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PON1 DNA methylation and neurobehavior in Mexican-American children with prenatal organophosphate exposure

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Abstract

PON1 is a multifunctional enzyme involved in oxidative stress and detoxification of some organophosphate (OP) pesticides. It has been associated with nervous system diseases like Alzheimer's disease, amyotrophic lateral sclerosis, Parkinson's disease, and autism. We previously found that *PON1* susceptible genotypes were associated with lower IQ scores in children. Epigenetic marks, such as DNA methylation, can regulate gene expression. Yet, data on whether DNA methylation may influence the relationship between *PON1* levels and neurobehavior are limited. In this study, we used Illumina 450K and EPIC BeadChip arrays to assess *PON1* DNA methylation in blood specimens collected from children (n=238) at birth (cord blood) and age 7 years and examined their relationship with cognitive outcomes. The Wechsler Intelligence Scale for Children was used to assess Full Scale IQ and four composite measures (Verbal Comprehension, Perceptual Reasoning, Working Memory, and Processing Speed Indexes) in 7-year-old children. We observed a consistent yet nonsignificant inverse relationship of methylation at several CpG sites close to the *PON1* transcription start site with Full Scale IQ and other composite measures of cognition. We also found an inverse relationship between cord blood methylation at cg15887283 with working memory and a positive association of 7-year-old methylation at cg22798737 with processing speed, independent of OP exposure. However, none of the associations remained significant after accounting for multiple comparisons. This study provides some evidence of the role DNA methylation may play in the known relationship between *PON1* and neurobehavior in children, however it appears to be only suggestive and warrants additional research.

Keywords

Epigenetics; cognition; IQ

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1. Introduction

The paraoxonase 1 (*PON1*) gene encodes a multifunctional enzyme that is involved both in detoxification of certain organophosphate (OP) pesticides and oxidative stress pathways (Costa et al. 2013; Li et al. 2003). Studies have implicated oxidative stress as an important mechanism in the pathogenesis of neurodegenerative diseases and developmental programming of neurodevelopmental deficits (Gandhi and Abramov 2012; Wells et al. 2009). *PON1* genetic polymorphisms and/or enzyme measurements have been associated with a number of diseases of the nervous system, including Alzheimer's disease (Erlich et al. 2006; Leduc and Poirier 2008; Paragh et al. 2002), amyotrophic lateral sclerosis,(Saeed et al. 2006; Slowik et al. 2006) Parkinson's disease (Zintzaras and Hadjigeorgiou 2004), and brain tumors (Kafadar et al. 2006). In children, PON1 enzyme activity levels were lower in children with autism (Pasca et al. 2010; Pasca et al. 2006). Additionally, in a prospective cohort of children (ages 6–9 yr) from New York City, urinary OP metabolite levels were associated with poorer scores in Perceptual Reasoning and Full Scale IQ (FSIQ) only among children whose mothers had the susceptible $PON1_{192\ QQ}$ genotype (Engel et al. 2011). We previously found that maternal urinary OP metabolite levels were associated with poorer Bayley Mental Development Index Scores in 2year-olds (Eskenazi et al. 2010) and IQ at age 7 (Eskenazi et al. 2014) among MexicanAmerican children and their mothers from the Center for the Health Assessment of Mothers and Children of Salinas (CHAMACOS) birth cohort. Associations between maternal OP exposure and MDI were strongest in children with the $PON1_{-108T}$ allele. Furthermore, the relationship between OPs and FSIQ was strongest in children of mothers with the lowest tertile of arylesterase (AREase) activity, a measure of PON1 enzyme quantity. We also found a direct relationship of *PON1* with neurodevelopment, demonstrating associations of the child $PON1_{-108T}$ allele (the allele linked to lower PON1 activity) with lower MDI scores at age 2 and maternal pregnancy AREase levels with lower WISC scores at age 7. The relationship between PON1 and adverse neurodevelopment likely involves both its ability to detoxify OP pesticides and its role in oxidative stress.

We have reported a broad variability (>100-fold range) of PON1 levels and substratespecific activities among CHAMACOS children and mothers (Furlong et al. 2006; Holland et al. 2006) that could affect differential susceptibility among individuals. Although genetic variants, particularly *PON1* promoter polymorphism ($PON1_{-108}$; rs705379), influence *PON1* gene expression and protein levels, they explain less than 25% of the variability of PON1 protein levels (Huen et al. 2010). Epigenetic marks, like DNA methylation, can affect gene expression without changes in DNA sequence. We previously showed that DNA methylation in the *PON1* gene can mediate the effect of the $PON1_{-108}$ genotype on PON1 protein levels (Huen et al. 2015). One recent study reported inverse associations between *PON1* DNA methylation in cord blood and cognition in young children aged 2 to 5 years exposed to prenatal mercury (Cardenas et al. 2017) but there is no research examining relationships of *PON1* DNA methylation with cognition in older school-aged children when PON1 enzyme levels approach that of adults (Gonzalez et al. 2012; Huen et al. 2010). We hypothesize that increased *PON1* DNA methylation levels may detrimentally influence children's cognitive function. In this study, we examined associations of *PON1* DNA methylation in blood

assessed at birth and age 7 with children's cognitive abilities as measured by Wechsler Intelligence Scale for Children (WISC) at age 7.

2. Materials and Methods

2.1. Study subjects

CHAMACOS is a longitudinal birth cohort study of mothers and their children living in the agricultural region of Salinas Valley, California. Pregnant women enrolled in the study (1999–2000) were at least 18 years of age, less than 20 weeks gestation, Spanish- or Englishspeaking, eligible for low-income health insurance, receiving prenatal care at one of the participating community clinics, and planning to deliver at the local public hospital. Six hundred and one pregnant women were enrolled and 526 remained in the study at delivery of live, singleton newborns (Eskenazi et al. 2003). In this study, we restricted analysis to children who had blood samples available for analysis at birth (cord blood) and/or age 7 years and who also completed neurobehavioral assessments at age 7 years. In total, 238 children were included in the analysis. Of these, 185 had methylation data at both time points and 53 children had methylation data available at birth only. Children included in the study did not differ from all children in the cohort by other demographic and exposure variables (e.g. poverty level, maternal marital status, or maternal prenatal farm work status, maternal OP urinary metabolite levels, or use of alcohol or tobacco).

Study protocols were approved by the University of California, Berkeley and the Centers for Disease Control and Prevention (CDC) Committees for the Protection of Human Subjects. Written informed consent was obtained from all mothers, and children provided verbal assent at age 7.

2.2. Blood collection and processing

Blood specimens from CHAMACOS children were collected from umbilical cords after delivery and by venipuncture when children were approximately 7 years old. Heparinized whole blood was collected in BD vacutainers® (Becton, Dickinson and Company, Franklin Lakes, NJ). Samples were then centrifuged, separated into aliquots of plasma, buffy coats and red blood cells, and then stored at −80°C at the School of Public Health Biorepository, University of California, Berkeley. Whole blood was also collected in BD vacutainers® (Becton, Dickinson and Company, Franklin Lakes, NJ) containing no anticoagulant. These samples were centrifuged, divided into serum and clot, and then stored at –80°C.

2.3. Bisulfite treatment and DNA methylation analyses

DNA isolation from clots was performed using a QIAamp Blood DNA Maxi kit (Qiagen, Inc., Santa Clarita, CA) as previously described (Holland et al. 2006). DNA was normalized to 55 ug/ml and bisulfite conversion was performed on 1ug aliquots of DNA using Zymo Bisulfite conversion Kits (Zymo Research, Orange, CA). Methylation levels at 18 *PON1* CpG sites in cord blood were analyzed as part of a genome-wide methylation assessment using the Illumina Infinium 450K DNA methylation BeadChip as previously described (Huen et al. 2015). The EPIC BeadChip is the most recently released version of Illumina's methylation array and replaces the previous 450K BeadChip. It covers >90% of the CpG

sites assessed by 450K and extends coverage to an additional >400,000 sites. The EPIC BeadChip, was used to assess DNA methylation in samples collected from 7-year-old children because the 450K BeadChip was no longer available at the time of the experiment. EPIC includes 19 *PON1* CpG sites, 15 of which were also included in the 450K BeadChip. All *PON1* CpG sites assessed by either platform are shown graphically in Figure 1 and described in Supplemental Table 1. Pidsley et al. reported that methylation levels assessed by EPIC and 450K BeadChip arrays were very highly correlated with Spearman Rank Correlation coefficients of 0.99 and it was suggested that data from the two platforms could easily be integrated for analysis (Pidsley et al. 2016). We recently reported similar findings in CHAMACOS children (Solomon et al. *In Press*) finding a strong correlation of overall methylation between both platforms. However, correlations at individual CpG sites, including those located in the *PON1* gene, tended to be weaker (Pearson's r: 0.02–0.18) at the extreme levels of methylation (highly methylated or highly unmethylated) compared to moderately methylated CpG sites (Pearson's r: 0.27–0.59). When we assessed differential methylation by sex using data from both assays, we confirmed good reproducibility, replicating the majority of significant hits with correlated effect sizes between platforms.

DNA samples were whole genome amplified, enzymatically fragmented, purified, and applied to the Infinium BeadChips according to the Illumina methylation protocol (Bibikova et al. 2011; Pidsley et al. 2016; Sandoval et al. 2011). BeadChip processing was performed using robotics and the Illumina Hi-Scan system was used for analysis. Samples included in the analysis had detection p-values below 0.01 for 95% of CpG sites. Three *PON1* CpG sites (2 in 450K and 3 in EPIC) with common SNPs (minor allele frequency >5%) within 50bp of the target identified in the MXL (Mexican ancestry in Los Angeles, California) HapMap population were excluded, resulting in a total of 16 450K and 16 EPIC *PON1* CpG sites included in the subsequent analyses. Raw signal intensities were background corrected and then normalized for colorchannel bias using the All Sample Mean Normalization (ASMN) method as described previously by Yousefi et al (2013). We also applied beta mixture quantile (BMIQ) normalization to make interpretation between type I and type II probes comparable (Teschendorff et al. 2013). Methylation data were expressed as M-values, which are calculated as the $\log_2$ ratio of the intensities of methylated to unmethylated probes (Du et al. 2010). Quality assurance procedures included use of repeats and internal standards to minimize technical variability.

2.4. Cell Composition

Cell type proportions were calculated in the R-package *minfi* (Aryee et al. 2014). For cord blood samples, we estimated cell-type proportions using a recently published cord blood reference dataset that included nucleated red blood cells (Bakulski et al. 2016). For blood samples collected from seven-year-old-children, we used an adult reference data set (Houseman et al. 2012).

2.5. Determination of *PON1*$_{-108}$ genotype

The promoter SNP, *PON1*$_{-108}$, was genotyped using a fluorogenic allele-specific assay (Amplifluor, Chemicon, Temecula, CA). The assay used a two-part nested PCR strategy where the region surrounding the SNP was pre-amplified using non-allelic flanking primers

and then the amplicon was diluted and used as the template for the Amplifluor assay. Quality assurance procedures for genotyping of this SNP included assessment of randomly distributed blank samples in each plate and duplicates of randomly selected samples (4% of samples) with independently isolated DNA from the same subjects. We observed a high degree (>99%) of concordance among repeated samples. All discrepancies were resolved with additional genotyping.

2.6. Determination of PON1 arylesterase activity in blood of newborns and 7-year old children

The AREase assay, which measures the rate of phenyl acetate hydrolysis in plasma, is considered a reliable measure of PON1 enzyme quantity. The assay uses spectrophotometric methods as described previously (Richter and Furlong 1999) and is highly correlated ($r > 0.85$) with other measures of PON1 quantity such as ELISA and Western blot based methods utilizing PON1 antibodies (Connelly et al. 2008; Kujiraoka et al. 2000). All assays were performed in triplicate. Quality assurance included use of internal controls (aliquots of the same sample run on all assay plates) and assessment of repeat samples (separate aliquots of the same sample run on different days) (2009b). The average inter assay coefficient of variation (CV) for internal controls run on all assay plates was 8.7%.

2.7. Assessment of neurobehavioral outcomes

Children's cognition was assessed at age 7 as previously described (Eskenazi et al. 2010; Stein et al. 2016). Children's cognitive abilities were determined using the Wechsler Intelligence Scale for Children – Fourth Edition (WISC-IV)(Wechsler 2003) and its Spanish equivalent (Wechsler 2005). All assessments were performed by the same bilingual psychometrician, who was trained and supervised by a pediatric neuropsychologist. They were administered in the dominant language of the child, which was determined by an oral vocabulary subtest (Woodcock and A. 1990). The WISC-IV provides a Full Scale IQ score that reflects overall performance on the subtests presented. It also generates four standardized composite measures: Verbal Comprehension Index (VCI), Perceptual Reasoning Index (PRI), Working Memory Index (WMI), and Processing Speed Index (PSI). All Wechsler composite scores (*e.g.,* VCI, FSIQ) are standardized by age to a mean of 100 (SD: 15).

Maternal education at enrollment and verbal comprehension were assessed via maternal interview at enrollment and by assessment using the Peabody Picture Vocabulary Test - Revised (PPVT) (Dunn and Dunn 1981) or its Spanish adaptation (Dunn et al. 1986) at six months postpartum, respectively.

2.8. Organophosphate pesticide exposure measurements

Six non-specific dialkyl phosphate (DAP) metabolites were measured in maternal urine collected twice during pregnancy around 13 and 26 weeks gestation as previously described (Eskenazi et al. 2014). These DAP metabolites were comprised of three dimethyl (DM) phosphate metabolites (dimethylphosphate, dimethylthiophosphate, dimethyldithiophosphate) and three diethyl (DE) phosphate metabolites (diethylphosphate, diethylthiophosphate, and diethyldithiophosphate) (Bradman et al., 2005). Additional details

on urine collection, limits of detection for each metabolite, and quality control measures are described elsewhere (Bradman et al. 2005). DAP metabolites were measured using gas chromatography-tandem mass spectrometry and quantified using isotope dilution calibration (Bravo et al., 2002). Concentrations below the limit of detection (LOD) were randomly imputed based on a log-normal probability distribution, estimated using maximum likelihood estimation, and molar concentrations were summed to yield total DM, total DE, and total DAP concentrations (nmoles/L). Detection frequencies for total DM, total DE, and total DAP concentrations were 80.2, 74.3, and 88.5% at 13 wks gestation and 99.6, 98.8, and 100.0% at 26 weeks gestation.

2.9. Statistical analysis

We performed linear regression models to determine the relationship of *PON1* DNA methylation at birth and age 7 years with (1) AREase activity and (2) measures of cognition. Separate models were constructed for each CpG site at each age for AREase activity and each neurodevelopment outcome. Models of *PON1* DNA methylation and AREase activity were adjusted for batch and cell composition. All models of *PON1* DNA methylation and neurodevelopment were adjusted for maternal education, maternal verbal cognition scores, batch, and cell composition. We chose to control for covariates used in our previous studies of maternal OP exposure, PON1, and cognition (Eskenazi et al. 2014) and also added cell composition since it can be a source of bias in DNA methylation studies. Additionally, we used linear regression models to examine associations of mean pregnancy OP metabolites (log 10 transformed DE, DM, and total DAPs) with DNA methylation at each *PON1* CpG site controlling for cell composition. For CpG sites with suggestive association with both OP metabolite concentrations and cognition outcomes, we used the PARAMED module (Emsley and Liu 2013)to do mediation analyses. We also performed secondary analyses which included running the same models with (1) stratification by sex, (2) adjustment for child $PON1_{-108}$ genotype, (3) stratification by maternal AREase activity, and (4) stratification by maternal $PON1_{-108}$ genotype. Controlling for $PON1_{-108}$ genotype should yield estimates of the relationship of methylation and cognition independent of the relationship between genotype and cognition. Unadjusted p-values < 0.05 were considered significant. However, to account for multiple testing, we also used the Benjamini-Hochberg (BH) method for false discovery rate (FDR) with an FDR $q < 0.05$ threshold for significance (Benjamini and Hochberg 1995). Statistical analyses were performed in STATA (version 12.0; StataCorp, College Station, TX). Power and sample size calculations were estimated using PS: Power and Sample Size Calculation version 3.1.2, 2014 (Dupont and Plummer 1998).

3. Results

Characteristics of CHAMACOS mothers and their children are shown in Table 1. The average maternal age at enrollment was 25.7 years (SD = 5.0). The majority of mothers (79%) had less than a high school education and nearly all lived within 200% of the poverty level. Maternal verbal comprehension scores assessed by PPVT ranged from 42 to 120 with an average of 86 (SD=20.6). There were slightly more girls (n=125) than boys (n=113) included in this study. On average, children were assessed at 7.1 years of age (SD=0.22). The majority of children (67%) were tested in Spanish while all other children were tested in

English at the 7 year visit. The average WISC Full-Scale IQ in 7-year old children was 104.1 (SD = 15.2). The geometric mean of the average total DAP, DM and DE concentrations in maternal urine during pregnancy was 124.6 nmol/L, 90.6 nmol/L and 20.3 nmol/L, respectively (Supplemental Table 2). As previously reported, average prenatal urinary DAP concentrations in the CHAMACOS population are higher than those observed in the general U.S. population (Bradman et al. 2005).

3.1. *PON1* DNA Methylation

DNA methylation in *PON1* CpG sites among CHAMACOS newborns and 7-year old children is shown in Figure 2. We observed similar patterns of methylation at both ages as was previously described in CHAMACOS newborns (Huen et al. 2015). Specifically, CpG sites in the Open Sea and North Shelf regions (CpG Sites 1 to 7) had very high levels of methylation, with average β values at these sites ranging from 0.84–0.90 and 0.91 to 0.99 in cord and 7-year old blood, respectively. Furthermore, CpG sites that were closer in proximity to the transcription start site (TSS) (CpG Sites 8 to 15) had lower levels of methylation with β's averaging from 0.51 to 0.77 and 0.53 to 0.66, in cord and age 7 blood, respectively.

To confirm the relationship of *PON1* DNA methylation with gene expression at the protein level, we examined associations of methylation at each CpG site with AREase activity. Mean AREase activity (Supplemental Table 3) was lowest in newborns (34.8 U/mL) and higher in 7-year old children and their mothers (123.1 and 126 U/mL, respectively), which has been described extensively in CHAMACOS children and mothers elsewhere (Holland et al. 2006; Huen et al. 2010). As in previous analyses (Huen et al. 2015), cord blood methylation at sites close to the TSS (CpG sites 8 to 15) were inversely associated with AREase activity after controlling for cell composition and batch. The relationship of 7-year old methylation with 7year old AREase activity were similar for CpG sites 8 to 15 however the associations were much stronger with larger beta coefficients and much smaller p-values (Table 2), a trend we also previously reported in 9-year old CHAMACOS children (Huen et al. 2015). In contrast, however, methylation of CpG site 19 in 7-year old children was positively associated with AREase activity (β(95%CI): 9.54(1.53,17.5)).

2.2. Cord blood *PON1* DNA methylation and neurodevelopment

The relationships of cord blood DNA methylation with cognition in 7-year-old children are shown in Table 3. DNA methylation at CpG site 19 was positively associated with verbal comprehension (β(95%CI): 7.6(0.10,15.0)) and similar trends were observed with working memory and FSIQ. Additionally, we observed a consistent overall trend among CpGs close to the TSS (sites 8 to 15). Their methylation levels were inversely associated with Full Scale IQ as well as the four composite measures (verbal comprehension, perceptual reasoning, working memory, and processing speed). However, none of these relationships reached statistical significance. Stratification by sex did not appreciably change results (Supplemental Tables 4 and 5).

Given that *PON1* DNA methylation in sites 8 to 15 can mediate the effect of the *PON1108* genotype on expression (Huen et al. 2015), we also performed regression models controlling

for *PON1*$_{-108}$ genotype to identify relationships of methylation with cognition that were independent of this promoter SNP. These results are shown in Supplemental Table 6. DNA methylation at CpG site 10 (located in the *PON1* CpG Island) was negatively associated with working memory after controlling for potential confounders and *PON1*$_{-108}$ genotype (β(95%CI):5.14(−9.70,−0.57). The direction of effect was the same for other CpG sites located near the TSS (CpG sites 8 to 15) although these associations did not reach statistical significance. All of the associations observed between cord *PON1* DNA methylation and IQ scores were no longer significant after accounting for multiple comparisons using the FDR.

We previously found that relationships of maternal OP exposure with child cognition were modified by maternal AREase activity. Therefore, we also performed models of *PON1* DNA methylation and child cognition stratified by maternal PON1 factors (AREase activity during pregnancy and maternal *PON1*$_{-108}$ genotype). We found suggestive evidence of an interaction between cord blood DNA methylation and maternal AREase activity on cognition for several CpG sites including sites 9, 10, 11, and 16 (Supplemental Table 7). In the stratified models, we observed a consistent trend of positive associations of DNA methylation with cognition in children with low maternal AREase activity and inverse associations in children with high maternal AREase activity. Trends were similar but weaker in the model stratifying by *PON1*$_{-108}$ genotype (Supplemental Table 8) where relationships of PON1 DNA methylation were positive in children with the maternal *PON1*$_{-108CC}$ genotype (higher expression genotype) and negative in those with maternal *PON1*$_{-108CT}$ and *PON1*$_{-108TT}$ genotypes (lower expression genotypes). None of these associations remained significant after accounting for multiple comparisons however.

2.3. 7 year old *PON1* DNA methylation and neurodevelopment

Results from the regression models performed for 7-year-olds' DNA methylation and cognition are shown in Table 4. Higher methylation levels at CpG site 1 were associated with higher processing speed scores (β(95%CI): 6.5(0.6,12.4)). Similar to findings for cord blood methylation, we also observed a consistent though nonsignificant inverse relationship between methylation levels at sites 8 to 15 with Full Scale IQ as well as with all four composite scores. Results remained similar after stratification by sex (Supplemental Tables 9 and 10). However, the positive association of CpG site 1 was stronger in girls. Furthermore, in boys, we observed a positive association of CpG site 6 with verbal comprehension and Full-Scale IQ scores as well as negative association of CpG site 17 with perceptual reasoning scores. After controlling for *PON1*$_{-108}$ genotype (Supplemental Table 11), the positive association of *PON1* CpG site 1 (cg22798737) with processing speed persisted (β(95%CI):6.57(0.45,12.69). However, none of the associations observed remained significant after controlling for FDR.

2.4. Mediation analysis of OPs, *PON1* DNA methylation, and child cognition

We previously reported that urinary DAP concentrations during pregnancy were, at varying degrees, associated with poorer working memory, processing speed, verbal comprehension, perceptual reasoning and full-scale IQ scores in 7-year old children (Bouchard et al. 2011). Therefore, to consider whether *PON1* DNA methylation mediates the relationship of prenatal OP exposure on child cognition, we performed mediation analysis (Supplemental

Table 12) for *PON1* CpG sites that were at least marginally associated both with prenatal DAP concentrations and 7-year cognition outcomes (Supplemental Tables 13 and 14). We found evidence of a direct effect of prenatal diethyl DAPs on verbal comprehension IQ (Controlled Direct Effect estimate (95%CI):−5.2(−10.1, −0.2) but no statistically significant indirect effect of cord blood methylation at CpG site 19 suggesting that *PON1* DNA methylation at site 19 does not mediate the relationship of prenatal OP exposure on child cognition in 7-year old children. We did not find evidence of a significant direct effect of total DAP concentrations on processing speed IQ but we did observe a significant indirect effect (estimate (95%CI):−0.42(−1.77,−0.002) of 7-year old blood methylation at CpG site 1, suggesting that the relationship of methylation at CpG site 1 with processing speed is independent of prenatal OP exposure.

3. Discussion

In this study, we sought to explore whether epigenetic marks, specifically DNA methylation, play a role in the relationship between *PON1* and child cognition. Previously, we reported that maternal *PON1* levels and prenatal exposure to OPs were associated with child IQ at age 7 in the same cohort (Eskenazi et al. 2014). In the current study, we identified one *PON1* CpG site for which increasing cord methylation levels were related with higher verbal comprehension IQ and one CpG site at age 7 which was positively associated with processing speed IQ. However, neither relationship remained statistically significant after controlling for multiple comparisons. Furthermore, CpG sites close to the TSS showed a consistent yet nonsignificant inverse relationship with FSIQ and other composite measures of cognition. Our data provide some suggestive evidence linking *PON1* DNA methylation with child IQ.

In our previous molecular studies of PON1 in the CHAMACOS cohort, we found that PON1 enzyme levels were most highly influenced by age and genetics (Holland et al. 2006; Huen et al. 2010; Huen et al. 2009a). However, these factors explained only a portion of the variability of PON1 enzyme levels. More recently, we reported highly significant associations of *PON1* DNA methylation both with *PON1* genotype and with PON1 enzyme levels measured by AREase activity in newborns and 9-year old children and provided evidence that the relationship of $PON1_{-108}$ genotype on AREase activity may be mediated through *PON1* DNA methylation (Huen et al. 2015). In this study, we observed similar relationships of *PON1* DNA methylation with AREase activity in 7-year old children. We also expanded upon our previous studies and identified suggestive evidence of associations between *PON1* DNA methylation with child cognition, including some that were independent of child $PON1_{-108}$ genotype.

Only one other study has examined *PON1* DNA methylation in relation to children's cognition. Cardenas and colleagues identified an inverse association between cord blood *PON1* DNA methylation at four CpG sites (sites 9 and 12–14) and Peabody Picture Vocabulary Test (PPVT) scores in 135 boys of different races or ethnicities (77% White, 9% Black, and 7% Hispanic, 7% Other) during early childhood (ages 2 to 5 years) although they did not account for multiple comparisons (Cardenas et al. 2017). PPVT scores have previously been strongly correlated with verbal and Full Scale IQ scores (Dunn L et al.

1997). In our study conducted in older children (age 7 years), we used WISC-IV to assess cognition and also observed an inverse relationship between *PON1* DNA methylation and cognition scores at the same CpG sites reported by Cardenas and colleagues, but the associations did not reach statistical significance. Neither study (ours or Cardenas et al.) observed differences in the association of *PON1* DNA methylation with cognition by sex.

Our study was also one of the first to examine the role of *PON1* DNA methylation as a potential mediator of OP exposure on children's cognitive outcomes. For CpG site19, cord blood methylation did not appear to mediate the relationship of prenatal diethyl DAPs on verbal comprehension scores. Instead, the total effect seemed primarily driven by prenatal OP exposure. In contrast, we identified a significant natural indirect effect of 7-year old blood methylation at CpG site 1 on processing speed while the direct effect of prenatal total DAPs was not statistically significant. In this case, the relationship of CpG site 1 methylation with cognition did not seem to be related to prenatal OP exposure. PON1 however is a multifunctional enzyme, and the relationship of site 1 methylation with cognition may very well involve a different pathway such as oxidative stress.

Although we did identify two CpG sites that were associated with cognitive outcomes in school-age children, neither relationship remained statistically significant after adjusting for multiple comparisons. For cord blood methylation at CpG site 19, our post-hoc power calculations show we would have greater than 80% power to detect differences in verbal comprehension scores of 7 points given our study sample size of 231, suggesting that our study was adequately powered and the relationship identified was likely non-significant. In contrast, for 7-year old methylation at CpG site 1, we calculated that a sample size of 373 would be needed to detect differences in processing speed IQ scores of about 6 points with 80% power, indicating that our study (n=167) may have been slightly underpowered to detect a statistically significant association in this case. Thus, it is possible that this association could reach statistical significance in a larger study.

This study does have several limitations. Given that DNA methylation is tissue-specific, examination of blood rather than brain tissue may not be the most relevant for neurodevelopmental outcomes, in spite of observed relationships between cord blood methylation and cognitive outcomes in study by Cardenas et al. (2017). Furthermore, tissue specificity may be less of an issue for a metabolic enzyme like PON1, which is known to circulate in the bloodstream as opposed to a protein specific to the brain (e.g. GABA receptors or neurotransmitter reuptake proteins). Additionally, free radicals circulate in blood through the body -- including the brain -- affecting levels of oxidative stress, an important mechanism through which PON1 can affect neurodevelopment in children. Another limitation related to tissue-specificity is that although we examined *PON1* DNA methylation in blood samples, plasma PON1 is primarily synthesized in the liver suggesting that blood methylation may not be the most relevant measure for PON1. However, we found strong associations of *PON1* DNA methylation in blood with AREase activity, a measure of plasma protein levels, which should be reflective of liver expression. We did not determine associations of *PON1* DNA methylation with gene expression. However, we did demonstrate relationships of *PON1* DNA methylation at several CpG sites with AREase activity, a well-known marker of PON1 protein expression, providing evidence for the functional

significance of *PON1* DNA methylation. Finally, this study was performed in a Mexican-American cohort living in an agricultural region and may not be fully generalizable to the general population.

This study provides suggestive evidence that *PON1* DNA methylation may be involved in children's cognitive development, via a pathway independent of OP exposure. Additional studies in larger diverse cohorts are warranted to obtain more definitive answers as to how *PON1* DNA methylation contributes to the relationship of *PON1* genotypes and levels with child IQ.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

ASMN	All Sample Mean Normalization
AREase	arylesterase
BMIQ	beta mixture quantile
CDC	Centers for Disease Control and Prevention
CHAMACOS	Center for the Health Assessment of Mothers and Children of Salinas
CV	coefficient of variation
DAP	dialkyl phosphate
DE	diethyl
DM	dimethyl
FSIQ	full-scale IQ
LOD	limit of detection
OP	organophosphate
PON1	paraoxonase 1
PPVT	Peabody Picture Vocabulary Test

PRI	Perceptual Reasoning Index
PSI	Processing Speed Index
TSS	transcription start site
VCI	Verbal Comprehension Index
WISC	Wechsler Intelligence Scale for Children
WMI	Working Memory Index

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Highlights

- *PON1* DNA methylation was inversely associated with PON1 protein levels.
- Methylation levels at some *PON1* CpG sites were weakly associated with cognition.
- Links between child *PON1* DNA methylation and cognition may depend on maternal PON1 levels and genotypes.
- The relation of prenatal OPs and cognition was not mediated by *PON1* DNA methylation.

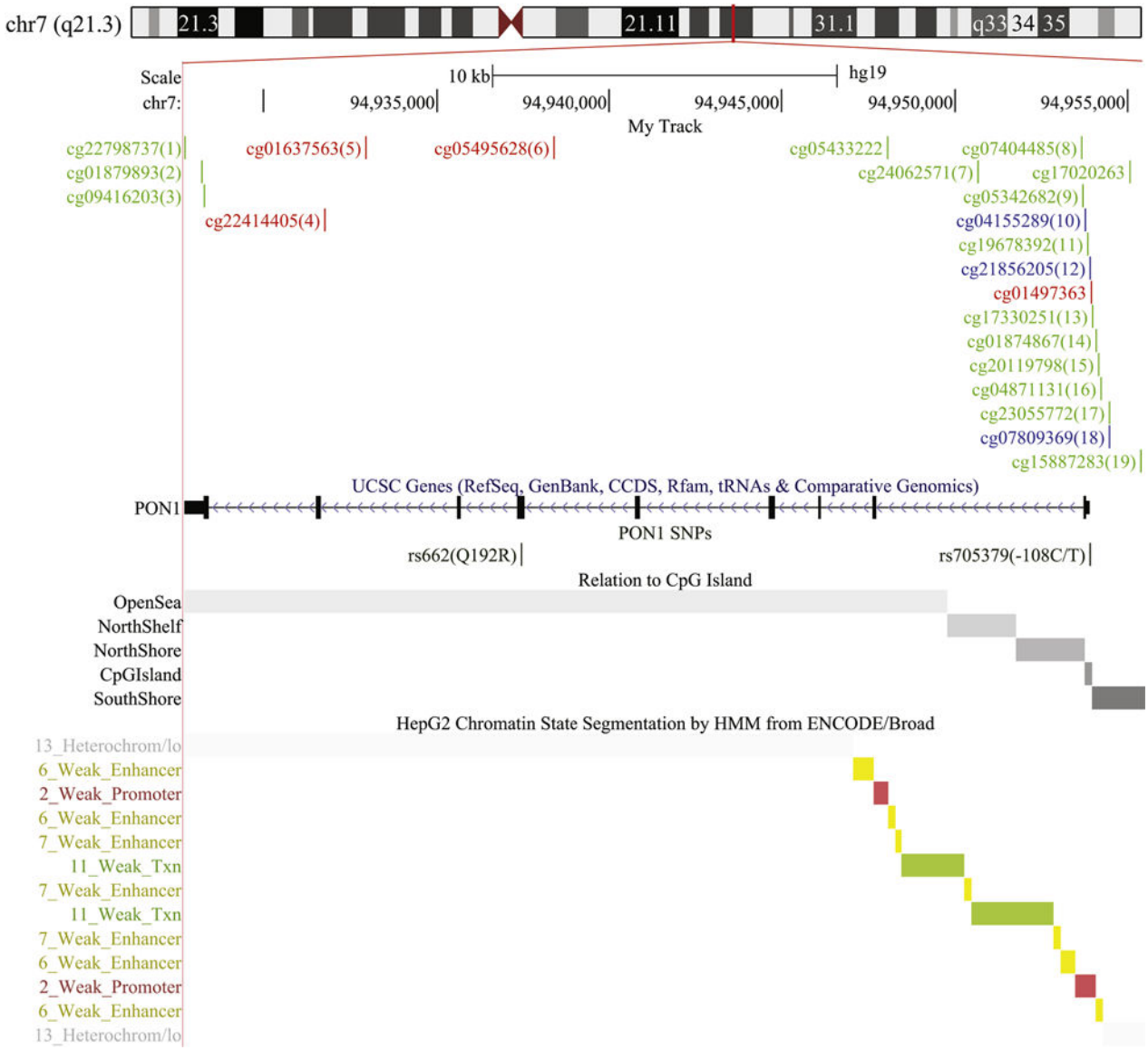

Figure 1. Schematic of the *PON1* gene.

Locations of PON1 CpG sites are in indicated in green (sites assessed by 450K and EPIC), red (sites assessed by EPIC only), and blue (sites assessed by 450K only). Common SNPs *PON1*$_{-108}$ and *PON1*$_{192}$ are also shown. The grey shaded areas indicate the regions in relation to the CpG island: open sea, shelves, shores, and island. Epigenetic states in liver cells as predicted by ChromHMM are shown in the bottom panel.

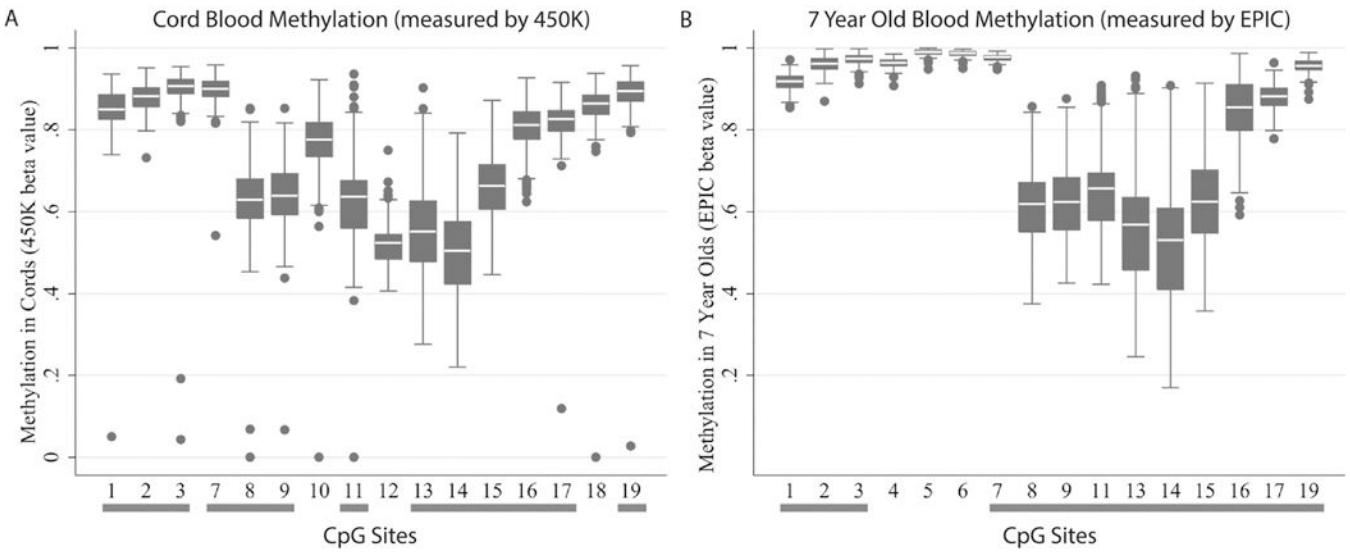

Figure 2. Box plots showing the distribution of methylation betas in cord blood and in 7- year old children.

PON1 DNA methylation in (a) cord blood measured by 450K and (b) 7-year old children measured by EPIC. Methylation levels are shown as beta values for ease of interpretation. At both ages, CpG sites 1 to 8 and 16 to 19 are highly methylated, while methylation levels are lower in CpG sites 8 to 15. CpG sites common to both 450K and EPIC assays are indicated with a grey bar underneath. CpG sites 4, 5, and 6 were not shown in (a) because they were not assessed by 450K and sites 10 and 18 were not shown in (b) because they were not assessed by EPIC.

Table 1.

Characteristics of CHAMACOS Mothers and Children

	n(%)	Mean (SD)
Maternal Characteristics		
Maternal Education		
<6th grade	111(45)	
7th-12th grade	82(34)	
Completed high school	52(21)	
Poverty level during pregnancy		
<Poverty level	147(60)	
Within 200% of poverty level	90(37)	
>200% poverty level	8(3)	
Maternal age at baseline 18–24	106(43)	
25–29	88(36)	
30–34	35(14)	
35–45	16(7)	
Maternal intelligence (PPVT Score)		85.9(20.7)
Child Characteristics		
Sex		
Boy	117(48)	
Girl	128(52)	
Gestational Age (wks)		38.9(1.4)
Child Age at 7-year assessment (yr)		7.1(0.2)
Language of 7-year WISC testing		
English	167(68)	
Spanish	78(32)	

Table 2.

Relationship of 7-year old blood *PON1* DNA methylation and 7–year old AREase activity (U/mL)

CpG Site Number	β(95%CI)	p-value
1	6.31(−5.68,18.30)	0.30
2	3.01(−2.28,8.31)	0.26
3	6.51(−0.06,13.10)	0.05
4	−0.40(−11.40,10.60)	0.94
5	0.61(−3.46,4.68)	0.77
6	7.64(1.72,13.60)	0.01
7	−0.41(−10.10,9.33)	0.93
8	−19.60(−26.50,−12.80)	5.68E-08
9	−20.00(−27.10,−12.90)	9.25E-08
11	−15.10(−20.50,−9.59)	1.75E-07
13	−11.10(−14.90,−7.22)	5.67E-08
14	−12.00(−16.00,−8.03)	1.28E-08
15	−14.10(−19.10,−8.97)	1.71E-07
16	−9.80(−13.50,−6.08)	5.48E-07
17	−3.85(−14.10,6.43)	0.46
19	9.54(1.53,17.50)	0.02

All models adjusted for cell composition and batch.

*Methylation in 7-year old blood samples were assessed by EPIC BeadChip. CpG sites 10 and 18 are not shown because they were not assessed by EPIC.

Table 3.

Regression models of cord blood *PON1* DNA methylation and WISC-IV IQ at age 7 years

	Verbal Comprehension n=231		Perceptual Reasoning n=232		Working Memory n= 213		Processing Speed n=214		Full Scale IQ n=211	
CpG Site Number	**β(95%CI)**	**p-value**	**β(95%CI)**	**p-value**	**β(95%CI)**	**p-value**	**β(95%CI)**	**p-value**	**β(95%CI)**	**p-value**
1	5.18(−3.83,14.20)	0.26	0.65(−7.30,8.61)	0.87	5.00(−2.04,12.00)	0.16	−0.60(−7.40,6.20)	0.86	3.56(−4.13,11.20)	0.36
2	2.03(−6.51,10.58)	0.64	−0.81(−8.31,6.69)	0.83	−4.66(−11.20,1.88)	0.16	−1.57(−7.98,4.85)	0.63	−1.40(−8.67,5.86)	0.70
3	2.24(−6.36,10.84)	0.61	2.16(−5.39,9.71)	0.57	3.98(−2.62,10.60)	0.24	−0.84(−7.20,5.53)	0.80	2.53(−4.68,9.75)	0.49
7	6.62(−3.07,16.30)	0.18	−0.17(−8.72,8.37)	0.97	−0.37(−8.05,7.31)	0.92	0.61(−6.83,8.04)	0.87	3.90(−4.51,12.30)	0.36
8	−3.52(−9.29,2.26)	0.23	−1.18(−6.29,3.93)	0.65	−0.09(−4.57,4.39)	0.97	−1.60(−5.86,2.66)	0.46	−2.32(−7.18,2.54)	0.35
9	−2.97(−8.41,2.48)	0.28	0.05(−4.75,4.85)	0.98	−0.17(−4.42,4.09)	0.94	−1.15(−5.14,2.85)	0.57	−1.46(−6.07,3.14)	0.53
10	−3.96(−8.81,0.88)	0.11	−2.09(−6.41,2.23)	0.34	−2.31(−6.09,1.47)	0.23	−0.68(−4.35,2.99)	0.72	−3.17(−7.24,0.91)	0.13
11	−1.45(−4.99,2.08)	0.42	−1.07(−4.20,2.07)	0.50	−0.15(−2.93,2.64)	0.92	−0.85(−3.48,1.77)	0.52	−1.29(−4.29,1.71)	0.40
12	−4.51(−12.40,3.42)	0.26	−2.11(−9.10,4.88)	0.55	−0.36(−6.65,5.94)	0.91	−2.56(−8.51,3.39)	0.40	−4.08(−10.90,2.73)	0.24
13	−1.57(−4.81,1.68)	0.34	−0.80(−3.66,2.06)	0.58	−0.12(−2.66,2.42)	0.93	−0.69(−3.08,1.71)	0.57	−1.24(−3.98,1.51)	0.38
14	−1.79(−5.19,1.62)	0.30	−1.29(−4.29,1.71)	0.40	−0.52(−3.19,2.14)	0.70	−0.56(−3.14,2.01)	0.67	−1.59(−4.46,1.28)	0.28
15	−2.15(−6.96,2.66)	0.38	0.030(−4.23,4.28)	0.99	0.32(−3.41,4.06)	0.86	−1.38(−4.98,2.22)	0.45	−1.23(−5.27,2.82)	0.55
16	−3.47(−8.74,1.79)	0.20	−1.49(−6.14,3.16)	0.53	−0.01(−4.10,4.08)	0.99	0.34(−3.62,4.29)	0.87	−1.57(−5.99,2.85)	0.49
17	−8.51(−18.60,1.60)	0.10	−0.90(−9.85,8.05)	0.84	−2.66(−10.90,5.53)	0.52	−0.57(−8.43,7.29)	0.89	−4.02(−12.90,4.87)	0.37
18	−4.05(−11.90,3.80)	0.31	−2.09(−8.99,4.82)	0.55	−1.10(−7.23,5.02)	0.72	−1.22(−7.20,4.76)	0.69	−2.27(−8.88,4.34)	0.50
19	**7.57(0.09,15.00)**	**4.70 E-02**	2.97(−3.63,9.58)	0.38	5.58(−0.31,11.50)	0.06	2.22(−3.45,7.88)	0.44	6.32(−0.07,12.70)	0.05

All models adjusted for maternal education, maternal scores for verbal cognition, and cell composition, and batch.

Unadjusted p-values <0.05 are bolded.

None of the associations remained significant after controlling for FDR.

*Methylation in cord blood samples were assessed by 450K BeadChip. CpG sites 4,5, and 6 are not shown because they were not assessed by 450K.

Table 4.

Regression models of 7-year blood *PON1* DNA methylation and WISC-IV IQ at age 7 years

	Verbal Comprehension n=179		Perceptual Reasoning n=179		Working Memory n= 167		Processing Speed n=167		Full Scale IQ n=167	
CpG Site Number	**beta(95%CI)**	**p-value**	**beta(95%CI)**	**p-value**	**beta(95%CI)**	**p-value**	**beta(95%CI)**	**p-value**	**beta(95%CI)**	**p-value**
1	6.30(−1.36,14.00)	0.11	2.52(−4.47,9.51)	0.48	2.83(−3.16,8.82)	0.35	**6.50(0.56,12.40)**	**0.03**	4.41(−2.23,11.10)	0.19
2	−1.22(−4.74,2.31)	0.50	−1.33(−4.51,1.85)	0.41	−1.71(−4.43,1.02)	0.22	−0.83(−3.56,1.90)	0.55	−1.96(−4.97,1.06)	0.20
3	3.12(−0.99,7.24)	0.14	−0.07(−3.82,3.68)	0.97	1.98(−1.31,5.27)	0.24	1.95(−1.31,5.21)	0.24	1.52(−2.11,5.14)	0.41
4	2.50(−4.03,9.02)	0.45	1.46(−4.48,7.41)	0.63	−0.69(−5.69,4.31)	0.79	−0.87(−5.86,4.13)	0.73	0.26(−5.24,5.76)	0.93
5	−0.04(−2.70,2.62)	0.98	−0.70(−3.12,1.72)	0.57	−0.36(−2.45,1.73)	0.73	0.42(−1.62,2.46)	0.69	−0.65(−2.92,1.63)	0.57
6	2.26(−1.22,5.74)	0.20	0.01(−3.17,3.19)	1.00	0.93(−1.77,3.63)	0.50	1.80(−0.86,4.47)	0.18	1.28(−1.69,4.24)	0.40
7	2.84(−3.45,9.13)	0.38	1.75(−3.97,7.47)	0.55	−0.74(−5.67,4.19)	0.77	0.00(−4.78,4.78)	0.76	1.34(−4.07,6.74)	0.17
8	−0.25(−4.69,4.18)	0.91	−1.61(−5.63,2.42)	0.43	−0.72(−4.11,2.66)	0.67	−1.74(−5.06,1.59)	1.00	−1.50(−5.20,2.20)	0.63
9	−1.57(−6.32,3.18)	0.51	−1.83(−6.18,2.51)	0.41	−1.21(−4.88,2.47)	0.52	−1.92(−5.54,1.69)	0.30	−2.49(−6.50,1.52)	0.43
11	−1.15(−4.71,2.41)	0.52	−1.50(−4.73,1.73)	0.36	−1.28(−4.00,1.43)	0.35	−1.83(−4.50,0.84)	0.30	−1.80(−4.78,1.17)	0.22
13	−0.97(−3.66,1.71)	0.48	−0.39(−2.83,2.05)	0.75	−0.66(−2.73,1.41)	0.53	−1.45(−3.47,0.58)	0.18	−1.08(−3.34,1.19)	0.23
14	−0.53(−3.14,2.07)	0.69	−1.31(−3.67,1.05)	0.28	−0.80(−2.80,1.19)	0.43	−1.19(−3.15,0.77)	0.16	−1.25(−3.44,0.93)	0.35
15	−0.77(−4.26,2.71)	0.66	−1.06(−4.21,2.09)	0.51	−0.73(−3.39,1.94)	0.59	−1.28(−3.90,1.33)	0.23	−1.33(−4.24,1.59)	0.26
16	−0.17(−2.58,2.24)	0.97	−0.69(−2.88,1.50)	0.54	−0.85(−2.70,1.00)	0.37	−0.94(−2.77,0.88)	0.33	−0.85(−2.88,1.18)	0.37
17	0.13(−6.23,6.49)	0.97	−2.87(−8.64,2.90)	0.33	−2.56(−7.48,2.36)	0.31	−2.52(−7.39,2.35)	0.31	−2.32(−7.74,3.10)	0.41
19	−3.65(−8.62,1.33)	0.15	−1.14(−5.69,3.41)	0.62	−0.16(−4.06,3.74)	0.94	0.64(−3.18,4.46)	0.31	−2.30(−6.56,1.96)	0.40

All models adjusted for maternal education, maternal scores for verbal cognition, cell composition, and batch.

None of the associations remained significant after controlling for FDR

*Methylation in 7-year old blood samples were assessed by EPIC BeadChip. CpG sites 10 and 18 are not shown because they were not assessed by EPIC.