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Research Article

High Pesticide Exposure Events and DNA Methylation among Pesticide Applicators in the Agricultural Health Study

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Pesticide exposure has been associated with acute and chronic adverse health effects. DNA methylation (DNAm) may mediate these effects. We evaluated the association between experiencing unusually high pesticide exposure events (HPEEs) and DNAm among pesticide applicators in the Agricultural Health Study (AHS), a prospective study of applicators from Iowa and North Carolina. DNA was extracted from whole blood from male AHS pesticide applicators ($n = 695$). Questionnaire data were used to ascertain the occurrence of HPEEs over the participant's lifetime. Pyrosequencing was used to quantify DNAm in *CDH1*, *GSTp1*, and *MGMT* promoters, and in the repetitive element, *LINE-1*. Linear and robust regression analyses evaluated adjusted associations between HPEE and DNAm. Ever having an HPEE ($n = 142$; 24%) was associated with elevated DNAm in the *GSTp1* promoter at CpG7 (chr11:67,351,134; $P < 0.01$) and for the mean across the CpGs

measured in the *GSTp1* promoter ($P < 0.01$). In stratified analyses, elevated *GSTp1* promoter DNAm associated with HPEE was more pronounced among applicators >59 years and those with plasma folate levels ≤ 16.56 ng/mL (p-interaction < 0.01); HPEE was associated with reduced *MGMT* promoter DNAm at CpG2 (chr10:131,265,803; $P = 0.03$), CpG3 (chr10:131,265,810; $P = 0.05$), and the mean across CpGs measured in the *MGMT* promoter ($P = 0.03$) among applicators >59 years and reduced *LINE-1* DNAm ($P = 0.05$) among applicators with ≤ 16.56 ng/mL plasma folate. Non-specific HPEEs may contribute to increased DNAm in *GSTp1*, and in some groups, reduced DNAm in *MGMT* and *LINE-1*. The impacts of these alterations on disease development are unclear, but elevated *GSTp1* promoter DNAm and subsequent gene inactivation has been consistently associated with prostate cancer. Environ. Mol. Mutagen. 58:19–29, 2017. © 2016 Wiley Periodicals, Inc.

Key words: DNA methylation; pesticides; exposure; Agricultural Health Study; epigenetics

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INTRODUCTION

It is well-documented that farmers and agricultural workers exposed to various pesticide classes and pesticides currently on the market are at increased risk for certain cancers, including leukemia, multiple myeloma, non-Hodgkin lymphoma (NHL), and prostate cancer [Blair et al., 1985; Hoar et al., 1986; Blair and Zahm, 1991; Brown et al., 1993; Zahm et al., 1993; Dich and Wiklund, 1998; Alavanja et al., 2003]. The biological mechanisms underlying this increased risk are incompletely understood [Alavanja et al., 2004; Alavanja and Bonner, 2005; Jaga and Dharmani, 2005; Alavanja et al., 2013a,b]. Multiple mechanisms are likely involved. Direct genotoxicity is an important mechanism but non-genotoxic mechanisms could be operating as well. Some suspected, non-genotoxic mechanisms described in the literature include oxidative stress and receptor-mediated toxicities [De Coster and van Larebeke, 2012; Sesti et al., 2012; Alavanja et al., 2013a,b]. There is emerging evidence that epigenetic mechanisms may also play an important role in pesticide-related carcinogenesis, based on findings in experimental studies [Tao et al., 2000; Das and Singal, 2004; Anway et al., 2005; Laird, 2005; Anway and Skinner, 2006; Skinner and Anway, 2007; Guerrero-Bosagna et al., 2012; Zhang et al., 2012], however, there have been very few human studies to date which have investigated this link.

Epigenetic mechanisms involve heritable changes in phenotype or gene expression that do not result from changes in the primary DNA sequence. An epigenetic mechanism, DNA methylation (DNAm), is a process of adding a methyl group to DNA via DNA methyltransferase; it influences gene expression and is linked to both transcriptional silencing and activation. Alterations in DNAm are recognized as key epigenetic changes in cancer [Bremnes et al., 2005; Kerr et al., 2007], and it is hypothesized that cells with aberrant DNAm acquire an overall gene expression pattern that favors proliferation and differentiation, leading to neoplastic transformation [Moore et al., 2003]. There is a paucity of evidence evaluating alterations in DNAm associated with exposure to pesticides. Most studies to date have been carried out in experimental animals or in human cell lines [Tao et al., 2000; Das and Singal, 2004; Anway et al., 2005; Laird, 2005; Anway and Skinner, 2006; Skinner and Anway, 2007; Guerrero-Bosagna et al., 2012; Zhang et al., 2012; Collotta et al., 2013]. The few human studies published have focused on persistent organic pollutants [Rusiecki et al., 2008; Kim et al., 2010; Lind et al., 2013] and arsenic [Pilsner et al., 2007, 2009; Chervona et al., 2012; Tajuddin et al., 2013]. We had the opportunity to evaluate epigenetic patterns potentially associated with pesticide exposure in a subgroup of pesticide applicators from a large cohort study, the Agricultural Health Study (AHS).

High pesticide exposure events (HPEEs), self-reported incidents of unusually high, non-specific exposure to pesticides, fertilizers, or other chemicals, are common within the agricultural community. HPEEs typically result from spills and other accidents and can involve high acute pesticide exposures; they are sometimes associated with various acute and chronic symptoms [Ogilvie et al., 1990]. In the AHS cohort at the time of enrollment, 14% of licensed pesticide applicators reported having at least one HPEE in their working lifetime [Alavanja et al., 1999]. Although the majority (87%) of these HPEE reports did not result in a health care visit [Bell et al., 2006], HPEEs may involve toxicologically and mechanistically relevant pesticide exposures, and long-term adverse health effects may result from pesticide exposures at levels associated with these events [O'Malley, 1997; Alavanja et al., 1999].

In this study we evaluate the association between HPEEs and levels of DNAm in blood in a population of male pesticide applicators [Starks et al., 2012a,b]. We focused on critical target loci within genes whose expression is regulated by DNAm, *E-cadherin* (*CDH1*), *glutathione-S-transferase-p1* (*GSTP1*), and *O⁶-alkylguanine-DNA alkyltransferase* (*MGMT*). These genes are commonly found to be aberrantly methylated in cancers which have been found to have positive associations with specific pesticides in the AHS, including lymphohematopoietic cancers, prostate cancer, and lung cancer [Melki et al., 1999; Goessl et al., 2002; Jeronimo et al., 2002; Jeronimo et al., 2002; Esteller, 2003; Nagasaka et al., 2003; Chim et al., 2004; Ekmekci et al., 2004; Kang et al., 2004; Rossi et al., 2004; Seidl et al., 2004; Bastian et al., 2005; Enokida et al., 2005; Hoque et al., 2005; Russo et al., 2005; Aggerholm et al., 2006; Gu et al., 2006; Nakata et al., 2006; Papadopoulou et al., 2006; Chuang et al., 2007; Alavanja et al., 2013a,b]. We also evaluated DNAm in a repetitive element, long interspersed nucleotide element (*LINE-1*), since reduced DNAm of *LINE-1* may indicate chromosomal instability [Rothenberg et al., 1972].

MATERIALS AND METHODS

Study Population

The AHS is a prospective cohort study that includes 57,310 private and commercial applicators licensed to apply restricted use pesticides in Iowa or North Carolina at the time of enrollment (1993–1997) [Alavanja et al., 1996]. Each participant completed a self-administered enrollment questionnaire, and 44% of the cohort also completed a “take home” questionnaire within one month of enrollment. The enrollment and take home questionnaires (1993–1997) comprised Phase 1 of the data collection. Two follow-up phone interviews were administered to the study participants at approximately 5-year intervals (1999–2003, 2005–2010), comprising Phases 2 and 3 of the data collection. The questionnaires from each phase of the study included questions about HPEEs.

A random sample of AHS applicators who completed all three phases of data collection was recruited ($n = 1,807$) for a study of neurobehavioral

outcomes and multiple pesticide exposure metrics, including HPEEs [Starks et al., 2012a,b]. The study population was chosen based on lifetime use of organophosphate pesticides (OPs). Specifically, a stratified random sample was selected from eligible AHS participants with equal sampling from the upper 25% and the lower 75% of the distribution of lifetime OP pesticide use [Starks et al., 2012a,b]. AHS participants who had reported a stroke, amyotrophic lateral sclerosis, multiple sclerosis, Parkinson's disease, retinal or macular degeneration, hypothyroidism, diabetes, or who were excessive drinkers were not included in the study [Starks et al., 2012a,b].

Whole blood samples collected at the time of neurobehavioral testing were used in the current study for extraction of DNA and quantification of DNAm. Of the total 701 applicators included in the neurobehavioral outcomes study, 693 did not report a past physician-diagnosed pesticide poisoning event. Of those, 598 were cancer-free and provided a whole blood sample. For two samples, we were unable to measure DNA methylation. The final number of applicators included in this study was 596.

This study was approved by the Institutional Review Boards of the National Institutes of Health, the University of Iowa, Harvard University, and the Uniformed Services University.

High Pesticide Exposure Event Assessment

Data on ever having an HPEE was based on the AHS questionnaires and was not specific to particular pesticides or other exposures. Each phase assessed HPEE somewhat differently, using the following questions:

Phase 1: Have you ever had an incident or experience while using any type of pesticide which caused you unusually high personal exposure?

Phase 2: Since (year of enrollment) did you have any incidents with fertilizers, herbicides, or other pesticides that caused you an unusually high personal exposure?

Phase 3: Since (date of last interview) have you had any incidents or pills that resulted in an unusually high exposure to pesticides from contact with your skin, from breathing fumes or dust, or from accidental ingestion?

Information regarding HPEEs was obtained prior to blood collection, thus providing a prospective design for the current study. Based on the HPEE data collected in the three phases, a metric of having ever reported an HPEE was constructed; if an applicator answered "yes" to having an HPEE in at least one of the three phases, they were considered to have ever reported an HPEE.

DNAm Quantification

DNA was extracted from whole blood using the QIAamp DNA blood mini/maxi kit (Qiagen, MD, USA). One microgram of genomic DNA was used for bisulfite conversion. The bisulfite-modified DNA was then amplified using PCR primers that flank the target CpGs. The biotinylated PCR primer enabled selection of one strand for pyrosequencing reaction. The single strand biotinylated PCR products were then pyrosequenced using a sequencing primer to measure the DNA methylation from the target CpGs. We performed bisulfite conversion using the EZ DNA Methylation-Gold™ Kit (Zymo Research, CA) followed by pyrosequencing using the PSQ Q96 MD Pyrosequencing system (QIAGEN, Valencia, CA) platform. Samples were run in duplicate and internal controls used to assess the efficiency of the bisulfite-conversion treatment. Samples with conversion efficiency <95% were excluded from downstream analysis. The degree of methylation at each CpG within the PCR amplicon was analyzed individually or as an average over the multiple CpG sites. The degree of methylation is expressed here as the percentage of methylated cytosines divided by the sum of methylated and unmethylated cytosines (%5mC), which we refer to throughout this paper as DNAm. For the purpose of quality control, we included duplicate samples (6% of the total number of samples) to which the laboratory was blinded.

We measured DNAm at individual CpG loci promoter regions of: *CDHI*, *GSTp1*, and *MGMT*. For *CDHI* we measured DNAm at three CpG sites (CpG#1: chr16:68,770,944; CpG#2: chr16:68,770,947; CpG#3: chr16:68,770,960), for *GSTp1* we measured 10 CpG sites (CpG #1: chr11:67,351,089; CpG #2: chr11:67,351,099; CpG #3: chr11:67,351,101; CpG #4: chr11:67,351,104; CpG #5: chr11:67,351,110; CpG #6: chr11:67,351,124; CpG #7: chr11:67,351,134; CpG #8: chr11:67,351,138; CpG #9: chr11:67,351,141; CpG #10: chr11:67,351,145), and for *MGMT* we measured three CpG sites (CpG #1: chr10:131,265,796; CpG #2: chr10:131,265,803; CpG #3: chr10:131,265,810). To quantify promoter methylation, we used methods previously described for *CDHI* [Avila et al., 2010; Kordi-Tamandani et al., 2010], *GSTp1* [Ronneberg et al., 2008; Peng et al., 2009], and *MGMT* [Cheng et al., 2006]. Details of the promoters, amplicons, primers, and sequences analyzed are presented in Supporting Information Table 1. For the repetitive element, *LINE-1* we measured DNAm at four loci. To quantify methylation in the repetitive element, *LINE-1*, we used methods described by Yang et al. [2004]

Plasma Folate and Vitamin B₁₂

Because DNAm is influenced by folate and vitamin B₁₂, micronutrients involved in 1-carbon metabolism, we measured plasma levels of folate and vitamin B₁₂ in all participants. This was a one-time measurement, made in the same sample as the whole blood used as a source of DNA. For plasma folate, we used an assay commonly used for clinical use [Rothenberg et al., 1972]. Plasma vitamin B₁₂ was measured by a quantitative sandwich enzyme immunoassay technique on the 2010 Elecsys Immunoanalyzer (Roche Diagnostics) [Rothenberg, 1961]. All plasma folate and B₁₂ measurements were carried out at the Clinical & Epidemiologic Research Laboratory, in the Department of Laboratory Medicine, at Children's Hospital, Boston. Units for plasma folate and B₁₂ were reported in ng/mL and pg/mL, respectively.

Statistical Analysis

For the three genes (*CDHI*, *GSTp1*, and *MGMT*), we analyzed associations between HPEE and DNAm at individual promoter CpG loci. Because the *GSTp1* promoter typically has low levels of methylation, and the margin of detection/error for measurement of percent methylation is generally in the range of 3-6%, we limited our analyses of *GSTp1* to observations with levels of methylation greater than 6%. For the repetitive element, *LINE-1* we evaluated the mean of the four CpGs measured.

We calculated intra-class correlation coefficients (ICCs) for each CpG locus and for the average of four CpG sites in *LINE-1* loci, based on blinded duplicates randomly arranged in the plates run for this study (6% of total sample were included as duplicates). ICCs ranged between 0.61 and 0.78 for the CpG sites measured in *CDHI*, 0.57 to 0.74 for CpG sites measured in *MGMT*, and 0.45 and 0.66 for CpG sites measured in *GSTp1*. For *LINE-1*, the ICC for the mean across four CpG sites was 0.40, but the coefficient of variation (CV) was very low (3%).

We visually evaluated distribution plots for all DNAm loci measured. Visual inspection of plots for CpG sites in the promoters of *CDHI* and *MGMT* loci, as well as DNAm in *LINE-1* indicated normal distribution; visual inspection of plots for CpG sites in the *GSTp1* promoter, however, indicated a non-normal distribution. Therefore, we utilized t-tests to compare DNAm levels between applicators reporting ever and never having an HPEE across the three phases of the AHS study for *CDHI*, *MGMT*, and *LINE-1* and Wilcoxon Rank Sum tests to compare the DNAm levels in *GSTp1*.

We evaluated a common set of covariates, potentially associated with DNAm or with HPEE, for confounding: age at blood draw (continuous), race, state of residence (Iowa or North Carolina), education, consumption of fruits and vegetables, alcohol consumption, years lived on a farm, body mass index (BMI), smoking, ever use of personal protective equipment, skin

sensitivity to sun exposure, and still living on a farm (Phase 3). We also included plasma levels of vitamin B₁₂ and folate in our evaluation for confounding, because both these dietary nutrients are required to maintain 1 carbon pools for normal homocysteine remethylation, S-adenosyl methionine (SAM) formation, and DNA methylation [Shin et al., 2010; Crider et al., 2012].

We first evaluated the unadjusted association between each covariate and each methylation marker, based on the mean of the CpG sites in that marker (e.g., *CDH1*, *MGMT*, *GSTp1*, and *LINE-1*), using linear regression. We then evaluated the unadjusted association between each covariate and HPEE (binary variable—ever/never), using logistic regression. Covariates associated with both a methylation marker and HPEE based on a *P*-value of <0.20 were subsequently selected for inclusion into an initial full multiple linear regression base model for that marker. Using a backwards regression, we then removed covariates from the initial full base model if they had a *P*-value of ≥ 0.20 . The final base model for each methylation marker included only those covariates with a *P*-value <0.20. Based on our backwards regression models, the following variables were included into final models: *CDH1*: drinks per month of alcohol and skin sensitivity to sun; for *MGMT*: pack years in Phase 1 and having personally mixed, loaded, or applied pesticides in Phase 3; for *GSTp1*: no covariates met the inclusion criteria; *LINE-1*: Still living on a farm in Phase 3. However, we included age as a continuous variable into all final models because of its relevance with DNA methylation patterns in blood cells and likelihood of having an HPEE.

We estimated adjusted associations between *CDH1*, *MGMT*, and *LINE-1* DNAm and HPEE using linear regression models in which the outcome (DNAm) was regressed on ever having an HPEE, adjusting for the base model covariates. For *GSTp1*, because the residuals were not normally distributed, even after various transformations, we employed a robust regression analysis [Lawrence and Arthur, 1990] to evaluate the associations between DNAm in *GSTp1* and HPEE. We further evaluated this association using bootstrapping and quantile regression analyses.

Sensitivity Analyses

For the primary analyses described above, we analyzed data from all participants. To evaluate the robustness of our results, we conducted two sensitivity analyses where we evaluated results from (1) non-smokers and (2) people with a greater than high school education. Our assumption was that if these subgroups of our study population showed internal consistency, then our results for the overall population could be considered more robust. We also checked if there was internal consistency by state residence (Iowa and North Carolina).

Stratified Analyses

We carried out a series of stratified analyses to investigate the potential for effect modification from age, plasma folate, and plasma B₁₂. We adjusted stratified analyses by age at blood draw (cut at the median; 59 years), plasma folate levels (cut at the median; 16.56 ng/mL), and vitamin B₁₂ levels (cut at the median; 585.95 ng/mL). We used the same adjusted linear regression analyses for *CDH1*, *MGMT*, and *LINE-1* and the same adjusted robust regression analyses for *GSTp1* as described above and calculated a *P*-value for interactions.

We used the P1RE1071201, P2RE1071202, and P3RE10901 releases of the AHS dataset. All analyses were performed using SAS software, version 9.2 (SAS Institute, Cary, NC).

RESULTS

A total of 142 (24%) of the 596 men in this sample reported an HPEE in any of the three phases of the AHS. Characteristics of the study population stratified by ever

reporting an HPEE in any of the three phases of the AHS are presented in Table I. Applicators reporting ever having had an HPEE were younger at blood draw (mean = 56.9; s.d.=10.7) than those who never experienced an HPEE (mean = 60.9; s.d.=11.5). Sixty-four percent of applicators with an HPEE had greater than a high school education compared to 49% of those without an HPEE. Applicators who reported an HPEE also reported more total lifetime days of application of any pesticides (mean = 2,005.31; s.d.=1,829.66) compared with those who reported never having an HPEE (mean = 1,452.16; s.d.=1,544.84). Despite these differences, age, education, and lifetime days pesticide application did not meet the criteria for confounders and thus inclusion in regression models. However, as mentioned above, we included age as a continuous variable into all final models because of its relevance with DNA methylation patterns in blood cells and likelihood of having an HPEE.

Student's t-tests and Wilcoxon rank sum tests, comparing DNAm at each locus between applicators reporting ever and never having an HPEE across the three phases of the AHS study are presented in Table II. As described above, for *GSTp1* we limited our analyses to observations with greater than 6% DNAm. This resulted in small sample sizes for CpGs 1, 3, 4, and 5. We therefore present results only for CpGs 2, 6, 7, 8, 9, and 10. We found no statistically significant differences via t-tests for DNAm in *CDH1*, *MGMT*, and *LINE-1* between applicators reporting an HPEE and those not reporting an HPEE. We found via Wilcoxon rank sum test that those reporting ever having an HPEE had higher DNAm at *GSTp1* CpG 7 (chr11:67,351,134; mean = 11.96 (s.d. = 7.95); median = 9.74) than those never reporting an HPEE (mean = 9.82 (s.d. = 5.04); median = 7.92); *P* = 0.02. There were also non-statistically significant higher DNAm levels for those reporting an HPEE compared with those who did not at *GSTp1* CpGs 8 (chr11:67,351,138), 9 (chr11:67,351,141) and 10 (chr11:67,351,145). The mean DNAm of CpGs 2,6,7,8,9,10 in *GSTp1* was significantly higher for those reporting an HPEE (mean = 14.57 (s.d. = 8.76) median = 11.40) than for those not reporting an HPEE (mean = 8.90 (s.d. = 3.18) median = 7.96); *P* < 0.01.

We detected no statistically significant associations between HPEE and DNAm in *CDH1*, *MGMT*, and *LINE-1* via adjusted linear regression analyses (Table III). The results of adjusted robust regression analyses for *GSTp1* CpGs are presented in Table IV. We found a statistically significant elevation of DNAm for *GSTp1* CpG 7 ($\beta = 2.07$; *P* < 0.01) and for the mean across six CpGs measured in the promoter ($\beta = 2.68$; *P* < 0.01); applicators ever having an HPEE had, on average, over 2% higher DNAm than those without an HPEE. We also found a non-significant positive association of a similar magnitude for *GSTp1* CpG 8 ($\beta = 1.98$; *P* = 0.06). Results from bootstrapping analyses and quantile regression

TABLE I. General Characteristics of Study Population

Characteristic	Never had an HPEE (n = 454)				Ever had an HPEE (n = 142)			
	Mean	SD	N	%	Mean	SD	N	%
Age at blood draw	60.91	(11.46)	454		56.87	(10.71)	142	
Categorized Age at blood draw								
30–40			15	3.30			8	5.63
>40–50			65	14.32			25	17.61
>50–60			142	31.28			64	45.07
>60–70			127	27.97			29	20.42
>70–80			82	18.06			11	7.75
>80			23	5.07			5	3.52
Testing location								
Iowa			229	(50.44)			74	(52.11)
North Carolina			225	(49.56)			68	(47.89)
Race								
White			431	(97.51)			138	(99.28)
Black			7	(1.58)			1	(0.72)
Other			4	(0.90)			0	(0.00)
Missing			12				3	
Education								
≤High school			220	(50.93)			50	(35.97)
>High school			212	(49.07)			89	(64.03)
Missing			22				3	
Personal protective Equipment Use								
No			67	(14.76)			20	(14.08)
Yes			387	(85.24)			122	(85.92)
Alcohol Use in the last year (Phase 3)								
No			172	(37.89)			45	(31.91)
Yes			282	(62.11)			96	(68.09)
Alcohol consumption (drinks/week) (Phase 3)								
None			172	(37.89)			45	(31.91)
Daily			28	(6.17)			16	(11.35)
Weekly			114	(25.11)			34	(24.11)
Monthly			73	(16.08)			21	(14.89)
< 1 time per month			67	(14.76)			25	(17.73)
Total Lifetime Days of Pesticides ^a	1,452.16	(1544.84)			2,005.31	(1829.66)		
Plasma Vitamin B12	637.80	(307.50)			668.40	(312.40)		
Plasma Folate	19.19	(9.62)			18.59	(8.51)		
Body Mass Index at Enrollment	27.16	(3.67)			27.13	(3.69)		
Body Mass Index at Phase 3	28.45	(4.17)			28.65	(4.43)		
Pack years smoked at Enrollment (Phase 1)	8.65	(17.55)			6.47	(13.05)		
Years smoked at Enrollment (Phase 1)	7.43	(11.09)			6.16	(10.52)		
Number of cigarettes smoked per day (Phase 3)	6.56	(9.16)			6.78	(10.10)		
Vegetable servings per month (Phase 1)	5.01	(1.88)			5.31	(1.87)		
Fruit servings per month (Phase 1)	3.55	(1.72)			3.76	(1.88)		

^aSummed across Phases 1-3 of the Agricultural Health Study.

analyses for *GSTp1* yielded similar results (data not shown). In sensitivity analyses, when we restricted our population to non-smokers and those with at least a high school education, we saw no meaningful differences in the results of our linear regression analyses or robust regression analyses compared to analyses including the total study population (data not shown). Additionally, patterns were similar for residents of both Iowa and North Carolina.

In adjusted linear regression-based stratified analyses for *CDHI*, *MGMT*, and *LINE-1* (Supporting Information

Table 2), we found that among applicators older than the median age (59 years) at blood draw, there were statistically significant associations of HPEE with reduced DNAm for *MGMT* CpG 2 (chr10:131,265,803; $\beta = -3.59$; $P = 0.03$), CpG3 (chr10:131,265,810; $\beta = -2.55$; $P = 0.05$), and for the mean across the three *MGMT* CpGs measured ($\beta = -2.68$; $P = 0.03$). In contrast there were only non-significant, weakly positive estimates for younger applicators. Tests for interaction of age by HPEE were statistically significant for *MGMT* CpG 2 ($P = 0.02$) and the mean across the promoter's three CpGs

TABLE II. Comparison of Mean Levels of %5-mC (DNAm) at Each Locus Measured Between pesticide applicators Ever and Never Reporting an HPEE

DNAm Locus	Never had an HPEE				Ever had an HPEE				P-value
	N	Mean	SD	Median	N	Mean	SD	Median	
CDH1 CpG 1 (chr16:68,770,944)	430	26.57	(9.14)	26.11	136	25.56	(10.78)	25.33	0.33
CDH1 CpG 2 (chr16:68,770,947)	429	25.65	(8.88)	25.38	136	25.07	(14.42)	23.96	0.66
CDH1 CpG 3 (chr16:68,770,960)	430	15.85	(7.43)	14.86	136	15.55	(8.95)	14.06	0.72
CDH1 Mean CpG 1-3	430	22.71	(6.62)	22.48	136	22.06	(9.38)	21.69	0.45
GSTp1 CpG 2 (chr11:67,351,099)	82	8.99	(3.07)	8.02	24	8.62	(3.34)	6.90	0.29 [#]
GSTp1 CpG 6 (chr11:67,351,124)	81	11.24	(7.29)	7.90	23	13.04	(9.38)	9.47	0.29 [#]
GSTp1 CpG 7 (chr11:67,351,134)	70	9.82	(5.04)	7.92	21	11.96	(7.95)	9.74	0.02 ^{#**}
GSTp1 CpG 8 (chr11:67,351,138)	64	10.93	(7.05)	8.19	15	14.67	(9.34)	13.04	0.12 [#]
GSTp1 CpG 9 (chr11:67,351,141)	40	10.32	(7.07)	7.85	8	14.89	(13.20)	9.93	0.09 [#]
GSTp1 CpG 10 (chr11:67,351,145)	91	12.12	(8.84)	8.76	19	18.99	(22.34)	11.95	0.07 [#]
GSTp1 Mean CpG 2,6-10	76	8.90	(3.18)	7.96	13	14.57	(8.76)	11.40	<0.01 ^{#**}
MGMT CpG 1 (chr10:131,265,796)	445	25.15	(8.18)	24.03	139	24.93	(9.15)	24.62	0.79
MGMT CpG 2 (chr10:131,265,803)	444	37.73	(9.32)	37.49	139	37.33	(10.35)	37.50	0.66
MGMT CpG 3 (chr11:131,265,810)	443	16.33	(7.60)	14.87	139	15.33	(7.50)	15.10	0.18
MGMT Mean CpG 1-3	445	26.42	(7.27)	25.60	139	25.86	(7.85)	25.29	0.44
Line 1 Mean (CpG 1-4)	447	78.43	(2.48)	78.29	141	78.42	(2.83)	78.15	0.95

[#]Wilcoxon rank-sum test p-value - for GSTp1 loci only.

^{**}Indicates t-test P-value <0.05.

($P = 0.05$). We also found statistically significant reduced DNAm in *LINE-1* among responders with less than or equal to the median, ≤ 16.56 ng/mL plasma folate levels ($\beta = -0.70$; $P = 0.05$) and no associations among those with higher than the median plasma folate levels; however, the test for interaction p-value was 0.13. In analyses stratified by plasma levels of vitamin B₁₂ we found no significant differences between strata for associations between HPEE and *CDH1*, *MGMT*, and *LINE-1* DNAm.

In adjusted robust regression-based stratified analyses for GSTp1 (Supporting Information Table 2), we found mixed results after stratifying by age, folate, and vitamin B₁₂. In analyses stratified by age, HPEE was significantly associated with increased DNAm at CpG 6 ($\beta = 12.83$; $P < 0.01$) and CpG 9 ($\beta = 2.24$; $P = 0.02$) in younger but not older applicators, with statistically significant p-interactions (0.03, <0.01, respectively). However, in older applicators HPEE was non-significantly associated with increased DNAm for in CpGs 2, 7, 8, and 10, resulting in significantly increased DNAm for the mean across CpGs in GSTp1 ($\beta = 5.65$; $P < 0.01$). Associations were not significant in younger applicators, and the P-value for interaction of age by HPEE was significant ($P < 0.01$). Similar, mixed patterns by individual CpGs were found after stratifications by folate, but HPEE was associated with greater methylation for the mean across CpGs, HPEE was associated with greater DNAm among those with plasma folate ≤ 16.56 ng/mL ($\beta = 13.74$; $P < 0.01$), than among those with plasma folate > 16.56 ($\beta = 2.90$; $P < 0.01$); p-interaction < 0.01. For vitamin B₁₂, effects for individual CpGs were not very strong, but for the mean across CpGs HPEE was

significantly associated with increased DNAm for those with higher than the median plasma level of 585.95 ng/mL ($\beta = 2.85$; $P = 0.02$) but not for those with lower B₁₂; p-interaction was marginally significant ($P = 0.09$).

DISCUSSION

In this study, we found that ever having an HPEE was significantly associated with elevated DNAm for *GSTp1* promoter CpG 7 ($P < 0.01$) and the mean across CpGs measured in the *GSTp1* promoter ($P < 0.01$). *GSTp1* promoter DNAm was elevated particularly among applicants who were older (>59 years at blood draw), and those with lower plasma folate levels (≤ 16.56 ng/mL) (median) plasma folate levels. *MGMT* promoter DNAm was reduced for CpG2 ($P = 0.03$), CpG3 ($P = 0.05$), and the mean across CpGs measured in the *MGMT* promoter ($P = 0.03$) among older applicators only. *LINE-1* DNAm was lower ($P = 0.05$) among applicators who had ≤ 16.56 ng/mL plasma folate levels only.

The genes we focused on in this study are potentially relevant to disease processes associated with exposure to pesticides. *GSTp1* is a member of a family of enzymes involved in DNA protection from electrophilic metabolites of carcinogens and reactive oxygen species (ROS) [Henrique and Jeronimo, 2004]. It is commonly hypermethylated in prostate cancer [Goessl et al., 2002; Jeronimo et al., 2002; Jeronimo et al., 2002; Kang et al., 2004; Bastian et al., 2005; Enokida et al., 2005; Hoque et al., 2005; Papadopoulou et al., 2006; Chuang et al., 2007] and has been reported to be hypermethylated in B-cell

TABLE III. Adjusted* Linear Regression Analyses Evaluating the Association Between an HPEE and %5-mC (DNAm) in *CDHI*, *MGMT*, and *LINE-1*

Gene	CpG	Ever HPEE across 3 phases		
		<i>N</i>	β	<i>P</i> -value
<i>CDHI</i>	CpG 1 (chr16:68,770,944)	535	-0.55	0.57
	CpG 2 (chr16:68,770,947)	534	-0.63	0.56
	CpG 3 (chr16:68,770,960)	535	0.16	0.85
	Mean CpG 1-3	535	-0.36	0.64
<i>MGMT</i>	CpG 1 (chr10:131,265,796)	553	-0.54	0.53
	CpG 2 (chr10:131,265,803)	552	-0.62	0.53
	CpG 3 (chr11:131,265,810)	551	-1.08	0.16
	Mean CpG 1-3	553	-0.78	0.30
<i>LINE-1</i>	mean	476	-0.23	0.39

*Models adjusted for variables as described below:.

CDHI adjusted for age at blood draw (continuous), drinks per month of alcohol, and skin sensitivity to sun.

MGMT adjusted for age at blood draw (continuous), pack years in Phase 1, and personally mixed, loaded, or applied pesticides in Phase 3.

LINE-1 adjusted for age at blood draw (continuous), and still living on a farm in Phase 3.

malignancies [Rossi et al., 2004] and leukemia [Melki et al., 1999]. We found elevated DNAm (hypermethylation) of the *GSTp1* promoter DNAm for pesticide applicators who experienced an HPEE compared with those who never experienced an HPEE across the three phases of the AHS. These elevations were more pronounced for older applicators and those with lower plasma folate levels measured at the same time DNAm was measured. Statistically significant interactions for various loci in the *GSTp1* promoter may indicate effect modification by age and by plasma folate levels. Restricting our analysis to DNAm $\geq 6\%$ for the sake of minimizing measurement error, reduced our sample size, however, the distributions of HPEE and other baseline characteristics, were similar between those in the $<6\%$ range of *GSTp1* DNAm and those with levels $\geq 6\%$.

MGMT is a DNA repair protein that removes mutagenic and cytotoxic adducts from O^6 -guanine in DNA. The *MGMT* protein is widely regarded as a major contributor to the protection of cells against the mutagenic, carcinogenic, and cytotoxic effects of DNA-alkylating agents [Nagasaka et al., 2003]. Our study found that among older applicators (those greater than 59 years) only, CpG sites 2 and 3 and the mean of the three CpGs in the *MGMT* promoter had lower DNAm levels (hypomethylation) for those with an HPEE compared to those without an HPEE. Thus, age is potentially an effect modifier in the association between HPEE and *MGMT* DNAm.

CDHI, a member of the cadherin family of adhesion molecules, acts as a tumor and invasion suppressor, the loss of which has been associated with tumorigenesis and increased metastatic potential. It has been found to be hypermethylated in leukemia [Ekmekci et al., 2004;

TABLE IV. Adjusted* Robust Regression Analyses Evaluating the Association Between an HPEE and %5-mC (DNAm) in *GSTp1*

CpG	Ever HPEE across 3 phases		
	<i>N</i>	Robust Regression Estimate (β)	<i>P</i> -value
<i>GSTP1</i> CpG2	106	-0.83	0.12
<i>GSTP1</i> CpG6	104	0.58	0.40
<i>GSTP1</i> CpG7	91	2.07	<0.01**
<i>GSTP1</i> CpG8	79	1.98	0.06
<i>GSTP1</i> CpG9	48	0.97	0.27
<i>GSTP1</i> CpG10	110	1.35	0.14
<i>GSTp1</i> Mean (CpG 2,6,7,8,9,10)	89	2.68	<0.01**

*Adjusted for age at blood draw (continuous).

**indicates Robust Regression *P*-value <0.05 .

Aggerholm et al., 2006], multiple myeloma [Chim et al., 2004; Seidl et al., 2004], other blood neoplasms [Esteller, 2003], lung cancer [Russo et al., 2005; Gu et al., 2006; Nakata et al., 2006] and prostate cancer [Hoque et al., 2005]. We did not find differences in *CDHI* DNAm levels between applicators with and without an HPEE.

The repetitive element, *LINE-1*, has been found to be hypomethylated in the blood of cancer patients [Barchitta et al., 2014]. *LINE-1* hypomethylation has also been associated with elevated serum levels of some pesticides, namely chlorinated hydrocarbons [Rusiecki et al., 2008; Kim et al., 2010]. In this study, we found that there was an inverse association between HPEE and *LINE-1* DNAm (e.g., reduced DNAm/hypomethylation) among applicators in the low plasma folate group only. Folate deficiency has been associated with chromosomal damage in human lymphocytes *in vitro* and in human lymphocytes and buccal cells *in vivo* [Crott et al., 2001], and low dietary intake of folate can contribute to cancer risk by demethylation of DNA due to uracil misincorporation during DNA synthesis [Herbert, 1986]. Prior studies have shown that low folate levels can affect susceptibility to disease from exposures, such as As, where the odds of As-related skin lesions were higher among individuals with low folate [Pilsner et al., 2007, 2009].

In this study, our focus was on HPEE, which likely represents acute, high pesticide exposure. It is not clear whether any observed effects result directly from the HPEE, or whether HPEE may serve as a surrogate for having work practices or characteristics that might lead to overall higher pesticide exposures over time. Applicators who reported a history of physician-diagnosed pesticide poisoning were excluded from the study population, so HPEE is not a surrogate for poisoning. The prevalence of HPEE across the three phases of the AHS study in the study sub-population was higher (24%) than in the AHS

population which did not participate in this study (15%) [Starks et al., 2012a,b], because of the sampling methodology used to recruit applicators for the study. Since applicators were sampled equally from those in the upper 25% and the lower 75% of the distribution of lifetime days of organophosphate pesticide use, this higher proportion with an HPEE is not unexpected, since HPEE is more prevalent among those with greater lifetime days of pesticide exposure.

Various mechanisms may alter DNA methylation patterns from exposure to pesticides which have been found to result in increased carcinogenic risk, including oxidative stress induction (ROS generation), alkylation of DNA, acetylcholinesterase (AChE) inhibition, endocrine disruption, and disruption of *S*-adenosyl-methionine (SAM) [Krieger, 2001]. Oxidative DNA damage can interfere with the ability of methyl-transferases to interact with DNA [Valinluck et al., 2004], resulting in an overall profile of reduced methylation of cytosines at CpG sites [Turk et al., 1995]. It has been shown that certain pesticides will allow promutagenic alkylation damage to DNA, which in turn can produce increased DNAm [Ray and Richards, 2001]. Organophosphates, which operate via AChE inhibition, have been found to decrease paraoxonase activity in chronically exposed workers [Hernandez et al., 2003]. Exposure to endocrine disruptors may promote an alteration in DNA methylation sequences, and this is associated with the development of trans-generational disease states [Chang et al., 2006]. Pesticide induced alterations in SAM may affect methyl donor availability [Shepherd et al., 2006].

Folate and vitamin B₁₂ play important roles in DNAm because these two cofactors are required for the synthesis of methionine and *S*-adenosyl methionine (SAM), the common methyl donor required for the maintenance of methylation patterns in DNA [Brunaud et al., 2003]. Folate and vitamin B₁₂ did not meet the criteria for model covariates. However, when we stratified by plasma folate levels, those with lower levels had higher *GSTp1* promoter and lower *LINE-1* DNAm than those with higher plasma folate levels.

Cell population heterogeneity in whole blood may have affected the findings in this study. However, DNAm levels have been found to be highly correlated between cell fractions, though there may be some regional differences [Adalsteinsson et al., 2012]. We were unable to account for different cell types in our study and thus were not able to address any potential confounding related to this. We were also unable to include the measurement of gene expression in our study, which would be useful to evaluate in future studies to understand more fully the impact of altered DNAm in people with high pesticide exposures.

Another limitation of this study is that recall may have been better among younger or more highly educated applicators, and this may have influenced the higher

prevalence of HPEE reported in those groups. Also, HPEE may possibly be more common in younger applicators due to riskier work or behaviors at a younger age. Since methylation alterations increase with age, and older applicators reported fewer HPEE in our study population, this could have driven some estimates towards the null, though we did not find potential effect modification by age for *CDH1* and *LINE-1* methylation, which had null estimates in analyses of the total population. Since the AHS applicators are predominantly white males, these results may not be relevant to females or people of other races, though race was not found to be a confounder in this study. Although we evaluated DNAm after HPEEs were reported, this study does not offer insight into the timing and persistence of modifications. A strength of the study is that it included a relatively large sample of pesticide applicators from two states, Iowa and North Carolina, who have a variety of farming practices and crops grown. Therefore, these results are potentially relevant to other farming populations.

Our study longitudinally assessed the association between high pesticide exposures and DNAm. Our exposure metric, HPEE, is a non-specific measure of pesticide exposure, which could reflect exposure to pesticides with different mechanisms of action. Our findings for HPEE, which we consider a surrogate for acute, high pesticide exposure, indicate that pesticide exposure at levels that do not produce acute pesticide poisoning has the potential to influence DNAm in humans in the promoter region of *GSTp1*, and in some subgroups reduced DNAm in the *MGMT* promoter and *LINE-1*. Since hypermethylation of *GSTp1* has been consistently associated with prostate cancer, and farmers have higher rates of prostate cancer than the general population, the associations we found with high pesticide exposures warrant further investigation. A better understanding of the timing and persistence of modifications could be ascertained via a study design that includes serial biomarkers over time.

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AUTHOR CONTRIBUTIONS

Jennifer Rusiecki conceived and executed the study, was the principal investigator, and wrote the manuscript; Jennifer Rusiecki, Andrea Baccarelli, and Matthew Bonner

designed the study and interpreted results; Jane Hoppin provided biologic samples, AHS/neurobehavioral study data, expertise on the exposure assessment, and assisted with interpreting results; Andrea Baccarelli and Hyan-Min Byun designed and carried out the Pyrosequencing assays and assisted with interpreting results; Melannie Alexander and Ligong Chen carried out statistical analyses; Melannie Alexander assisted with drafting the manuscript; Lee Moore provided expertise on DNA methylation and assisted with interpreting results; Laura Beane-Freeman, Gabriella Andreotti, Kathryn Barry, Michael Alavanja, and Freya Kamel provided expertise on the AHS, exposure assessment, and assisted with interpreting results. All co-authors assisted with editing the manuscript.

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Supplementary Table 1. Primer sequence and information

Assay	Name	Sequence	Genomic Location of the CpGs Human Feb. 2009 (GRCh37/hg19) Assembly	Annealing Temp. (°C)
CDH1	CDH1-F	TTTGATTTTAGGTTTTAGTGAGT		55
	CDH1-R (bio)	ACCACAACCAATCAACAA		
	CDH1-sp	GTTTTAGTGAGTTAT		
	Sequencing entry	CGGCGGGGTTGGGATTCGAATTT AGTGGA	chr16:68,770,944-68,770,961	
GSTP1	GSTP1-F	TTTGGGAAAGAGGGAAAGGT		50
	GSTP1-R (bio)	AACCTTATAAAAATAATCCC		
	GSTP1-sp	AGAGGGAAAGGTTTTTT		
	Sequencing entry	CGGTTAGTTGCGCGGCGATTTTCG GGGATTTTAGGGCGTTTTTTTTCG GTCGACGTTCCGGGTGTAG	chr11:67,351,089-67,351,146	
MGMT	MGMT-F	GTTGTAGTGATTGTGGATTGG		53.9
	MGMT-R (bio)	CAACATAAAAAAATAAAAAAACC C		
	MGMT-sp	GTTGTAGTGATTGTGGATTGG		
	Sequencing entry	CGTGTGGCGGGGTCGTGGTAGT TTTT	chr10:131,265,796-131,265,811	

CDH1

CpG #1: chr16:68,770,944

CpG #2: chr16:68,770,947

CpG #3: chr16:68,770,960

GSTP1

CpG #1: chr11:67,351,089

CpG #2: chr11:67,351,099

CpG #3: chr11:67,351,101

CpG #4: chr11:67,351,104

Supplementary Table 1. (continued)

CpG #5: chr11:67,351,110
CpG #6: chr11:67,351,124
CpG #7: chr11:67,351,134
CpG #8: chr11:67,351,138
CpG #9: chr11:67,351,141
CpG #10: chr11:67,351,145

MGMT

CpG #1: chr10:131,265,796
CpG #2: chr10:131,265,803
CpG #3: chr10:131,265,810

Supplemental Table 2. Results of stratified analyses by age at blood draw, plasma folate levels, and Vitamin B12 levels via adjusted1 Linear Regression analyses for *CDH1*, *MGMT*, and *LINE-1* and via adjusted1 Robust Regression analyses for *GSTP1*

Locus	Age at blood draw			Age at blood draw			p-interaction term	
	N	Estimate	p-value	N	Estimate	p-value		
	≤59 years			>59 years				
<i>CDH1</i>								
<i>CDH1</i> CpG 1 (chr16:68,770,944)	269	-0.05	0.97	266	-1.33	0.38	0.53	
<i>CDH1</i> CpG 2 (chr16:68,770,947)	269	0.65	0.68	265	-2.55	0.10	0.15	
<i>CDH1</i> CpG 3 (chr16:68,770,960)	269	0.38	0.72	265	-0.24	0.85	0.67	
<i>CDH1</i> Mean CpG 1-3	269	0.33	0.75	265	-1.41	0.23	0.26	
<i>GSTp1</i> [#]								
<i>GSTp1</i> CpG 2 (chr11:67,351,099)	52	-0.92	0.15	54	0.53	0.64	0.87	
<i>GSTp1</i> CpG 6 (chr11:67,351,124)	52	2.24	0.02	52	-0.85	0.42	0.03	*
<i>GSTp1</i> CpG 7 (chr11:67,351,134)	42	1.28	0.05	49	3.20	0.00	0.08	
<i>GSTp1</i> CpG 8 (chr11:67,351,138)	38	1.23	0.40	41	3.07	0.05	0.32	
<i>GSTp1</i> CpG 9 (chr11:67,351,141)	24	12.83	0.00	24	-0.96	0.64	<0.01	*
<i>GSTp1</i> CpG 10 (chr11:67,351,145)	53	0.60	0.76	57	1.54	0.13	0.82	
<i>GSTp1</i> Mean CpG 2,6-10	46	0.50	0.61	43	5.65	0.00	<0.01	*
<i>MGMT</i>								
<i>MGMT</i> CpG 1 (chr10:131,265,796)	280	0.19	0.87	273	-1.89	0.16	0.25	
<i>MGMT</i> CpG 2 (chr10:131,265,803)	279	1.07	0.38	273	-3.59	0.03	0.02	*
<i>MGMT</i> CpG 3 (chr11:131,265,810)	278	-0.16	0.87	273	-2.55	0.05	0.11	
<i>MGMT</i> Mean CpG 1-3	280	0.33	0.73	273	-2.68	0.03	0.05	*
<i>LINE-1</i>								
<i>LINE-1</i> Mean	280	-0.39	0.26	273	-0.02	0.97	0.55	

	Plasma Folate level (ng/mL)							
	≤16.56 ng/mL			>16.56 ng/mL				
<i>CDH1</i>								
<i>CDH1</i> CpG 1 (chr16:68,770,944)	279	-1.42	0.30	256	0.29	0.84	0.36	
<i>CDH1</i> CpG 2 (chr16:68,770,947)	278	-0.73	0.63	256	-0.63	0.70	0.92	
<i>CDH1</i> CpG 3 (chr16:68,770,960)	278	-0.26	0.83	256	0.75	0.50	0.79	
<i>CDH1</i> Mean CpG 1-3	278	-0.83	0.45	256	0.14	0.90	0.65	
<i>GSTp1</i> [#]								
<i>GSTp1</i> CpG 2 (chr11:67,351,099)	51	-1.87	0.02	55	0.31	0.66	0.05	*
<i>GSTp1</i> CpG 6 (chr11:67,351,124)	50	0.52	0.81	54	0.86	0.21	0.86	
<i>GSTp1</i> CpG 7 (chr11:67,351,134)	44	1.05	0.30	47	2.64	0.00	0.23	
<i>GSTp1</i> CpG 8 (chr11:67,351,138)	39	1.77	0.42	40	2.69	0.06	0.80	
<i>GSTp1</i> CpG 9 (chr11:67,351,141)	23	40.08	0.00	25	1.34	0.08	<0.01	*
<i>GSTp1</i> CpG 10 (chr11:67,351,145)	53	-1.53	0.40	57	2.72	0.00	0.03	*
<i>GSTp1</i> Mean CpG 2,6-10	42	13.74	0.00	47	2.90	0.00	<0.01	*
<i>MGMT</i>								
<i>MGMT</i> CpG 1 (chr10:131,265,796)	285	-0.60	0.63	268	-0.51	0.68	0.90	
<i>MGMT</i> CpG 2 (chr10:131,265,803)	285	0.04	0.98	267	-1.47	0.28	0.50	
<i>MGMT</i> CpG 3 (chr11:131,265,810)	284	-0.72	0.49	267	-1.43	0.21	0.81	
<i>MGMT</i> Mean CpG 1-3	285	-0.49	0.66	268	-1.15	0.28	0.77	
<i>LINE-1</i>								
<i>LINE-1</i> Mean	285	-0.69	0.05	268	0.19	0.66	0.13	

	Plasma B12 level (ng/mL)						
	≤ 585.95 ng/mL			> 585.95 ng/mL			
<i>CDH1</i>							
<i>CDH1</i> CpG 1 (chr16:68,770,944)	276	-1.64	0.23	259	0.23	0.87	0.34
<i>CDH1</i> CpG 2 (chr16:68,770,947)	276	-2.41	0.13	258	0.75	0.62	0.20
<i>CDH1</i> CpG 3 (chr16:68,770,960)	276	-1.13	0.32	258	1.25	0.27	0.12
<i>CDH1</i> Mean CpG 1-3	276	-1.73	0.11	258	0.71	0.52	0.13
<i>GSTp1</i> [#]							
<i>GSTp1</i> CpG 2 (chr11:67,351,099)	59	-1.16	0.17	47	-0.32	0.67	0.54
<i>GSTp1</i> CpG 6 (chr11:67,351,124)	59	0.81	0.40	45	0.43	0.77	0.44
<i>GSTp1</i> CpG 7 (chr11:67,351,134)	43	2.29	0.01	48	2.13	0.00	0.83
<i>GSTp1</i> CpG 8 (chr11:67,351,138)	41	-0.85	0.14	38	3.37	0.06	0.06
<i>GSTp1</i> CpG 9 (chr11:67,351,141)	25	1.41	0.23	23	0.06	0.97	0.01
<i>GSTp1</i> CpG 10 (chr11:67,351,145)	59	0.73	0.67	51	1.88	0.07	0.56
<i>GSTp1</i> Mean CpG 2,6-10	48	-0.02	0.98	41	2.85	0.02	0.09
<i>MGMT</i>							
<i>MGMT</i> CpG 1 (chr10:131,265,796)	281	-1.07	0.39	272	-0.03	0.98	0.54
<i>MGMT</i> CpG 2 (chr10:131,265,803)	280	-1.07	0.44	272	-0.10	0.94	0.63
<i>MGMT</i> CpG 3 (chr11:131,265,810)	280	-1.98	0.06	271	-0.44	0.70	0.32
<i>MGMT</i> Mean CpG 1-3	281	-1.38	0.20	272	-0.25	0.82	0.45
<i>LINE-1</i>							
<i>LINE-1</i> Mean	281	-0.66	0.10	272	0.10	0.78	0.15

*

Based on Robust Regression

* p-value for interaction ≤0.05

¹ Adjusted for same covariates as in Linear Regressions and Robust Regressions for the total population