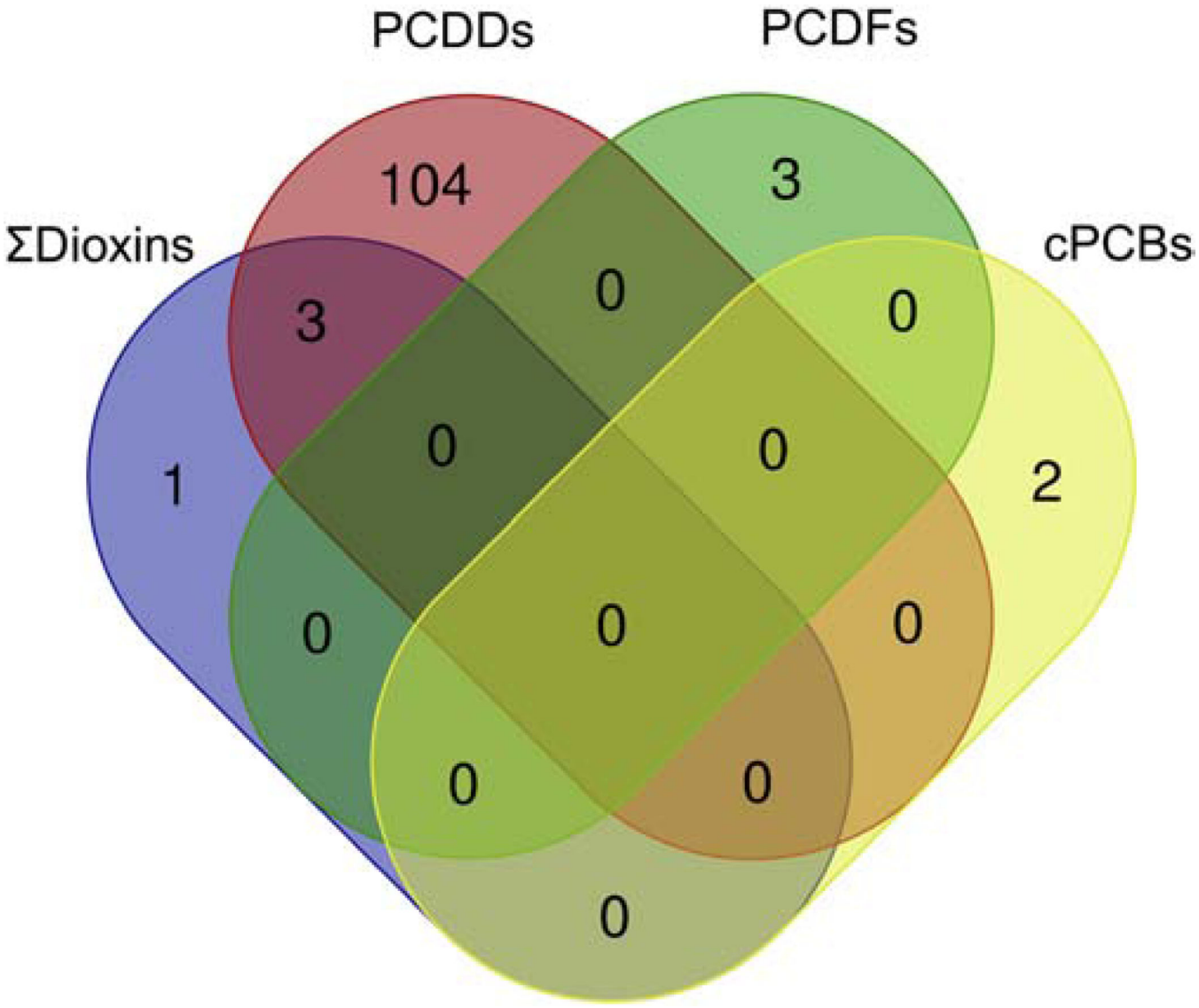

Figure 2.

Venn diagram of 113 significantly differentially methylated CpGs (FDR p≤5.00E-02) in ACHS-II for four exposure groups: the sum of all 28 compounds (ΣDioxins), polychlorinated dibenzodioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), and non-ortho substituted PCBs (cPCBs). There were no FDR significant CpGs for the mono-ortho substituted PCBs. Exposure measures were lipid-substituted toxic equivalencies (TEQs).

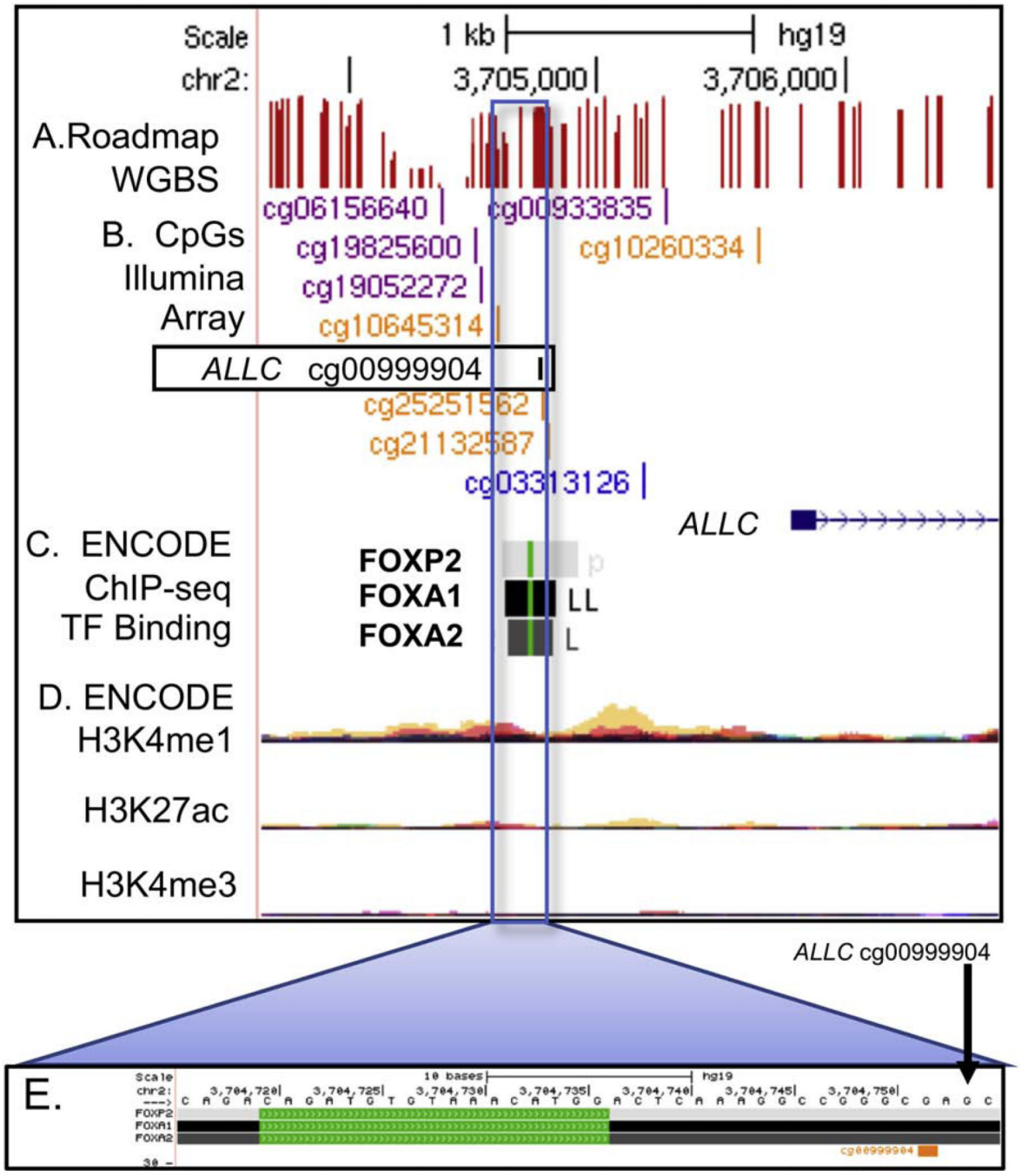

Figure 3.

ALLC genome browser view showing the altered CpG (boxed) in relation to transcription factor binding sites in the *ALLC* promoter region. Tracks listed from the top of browser: A. CpG sites as detected by whole genome bisulfite sequencing (Roadmap WGBS, methylation level indicated by height of red bar); B. CpGs on Illumina 450K array, reference cg numbers listed; C. ENCODE project identified transcription factor binding as detected by ChIP-seq, green line indicates position of the TF binding motif sequence; D. ENCODE project enhancer related histone modification measurements layered by color to show multiple cell

types (red=GM12878 B cell, tan=H1-hESC stem cell); E. Magnification of genome region containing TFBS.

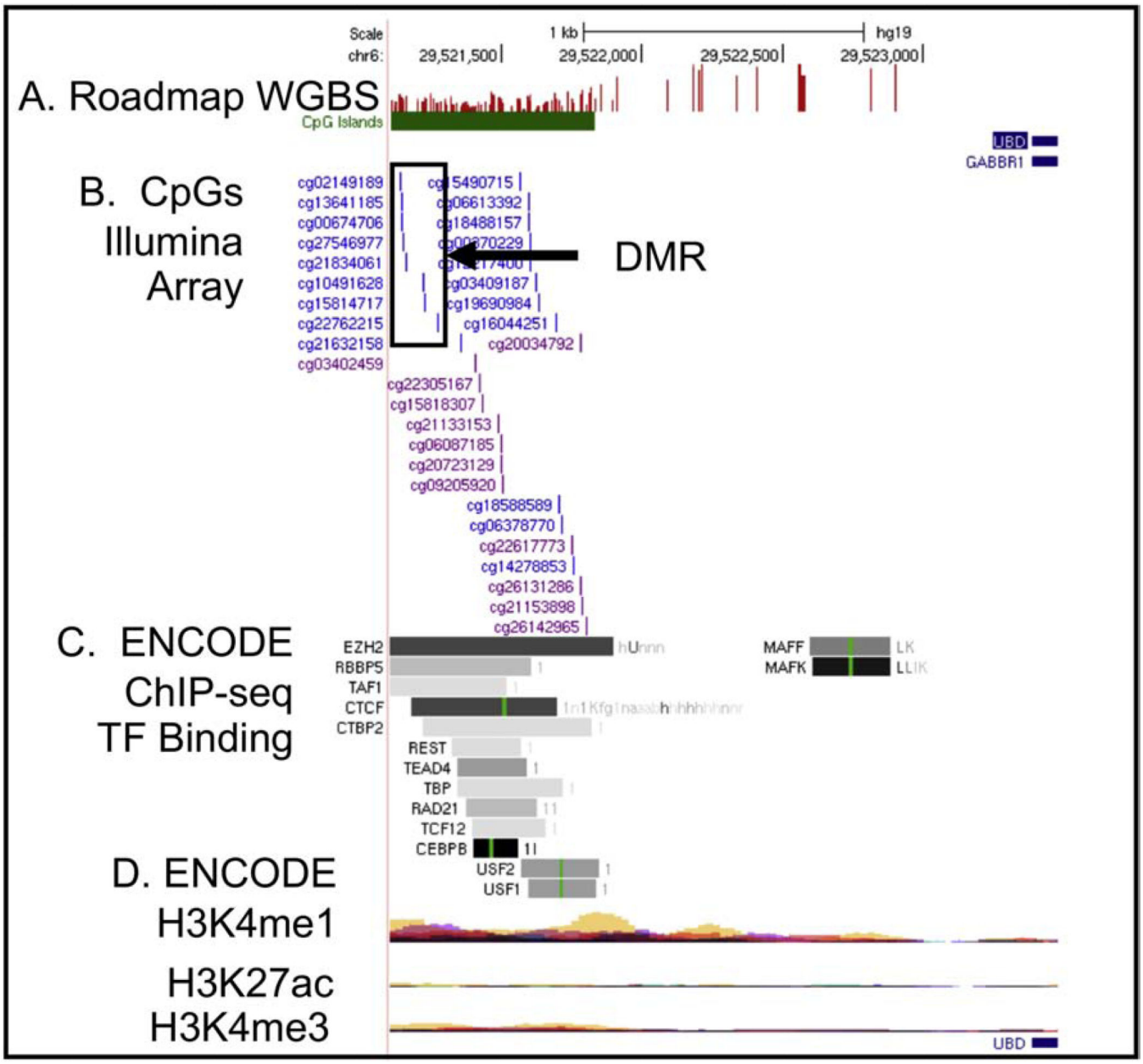

Figure 4.

Differentially methylated region (DMR, boxed CpGs) in CpG island near *UBD* and *GABBR1* genes. Tracks listed from the top of browser: A. CpG sites as detected by whole genome bisulfite sequencing (Roadmap WGBS, methylation level indicated by height of red bar); B. CpGs on Illumina 450K array, reference cg numbers listed; C. ENCODE project identified transcription factor binding as detected by ChIP-seq, green line indicates position of the TF binding motif sequence; D. ENCODE project enhancer related histone modification measurements layered by color to show multiple cell types (red=GM12878 B cell, tan=H1-hESC stem cell).

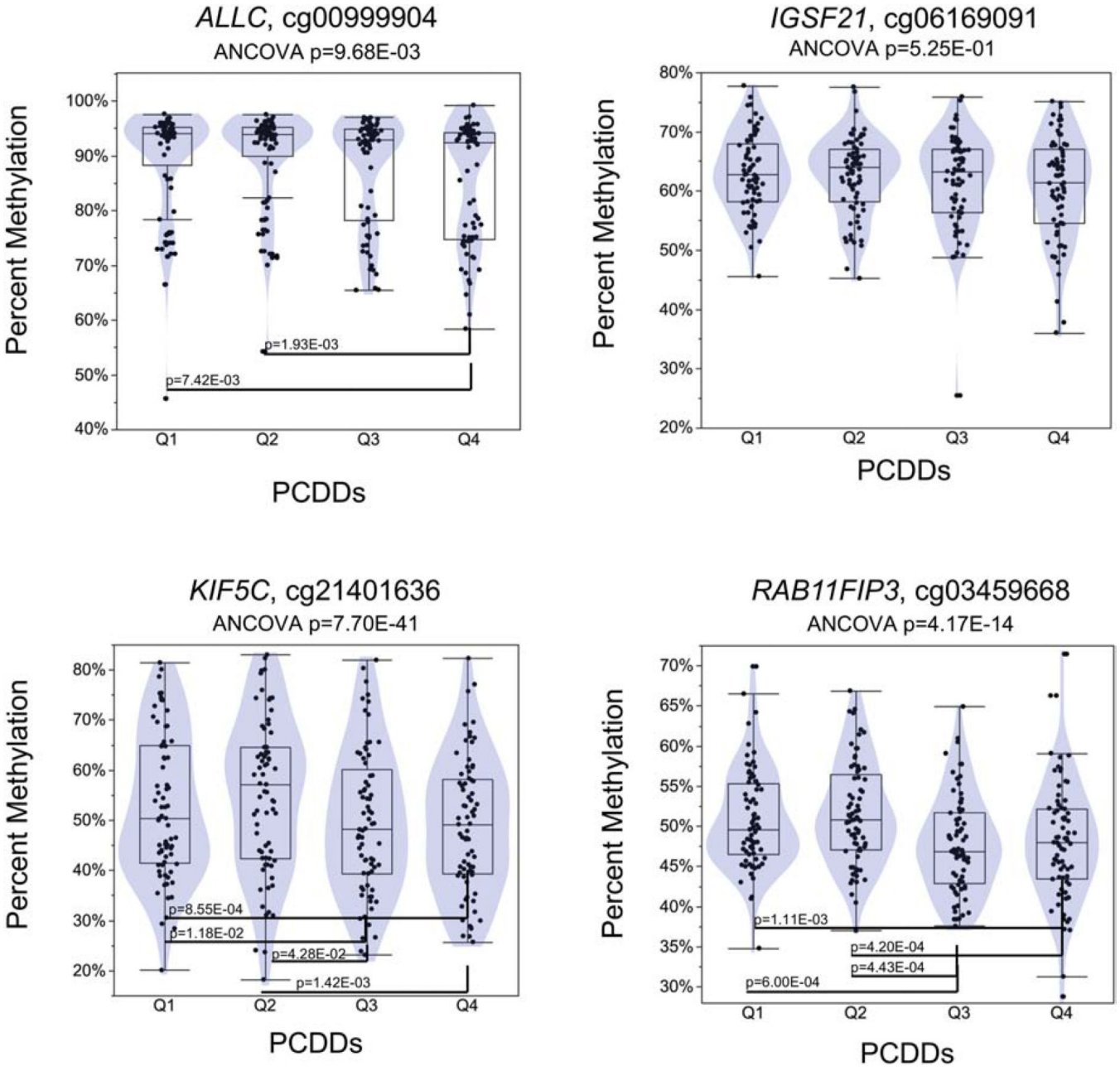

Figure 5.

Dioxin and dioxin-like compounds quartile distributions for CpGs with ≥1.0% absolute differential methylation. ANCOVA was adjusted for age, race, and sex. Pair-wise Student's t-test p-values between quartiles are presented.

Table 1.

Characteristics of the Anniston Community Health Survey, phase II.

Characteristic	African-Americans (n=150)	Whites (n=142)	Total (n=292)
	Mean ± SE		
Age[a]	60.9 ± 0.9	64.3 ± 1.2	62.6 ± 0.8
Body Mass Index (BMI)	32.3 ± 0.6	30.8 ± 0.7 n (%)	31.6 ± 0.5
	n (%)		
Female	115 (76.7)	102 (71.8)	217 (74.3)
Age Group (years)[b]			
<40	6 (4.0)	10 (7.0)	16 (5.5)
40–59	63 (42.0)	41 (28.9)	104 (35.6)
≥60	81 (54.0)	91 (64.1)	172 (58.9)
BMI Class (kg/m^2)			
<25	28 (18.7)	35 (24.6)	63 (21.6)
25–29	38 (25.3)	43 (30.3)	81 (27.8)
≥30	84 (56.0)	64 (45.1)	148 (50.7)
Current smoker	36 (24.0)	30 (21.1)	66 (22.6)
Residence in west Anniston[c]	140 (93.3)	113 (79.6)	253 (86.6)
Occupational PCB exposure	32 (21.3)	40 (28.2)	72 (24.7)

[a] p=2.91E-02 for African-Americans compared to whites using Welch's two-sided t-test.

[b] p=4.83E-02 for African-Americans compared to whites using using chi-square test.

[c] p=4.46E-04 for African-Americans compared to whites using chi-square test.

Table 2.

Dioxins and dioxin-like compounds measured in ACHS-II.

ΣDioxins		PCDDs	PCDFs[a]	cPCBs[a]	mPCBs[a]
2378-TCDD	PCB81	2378-TCDD	23478-PeCDF	PCB126	PCB105
12378-PeCDD	PCB105	12378-PeCDD	123678-HxCDF	PCB169	PCB114
123678-HxCDD	PCB114	123678-HxCDD	123478-HxCD		PCB118
123478-HxCDD	PCB118	123478-HxCDD	234678-HxCDF		PCB156
123789-HxCDD	PCB123	123789-HxCDD	1234678-HpCDF		PCB157
1234678-HpCDD	PCB126	1234678-HpCDD			PCB167
12346789-OCDD	PCB156	12346789-OCDD			PCB189
2378-TCDF	PCB157				
12378-PeCDF	PCB167				
23478-PeCDF	PCB169				
123678-HxCDF	PCB189				
123789-HxCDF					
123478-HxCDF					
234678-HxCDF					
1234678-HpCDF					
1234789-HpCDF					
12346789-OCDF					

Values (lipid-substituted, pg/g) below the limit of detection were imputed by dividing the limit of detection for the assay by the square root of 2.

[a]The following compounds from the PCDFs and PCB groups were excluded because ≥60% of participants had levels below the limit of detection: 2378-TCDF, 12378-PeCDF, 123789-HxCDF, 1234789-HpCDF, 12346789-OCDF, PCB81, and PCB123. The ΣDioxins included all 28 compounds.

Table 3.

Arithmetic means of exposure groups TEQs by age, race, and sex for ACHS-II (n=292).

Exposure group[a]	Age group (years)	Arithmetic mean ± SEM	p-value[b]	Race	Arithmetic mean ± SEM	p-value[b]	Sex	Arithmetic mean ±SEM	p-value[c]
ΣDioxins	<40	4.92 ± 1.70	1.98E-30	African-American	32.04 ± 2.34	1.00E-12	Female	29.53 ± 1.79	4.30E-09
	40–59	17.20 ± 1.40		White	19.70 ± 1.42		Male	15.94 ± 1.52	
	≥60	33.35 ± 2.08							
PCDDs	<40	3.38 ± 1.21	2.89E-37	African-American	12.54 ± 0.85	6.17E-04	Female	12.64 ± 0.63	1.27E-06
	40–59	8.58 ± 0.75		White	10.60 ± 0.52		Male	8.60 ± 0.66	
	≥60	14.19 ± 0.65							
PCDFs	<40	1.08 ± 0.73	7.13E-24	African-American	3.14 ± 0.25	5.34E-03	Female	3.04 ± 0.17	7.13E-04
	40–59	2.14 ± 0.15		White	2.53 ± 0.11		Male	2.26 ± 0.21	
	≥60	3.43 ± 0.15							
cPCBs	<40	0.21 ± 0.07	3.83E-35	African-American	12.01 ± 1.37	3.02E-17	Female	10.27 ± 1.07	1.74E-08
	40–59	4.55 ± 0.61		White	4.77 ± 0.78		Male	3.35 ± 0.55	
	≥60	11.64 ± 1.30							
mPCBs	<40	0.18 ± 0.06	6.87E-37	African-American	4.18 ± 0.36	8.02E-25	Female	1.60 ± 0.24	2.63E-08
	40–59	1.78 ± 0.21		White	1.72 ± 0.22		Male	3.46 ± 0.29	
	≥60	3.96 ± 0.34							

[a]Exposure groups: ΣDioxins = sum of 28 dioxin and dioxin-like compounds, PCDDs = polychlorinated dibenzodioxins (n=7), PCDFs = polychlorinated dibenzofurans (n= 5), cPCBs = non-ortho subsituted PCBs (n=2), mPCBs = mono-ortho substituted PCBs (n=7).

[b]TEQs were log10 transformed in a linear regression model adjusted for race and sex. Age was modeled as a continuous variable.

[c]TEQs were log10 transformed in a linear regression model adjusted for age and sex.

[d]TEQs were log10 transformed in a linear regression model adjusted for age and race.

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Table 4.

Top ACHS-II Dioxin-associated CpGs, selected by Bonferroni p≤6.74E-08.

ProbeID	Gene Symbol(s)	Coordinate	Exposure[a]	% Differential Methylation (Mean ± SEM)[b]	p-value[c]	Exposure Regression Coefficient[c]	Dioxin/Furan/PCB Association[d]
cg00999904	*ALLC*	chr2:3704751	PCDDs	−4.64 ± 0.21	1.05E-09	−1.8964	TCDD
cg11354991	*BBS4, HIGD2B*	chr15:72978685	PCDDs	0.09 ± 0.01	6.04E-09	0.8172	TCDD, PCB52, PCB180
cg03510117	*CYCS*	chr7:25164991	PCDDs	0.37 ± 0.01	3.01E-08	0.6423	TCDD, PCB153
cg06169091	*IGSF21*	chr1:18678286	PCDDs	−2.49 ± 0.16	3.08E-08	−0.5569	
cg21401636	*KIF5C*[e]	chr2:149629217	PCDDs	−3.73 ± 0.30	1.88E-08	−0.6405	TCDD
cg03459668	*RAB11FIP3*	chr16:545622	PCDDs	−2.86 ± 0.14	2.26E-08	−0.4876	TCDD, PCB126
cg07303330	*TAAR2*	chr6:132945515	PCDDs	−0.88 ± 0.06	7.85E-09	−0.9439	
cg26787894	*CD83*	chr6:14117621	PCDFs	0.29 ± 0.02	3.39E-08	0.4943	TCDD
cg06242879	*ERBB3, PA2G4*	chr12:56497745	PCDFs	0.36 ± 0.04	6.13E-09	0.3756	TCDD, PCB77
cg23269663	*BCL3*	chr19:45250480	cPCBs	−0.51 ± 0.03	5.15E-08	−0.2829	TCDD

CpGs are listed in order of exposure category and gene symbol. Individual exposures with ≥60% participants below the limit of detection

were excluded from the exposure groups with the exception of the ΣDioxins.

[a]Exposure groups: ΣDioxins = sum of 28 dioxin and dioxin-like compounds, PCDDs = polychlorinated dibenzodioxins (n=7), PCDFs = polychlorinated dibenzofurans (n= 5), cPCBs = non-ortho subsituted PCBs (n=2), mPCBs = mono-ortho substituted PCBs (n=7).

[b]Differential methylation was calculated by subtracting methylation in lowest exposure quartile from the highest.

[c]Robust linear regression models were adjusted for age, race, sex, smoking status, bisulfite-conversion batch, and estimated percentages of CD4+ and CD8+ T-cells, CD19+ B-cells, monocytes, granulocytes, and natural killer cells.

[d]Genes associated with dioxin, furan, or PCB exposure based on the 2019 Comparative Toxicogenomics Database.

[e]Effect modification by race was detected. See Results 3.6 and Supplemental Table 3 for details.