RESEARCH ARTICLE

Pesticide use and LINE-1 methylation among male private pesticide applicators in the Agricultural Health Study

Melannie Alexander1, Stella Koutros2, Matthew R. Bonner3, Kathryn Hughes Barry2,4,5, Michael C.R. Alavanja2, Gabriella Andreotti2, Hyang-Min Byun6, Ligong Chen1, Laura E. Beane Freeman2, Jonathan N. Hofmann2, Freya Kamel7, Lee E. Moore2, Andrea Baccarelli8 and Jennifer Rusiecki1,*

1Department of Preventive Medicine and Biostatistics, Uniformed Services University of the Health Sciences, 4301 Jones Bridge Road, Bethesda, MD, USA, 2Division of Cancer Epidemiology and Genetics, National Cancer Institute, National Institutes of Health, Bethesda, MD, USA, 3Department of Epidemiology and Environmental Health, State University of New York, Buffalo, NY, USA, 4Department of Epidemiology and Public Health, University of Maryland School of Medicine, Baltimore, MD, USA, 5Program in Oncology, University of Maryland Marlene and Stewart Greenebaum Comprehensive Cancer Center, Baltimore, MD, USA, 6Institute of Cellular Medicine, Newcastle University, Newcastle, UK, 7Epidemiology Branch, National Institute of Environmental Health Sciences, Research Triangle Park, NC, USA and 8Department of Environmental Health Sciences, Mailman School of Public Health, Columbia University, New York, NY, USA

*Correspondence address. Uniformed Services University of the Health Sciences, 4301 Jones Bridge Road, Bethesda, MD 20814, USA.
Tel: 301-295-3712; Fax: 301-295-1933; E-mail: jennifer.rusiecki@usuhs.edu

Abstract

Cancer risk may be associated with DNA methylation (DNAm) levels in Long Interspersed Nucleotide Element 1 (LINE-1), a surrogate for global DNAm. Exposure to certain pesticides may increase risk of particular cancers, perhaps mediated in part through global DNAm alterations. To date, human data on pesticide exposure and global DNAm alterations are limited. The goal of this study was to evaluate alterations of LINE-1 DNAm by pesticides in a variety of classes. Data from 596 cancer-free male participants enrolled in the Agricultural Health Study (AHS) were used to examine associations between use of 57 pesticides and LINE-1 DNAm measured via Pyrosequencing in peripheral blood leucocytes. Participants provided information on pesticide use at three contacts between 1993 and 2010. Associations of ever/never pesticide use and lifetime days of application (years of use × days per year) and LINE-1 DNAm level were assessed using linear regression, adjusting for potential confounders (race, age at blood draw, and frequency of drinking alcohol) and other moderately correlated pesticides. After adjustment, ever application of 10 pesticides was positively associated and ever application of eight pesticides was negatively associated with LINE-1 DNAm. In dose–response analyses, increases in five pesticides (imazethapyr,
fenthion, EPTC, butylate, and heptachlor) were associated with increasing LINE-1 DNA methylation (p_trend < 0.05) and increases in three pesticides (carbaryl, chlordane, and paraquat) were associated with decreasing LINE-1 DNA methylation (p_trend < 0.05). This study provides some mechanistic insight into the pesticide–cancer relationship, which may be mediated in part by epigenetics.

Key words: pesticides; LINE-1; epigenetics; DNA methylation; Agricultural Health Study

Introduction

Epigenetic mechanisms have been implicated in aberrant biological processes, particularly carcinogenesis, due to their influence on phenotype. This influence is often exerted by modulating gene expression. Global DNA methylation (DNAm) is a commonly studied epigenetic mechanism, with lower levels of global DNAm often associated with chromosomal instability and increased mutation rates [1, 2]. Previous studies have found correlations between global DNAm and DNAm levels in repetitive elements such as Long Interspersed Nucleotide Element 1 (LINE-1); half a million such repetitive elements reside in the genome [3]. In addition, studies have found associations between reduced levels of LINE-1 DNAm and risk of various malignancies [4]. Taken together, these results suggest that LINE-1 DNAm may be used as a surrogate marker of global methylation in order to investigate the relationship of the latter to cancer, using either cancerous tissue or accessible tissue such as peripheral blood leukocytes (PBLs) [4–8].

Changes in global levels of DNAm may mediate associations between cancer and toxicant exposures, lifestyle factors, and other natural processes [9]. Although pesticide exposures may be related to cancer, the underlying biological mechanisms linking specific pesticides and certain cancers are not well understood. Genotoxicity has been suggested to mediate the association of pesticides and cancer risk, but other non-genotoxic mechanisms (e.g. endocrine disruption, oxidative stress) may also play a role [10–12]. Furthermore, prior studies have suggested that pesticides may influence carcinogenesis via epigenetics [13, 14]. However, little information has been published on alterations in DNAm associated with pesticide exposure in humans, as most prior studies have been conducted in animal models or human cell lines [13, 15, 16]. The few human studies of alterations of LINE-1 DNAm conducted to date have focused mainly on persistent organic pollutants (POPs), including organochlorine (OC) insecticides, and arsenic [17–21], but not to our knowledge on other pesticide classes.

Using information on a sub-group of pesticide applicators from a large cohort study, the Agricultural Health Study (AHS), we recently reported differences in DNAm patterns associated with self-reported high pesticide exposure events (HPEE). Decreases in LINE-1 DNAm were associated with HPEE among applicators with low plasma folate (<16.56 ng/ml) [22]. These results indicate that epigenetic changes, such as alterations in DNAm, may be an alternative to genotoxicity as a mechanism leading to cancer. Considering that application of specific pesticides and/or pesticide classes is associated with increased risk for certain cancers (e.g. leukemia, multiple myeloma, non-Hodgkin lymphoma [NHL], prostate, bladder) in the AHS [23–27], the relationship between application of specific pesticides and LINE-1 DNAm levels warrants investigation.

Results

Characteristics that were included in the final models are presented in Table 1. Most participants were 50 years of age or older (81.0%) and white (97.9%). In the year prior to Phase 1, one-third reported never drinking alcohol, one-third drank up to once a week, and one-third drank more than once a week. The average length of time between the last follow-up and blood collection was 1.2 years (standard deviation, SD = ±0.5, Table 1). The average LINE-1 DNAm level for the study sample was 78.4% (SD = ±2.6, Table 1), with minimum and maximum values of 66.1 and 93.5%, respectively (data not shown).

After adjusting for race, frequency of drinking in the 12 months prior to Phase 1, age at blood draw, and use of correlated pesticides (when applicable), ever application of 10 pesticides was significantly associated with increases in LINE-1 DNAm: atrazine (β = 0.85, P < 0.01), dicamba (β = 0.59, P = 0.01), imazethapyr (β = 0.63, P = 0.01), terbufos (β = 0.56, P = 0.01), fenthion (β = 1.14, P = 0.02), heptachlor (β = 0.58, P = 0.03), butylate (β = 0.45, P = 0.04), S-ethyl-N,N-dipropylthiocarbamate (EPTC; β = 0.63, P = 0.05), dichlorvos (DDVP; β = 0.56, P = 0.05), and metolachlor (β = 0.42, P = 0.05 [Table 2]). Among these pesticides, six were herbicides from five chemical classes (atrazine [chlorotriazine], dicamba [benzoic acid], imazethapyr [imidazolinone], butylate and EPTC [both thiocarbamates], and metolachlor [chloroacetanilide]); three were organophosphate (OP) insecticides (terbufos [aliphatic organophosphate], fenthion [phenyl organophosphate], and DDVP [organophosphate]), and one was an OC [specifically cyclodiene] insecticide, (heptachlor). Those who ever applied fenthion exhibited the strongest positive association for LINE-1 DNAm (β = 1.14, P = 0.02). This can be interpreted as applicators who ever applied fenthion had, on average, 1.14% higher 5-mC than those who never applied fenthion.

Table 1. Mean LINE-1 DNAm by selected characteristics of the cancer-free study population

| Characteristics                  | N   | %   |
|----------------------------------|-----|-----|
| **Age at blood draw**            |     |     |
| 30–39                            | 23  | 3.9 |
| 40–49                            | 90  | 15.1|
| 50–59                            | 206 | 34.6|
| 60–69                            | 156 | 26.2|
| ≥70                              | 121 | 20.2|
| **Race**                         |     |     |
| White                            | 569 | 97.9|
| Black                            | 8   | 1.4 |
| Other                            | 4   | 0.7 |
| **Alcohol intake in the 12 months prior to enrollment** | | |
| Never                            | 180 | 31.6|
| Up to once a week                | 200 | 35.2|
| More than one time a week        | 189 | 33.2|

| **Time between last follow-up and blood collection (years)** | Mean | SD |
|-------------------------------------------------------------|------|----|
|                                                             | 1.2  | 0.5|
| **Mean %5-mC LINE-1 Methylation**                           | 78.4 | 2.6|

SD, standard deviation.
### Table 2. Adjusted associations between select specific pesticide exposure (ever/never) and LINE-1 methylation in the AHS

| Functional class | Chemical class | Pesticide | \( N_{\text{ever}}/N_{\text{never}} \) | \( \beta \) (SE) | P-value |
|------------------|----------------|-----------|----------------------------------|----------------|---------|
| **Positive associations** | | | | | |
| Herbicides | Chlorotriazine herbicides | Atrazine* | 448/113 | 0.85 (0.27) | <0.01 |
| Herbicides | Benzoic acid herbicides | Dicamba | 283/278 | 0.59 (0.22) | 0.01 |
| Herbicides | Imidazolinone herbicides | Imazethapyr | 172/389 | 0.63 (0.24) | 0.01 |
| Insecticides | Aliphatic organothiophosphate insecticides | Terbufos | 280/281 | 0.56 (0.22) | 0.01 |
| Insecticides | Phenyl organonitrophenate insecticides | Fenthion | 28/533 | 1.14 (0.50) | 0.02 |
| Insecticides | Cycloidiene insecticides | Heptachlorb | 118/443 | 0.58 (0.27) | 0.03 |
| Herbicides | Thiocarbamate herbicides | Butylate | 225/336 | 0.45 (0.22) | 0.04 |
| Herbicides | Thiocarbanate herbicides | EPTC | 76/485 | 0.63 (0.32) | 0.05 |
| Insecticides | Organophosphate insecticides | DDVP | 467/94 | 0.56 (0.29) | 0.05 |
| Herbicides | Chloroacetanilide herbicides | Metolachlor | 307/254 | 0.42 (0.22) | 0.05 |
| Herbicides | Chlorotriazine herbicides | Cyazinane | 254/307 | 0.40 (0.22) | 0.07 |
| Herbicides | Phenoxyacetic acid herbicides | 2,4-D | 473/88 | 0.52 (0.30) | 0.08 |
| Insecticides | Pyrimidine organonitrophenate insecticides | Tebuquinimines | 58/503 | 0.54 (0.36) | 0.13 |
| Herbicides | Triazineone herbicides | Metribuzin | 241/320 | 0.31 (0.22) | 0.15 |
| Insecticides | Isoxindole organonitrophenate insecticides | Phosmet | 84/477 | 0.43 (0.32) | 0.17 |
| Insecticides | Aliphatic organonitrophenate insecticides | Phorate | 182/379 | 0.32 (0.23) | 0.17 |
| Insecticides | Phenyl ethylphosphonothioate insecticides | Fonofob | 159/402 | 0.31 (0.24) | 0.20 |
| Herbicides | Dinitroaniline herbicides | Trifuralin | 245/316 | 0.28 (0.22) | 0.21 |
| Insecticides | Pyrethroid ester insecticides | Permethrin (Animals) | 122/439 | 0.32 (0.27) | 0.22 |
| Insecticides | Heterocyclic organonitrophenate insecticides | Coumaphos | 61/500 | 0.40 (0.35) | 0.25 |
| Insecticides | Benzofuranyl methylcarbamate insecticides | Carbofuran | 233/328 | 0.26 (0.22) | 0.26 |
| Herbicides | Cycloidiene insecticides | Aldrinb | 147/414 | 0.26 (0.26) | 0.33 |
| Insecticides | Aliphatic amide organonitrophenate insecticides | Dimethoate | 47/514 | 0.35 (0.39) | 0.38 |
| Herbicides | Chloroacetanilide herbicides | Alachorb | 390/171 | 0.08 (0.24) | 0.75 |
| Fungicides | Phthalimide fungicides | Captan | 204/457 | 0.09 (0.28) | 0.76 |
| Insecticides | Aliphatic organonitrophenate insecticides | Malathion | 430/111 | 0.08 (0.32) | 0.77 |
| Herbicides | Pyrimidylsulfonylurea herbicides | Chlorimuron-ethyl | 232/329 | 0.06 (0.22) | 0.78 |
| Insecticides | OC insecticides | Lindaneb | 185/376 | 0.04 (0.23) | 0.85 |
| Insecticides | Pyridine organonitrophenate insecticides | Diazinon | 372/189 | 0.00 (0.23) | 0.99 |
| Insecticides | Cycloidiene insecticides | Dieldrinb | 55/506 | 0.00 (0.37) | 0.99 |
| **Inverse associations** | | | | | |
| Fumigants | Halogenated hydrocarbon fumigants | Methyl bromide*,b | 149/412 | −0.82 (0.29) | <0.01 |
| Insecticides | Carbamate insecticides | Carbaryl | 424/137 | −0.80 (0.25) | <0.01 |
| Herbicides | Cycloidiene insecticides | Chlorandeb | 213/348 | −0.64 (0.24) | 0.01 |
| Insecticides | Phenyl organonitrophenate insecticides | Methyl parathion | 154/407 | −0.52 (0.24) | 0.03 |
| Fumigants | Halogenated hydrocarbon fumigants | Ethylene dibromideb | 70/491 | −0.68 (0.33) | 0.04 |
| Herbicides | Quaternary ammonium herbicides | Paraquat | 202/359 | −0.42 (0.23) | 0.05 |
| Herbicides | Phenoxypropionic acid herbicides | Silveb | 87/474 | −0.59 (0.30) | 0.05 |
| Herbicides | Phenoxyacetic acid herbicides | 2,4,5-Tb | 188/373 | −0.46 (0.24) | 0.05 |
| Fungicides | Acylamino acid fungicides | Metalaxylb | 192/369 | −0.47 (0.26) | 0.07 |
| Insecticides | Phosphoramicthioate insecticides | Acephatea | 135/426 | −0.56 (0.31) | 0.07 |
| Insecticides | Aliphatic organonitrophenate insecticides | Ethopropa | 89/472 | −0.60 (0.34) | 0.07 |
| Insecticides | Oxime carbamate insecticides | Aldicarbaz | 135/426 | −0.45 (0.25) | 0.08 |
| Insecticides | OC insecticides | DDTb | 210/351 | −0.42 (0.25) | 0.10 |
| Fungicides | Polymeric dithiocarbamate fungicides | Maneb | 96/465 | −0.45 (0.29) | 0.12 |
| Insecticides | OC insecticides | Toxaphene | 165/396 | −0.38 (0.25) | 0.12 |
| Insecticides | Pyrethroid ester insecticides | Permethrin (Crops) | 121/440 | −0.39 (0.24) | 0.14 |
| Insecticides | Aliphatic organonitrophenate insecticides | Disulfotonb | 85/476 | −0.52 (0.36) | 0.15 |
| Fungicides | Aromatic fungicides | Chlorothalonilb | 102/459 | −0.42 (0.29) | 0.15 |
| Insecticides | Phenyl organonitrophenate insecticides | Parathion | 90/471 | −0.40 (0.30) | 0.18 |
| Herbicides | Organophosphate herbicides | Glyphosate | 52/40 | −0.52 (0.42) | 0.21 |
| Fumigants | Fumigant insecticides | Carbon Tetrachloride/Carbon disulfide | 60/501 | −0.34 (0.35) | 0.33 |
| Herbicides | Aromatic herbicides | Petroleum distillates | 331/230 | −0.14 (0.22) | 0.53 |
| Fungicides | Benzimidazole fungicides | Benomylb | 136/425 | −0.18 (0.28) | 0.53 |
| Herbicides | Dinitroaniline herbicides | Pendimethalin | 303/258 | −0.10 (0.22) | 0.66 |
| Herbicides | Pyridine organonitrophenate insecticides | Chlorpyrifos | 323/238 | −0.07 (0.22) | 0.75 |
| Insecticides | Phosphate | Aluminum phosphate | 43/518 | −0.06 (0.41) | 0.89 |
| Insecticides | Organophosphate insecticides | Tetrachlorvinphos | 49/512 | −0.05 (0.39) | 0.89 |

Selected pesticides have at least 20 people exposed.
Models adjusted for: age at blood draw, race, and alcohol frequency per week in the year prior to Phase 1.
*Further adjusted for correlated pesticides.
*Pesticide was banned, no longer used on or phased out prior to end of the last follow-up period. Source: https://iaspub.epa.gov/apex/pesticides/f?p=--chemicalsearch:1.
SE, Standard Error; EPTC, S-ethyl-N,N-dipropylthiocarbamate; DDVP, Dichlorvos; 2,4-D, 2,4-D dichlorophenoxyacetic acid; 2,4,5-T, 2,4,5-Trichlorophenoxyacetic acid; DDT, Dichlorodiphenyltrichloroethane.
*Bolded P-values indicate \( P \leq 0.05 \).
Adjusting for the same covariates, ever application of eight pesticides was significantly associated with decreases in LINE-1 DNAm: methyl bromide ($\beta = -0.82, P < 0.01$), carbaryl ($\beta = -0.80, P < 0.01$), chlordane ($\beta = -0.64, P = 0.01$), methyl parathion ($\beta = -0.52, P = 0.03$), ethylene dibromide ($\beta = -0.68, P = 0.04$), paraquat ($\beta = -0.45, P = 0.05$), silvex ($\beta = -0.59, P = 0.05$), and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T; $\beta = -0.46, P = 0.05$). Two of these pesticides (methyl bromide and ethylene dibromide) were fumigants from one chemical class [halogenated hydrocarbon], three were herbicides from three unique chemical classes (paraquat [quaternary ammonium], silvex [phenoxyproprionic acid], 2,4,5-T [phenoxyacetic acid]), and three were insecticides from three unique chemical classes (carbaryl [carbamate], chlordane [OC /cyclodiene], methyl parathion [phenyl organothiophosphate]). Those who ever applied methyl bromide exhibited the strongest negative association for LINE-1 DNAm ($\beta = -0.80, P < 0.01$); that is, applicators who ever applied methyl bromide had, on average, 0.80% lower 5-mc than those who never applied this particular pesticide. Since there was moderate correlation (e.g. 0.43–0.67) among the four CpG sites evaluated, we checked whether patterns of association differed by CpG site. We found that estimates for each of the four CpGs were generally in the same direction as those found for the mean of the four CpGs, though results were statistically significant mainly for CpG 1 findings (results not shown).

Linear regression analyses of the categorized lifetime days of specific pesticide use (none, low [$\leq$ median], or high [>$\text{median}$]) generally reflected the results of the ever/never analyses. Table 3 presents results for pesticides for which we found a significant dose-response relationship with LINE-1 DNAm. Additional pesticides that did not demonstrate a dose-response relationship with LINE-1 DNAm are presented in Supplementary Table S1. Significant positive trends were observed for increasing lifetime days of application of imazethapyr ($P_{\text{trend}} = 0.01$), heptachlor ($P_{\text{trend}} = 0.03$), fenthion ($P_{\text{trend}} = 0.04$), butylate ($P_{\text{trend}} = 0.04$), and EPTC ($P_{\text{trend}} = 0.05$). Applicators in the highest category (>$\text{median}$) of lifetime days for these pesticides had statistically significant increases in DNAm, with the exception of fenthion and EPTC. The largest increase in LINE-1 DNAm was for the estimate for the highest lifetime days category of heptachlor application ($\beta = 1.00, P = 0.03$), meaning that individuals who were in the highest lifetime days category for heptachlor exhibited, on average 1.00% higher 5-mc than those who never applied this pesticide.

Statistically significant inverse trends were detected for carbaryl ($P_{\text{trend}} < 0.01$), paraquat ($P_{\text{trend}} = 0.01$), and chlordane ($P_{\text{trend}} = 0.02$). Applicators with the highest levels of lifetime days of application of carbaryl ($P < 0.01$), paraquat ($P = 0.01$), and chlordane ($P = 0.04$) had lower LINE-1 DNAm levels compared with those who never used each of these pesticides, respectively. Applicators in the highest lifetime days group for paraquat experienced the strongest negative association with LINE-1 DNAm levels ($\beta = -0.88, P = 0.01$); that is, individuals who were in the highest lifetime days category for paraquat exhibited, on average 88% lower 5-mc than those who never applied this pesticide.

Analyses stratified by median plasma folate levels were conducted (data not shown). The test for interaction of plasma

### Table 3. adjusted associations between specific pesticide exposure (categories of lifetime days) with a significant dose–response relationship and LINE-1 methylation in the AHS

| Functional class | Chemical class | Pesticide | N | $\beta$ (SE) | P-value | $P_{\text{trend}}$ |
|------------------|----------------|-----------|---|-------------|---------|-----------------|
| **Positive associations** | | | | | |
| Herbicides | Imidazolinone herbicides | Imazethapyr | None | 389 | Ref | – |
| | | | Low | 90 | 0.47 (0.30) | 0.11 |
| | | | High | 82 | 0.81 (0.32) | 0.01 | 0.01 |
| Insecticides | Cyclodiene insecticides | Heptachlor$^*$ | None | 492 | Ref | – |
| | | | Low | 35 | 0.40 (0.45) | 0.37 |
| | | | High | 34 | 1.00 (0.46) | 0.03 | 0.03 |
| Insecticides | Phenyl organothiophosphate insecticides | Fenthion | None | 533 | Ref | – |
| | | | Low | 16 | 0.97 (0.65) | 0.14 |
| | | | High | 12 | 1.38 (0.75) | 0.07 | 0.04 |
| Herbicides | Thio carbamate herbicides | Butylate | None | 398 | Ref | – |
| | | | Low | 104 | 0.35 (0.28) | 0.21 |
| | | | High | 59 | 0.75 (0.36) | 0.04 | 0.04 |
| Herbicides | Thio carbamate herbicides | EPTC | None | 490 | Ref | – |
| | | | Low | 36 | 0.60 (0.44) | 0.17 |
| | | | High | 35 | 0.83 (0.45) | 0.06 | 0.05 |
| **Inverse associations** | | | | | |
| Insecticides | Carbamate insecticides | Carbaryl | None | 247 | Ref | – |
| | | | Low | 172 | –0.08 (0.25) | 0.76 |
| | | | High | 142 | –0.85 (0.28) | $<0.01$ | $<0.01$ |
| Herbicides | Quaternary ammonium herbicides | Paraquat | None | 422 | Ref | – |
| | | | Low | 74 | –0.48 (0.32) | 0.14 |
| | | | High | 65 | –0.88 (0.34) | 0.01 | 0.01 |
| Insecticides | Cyclodiene insecticides | Chlordane$^*$ | None | 423 | Ref | – |
| | | | Low | 88 | –0.52 (0.32) | 0.09 |
| | | | High | 50 | –0.80 (0.39) | 0.04 | 0.02 |

Pesticides were considered for inclusion into the table if they were found to be significant in the ever/never analyses and the P-test for trend was $\leq 0.05$.

Deldrin was not evaluated for lifetime days of application due to small cell counts ($\leq 20$ non-zero values).

Models adjusted for: age at blood draw, race, and alcohol frequency per week in the year prior to Phase 1.

$^*$Bolded P-values indicate $P \leq 0.05$.

SE, Standard Error; EPTC, S-ethyl-N,N-dipropylthiophosphoramide.

$^*$Pesticide was banned, no longer used on or phased out prior to end of the last follow-up period. Source: https://iaspub.epa.gov/apex/pesticides/f?p=chemicalSearch=1.
folic acid level by ever/never application of diazinon was significant \((P = 0.04)\). Among applicators with low folic acid levels, applicators who ever applied diazinon had significantly increased LINE-1 DNAm compared with those who had never applied this pesticide \((\beta = -0.60, P = 0.05)\). In the high folic acid group, increased LINE-1 DNAm was observed among those who ever applied diazinon compared with those who never applied this pesticide; however, this finding was not statistically significant \((\beta = 0.50, P = 0.15)\). No other significant interactions were detected for ever/never application or categories of lifetime days of use.

For the most recent exposures, few significant associations were found (Supplementary Table S2). Applicators who recently applied metolachlor had higher LINE-1 DNAm compared with those who never applied this pesticide \((\beta = 0.74, P = 0.03)\) after adjustment for age at blood draw, race, alcohol frequency per week in the year prior to Phase 1, and past metolachlor use in Phases 1 and 2. Two additional pesticides had positive associations with LINE-1 DNAm in the Phase 3 lifetime days analyses: we observed increased LINE-1 DNAm among those in the low exposure categories of malathion (an aliphatic organothiophosphate insecticide) and 2,4-D (a phenoxyacetic acid herbicide) \((\beta = 0.79, P = 0.04 \text{ and } \beta = 0.76, P < 0.01, \text{ respectively})\). No positive associations were found for those in the high exposure category of specific pesticides in Phase 3. Two pesticides exhibited a dose-response relationship with LINE-1 DNAm. First, increasing number of lifetime days of applying dicamba was positively associated with LINE-1 DNAm \((P_{\text{trend}} = 0.02)\); however, estimates for neither the low nor high lifetime days exposure categories achieved statistical significance (low lifetime days: \(\beta = 0.41, P = 0.31\); high lifetime days: \(\beta = 0.77, P = 0.09\)). Second, increasing number of lifetime days of lifetime days of paraquat was negatively related to LINE-1 DNAm \((P_{\text{trend}} = 0.01)\). Again, estimates for both low and high lifetime day exposure categories for paraquat application were not statistically significant (low lifetime days: \(\beta = -0.68, P = 0.34\); high lifetime days: \(\beta = -1.20, P = 0.09\)).

**Discussion**

In this investigation of 57 pesticides across several pesticide classes, we found that ever application of 10 pesticides was positively associated and ever application of 8 pesticides was negatively associated with LINE-1 DNAm levels after adjusting for potential confounders in a large sample of cancer-free, male pesticide applicators. In general, results from analyses of lifetime days application supported findings from the analyses of ever/never use of the same pesticide. As has been frequently done in prior studies [18, 20–22], our study used the mean across four CpG sites in LINE-1 as the measure of LINE-1 DNAm. As has been reported previously, this measure was reflective of the estimates found if we evaluated each of the four CpGs separately, i.e. the estimates were in the same direction, though statistical significance was reached only for CpG 1.

Currently, there are limited data on changes in DNAm associated with pesticide exposure, with the greater part of studies in the literature being based in animals or human cell lines and focusing on a few specific pesticides [13, 15, 16]. Though a few epidemiological studies have examined changes in LINE-1 DNAm after exposure to specific pesticides [17–21, 28], these studies have only focused on several OCs and arsenical pesticides. In contrast, this study evaluated associations of specific pesticides with LINE-1 DNAm. Pesticides for which a dose-response relationship with LINE-1 DNAm was observed in this study have been linked with various cancers in the AHS. For example, increased application of imazethapyr has been previously linked to bladder cancer [24], a cancer with a strong epigenetic etiology [29]. In additional, several pesticides exhibiting a dose-response relationship with LINE-1 DNAm in this study have been linked to other cancers in the AHS (e.g. cancers of the pancreas, colon, prostate, bladder, rectum, and skin, and various lymphohematopoietic cancers) [23, 24, 30, 31].

The effects of exposures to pesticides on LINE-1 DNAm have focused mainly on arsenicals and OC pesticides [17–20, 28]. Though this study does not focus on arsenical pesticides, a review by Bailey and Fry suggest that long-term exposure to arsenicals gives rise to a number of adverse health conditions, including cancers of the skin, lung, liver, bladder, and prostate. The authors suggest that the relationship between arsenicals and cancer is likely partially mediated through epigenetic alterations, where changes in global DNAm have been observed in experimental studies where cell lines were malignantly transformed after exposure to inorganic arsenic [28]. Rusiecki et al. found inverse associations between POPs and DNAm in another repetitive element, Alu, in plasma from a population of Greenlandic Inuit [18]; estimates were in the same direction for LINE-1, but they were not statistically significant. A study in a healthy Korean adult population observed significant inverse trends for metabolites of three OCs: oxychlordane, p,p’-DDE, and trans-nonachlor and LINE-1 DNAm [20]. Two other studies found similar associations between several OC pesticides and global hypomethylation; however, measurement of global DNAm was done via a Luminometric Methylation Assay, which is an estimation of global methylation, and not necessarily LINE-1, making results difficult to compare with the previous studies [17, 19]. Although this study identified relationships between certain OC pesticides (chlordane and heptachlor) and changes in LINE-1 DNAm, the direction of the association was not consistent within chemical class because exposure to chlor dane and heptachlor appear to decrease and increase DNAm, respectively. We previously reported that HPEEs were associated with DNAm patterns in the same population presented here [22]. Though no overall relationship between HPEE and LINE-1 DNAm was detected in the sample of applicators evaluated, an HPEE is a non-specific pesticide exposure, warranting this study which examines the effects of specific pesticide application. The lack of consistency in findings between the HPEE study and this study may be due to the non-specific nature of HPEE.

Inconsistencies between our findings for OC pesticides and those from previous studies that focused on OC pesticides may be largely due to different exposure assessment methods (e.g. levels of OCs in serum, self-reported exposures to specific pesticides), and differences in the underlying study population distribution with regard to age, gender, and race [17–21]. The participants of this study were primarily older, non-Hispanic white males; therefore, results from this study may not be entirely generalizable to other populations.

It is unclear how pesticide exposures could lead to changes in DNAm and potentially increased cancer risk, but several mechanisms have been proposed [32]. First, it is likely that several of these pesticides behave as endocrine disrupters, synthetic chemicals that mimic the actions of natural hormones due to structural similarity [33]. A review found that more than 100 pesticides are endocrine disrupters [33]. Five of the 18 pesticides associated with LINE-1 DNAm changes in this study (positive associations: atrazine, heptachlor, and metolachlor; negative changes: carbaryl and chlordane) are classified as endocrine disrupters. Prior studies have observed epigenetic changes after exposure to environmental endocrine disruptors.
and folate were also considered as potential confounders due to their relationships with DNA methylation. The role of these confounders in the relationship between pesticide exposure and cancer via epigenetic changes will be needed, as epigenetic alterations could potentially serve as biomarkers for cancer risk. Additionally, the presence of arsenic, SAM is depleted to facilitate arsenic excretion which may lead to inhibition of methyltransferases, enzymes which are responsible for the transfer of methyl groups to DNA. As a result of methyltransferase inhibition, changes in global DNA methylation may occur.

Regardless of the mechanism, it should be noted that exposures to some pesticides may induce changes in DNA methylation that may persist for long periods of time. Some pesticides that were associated with changes in DNA methylation in this study have been banned (e.g., 2,4,5-T and chlordane) or have not been used (e.g., methyl bromide) for many years. Because of the non-genotoxic/non-mutagenic designations of several of the studied pesticides, future studies aimed at elucidating the relationship between pesticide exposure and cancer via epigenetic changes will be needed, as epigenetic alterations could potentially serve as biomarkers for cancer risk.

This study has several strengths, including a large sample of cancer-free individuals and well-characterized pesticide exposures. In addition, lifelong and recent use of specific pesticides was assessed prior to blood draw for DNA methylation quantification. Furthermore, potential confounding by other pesticides was examined and adjusted for appropriately. Plasma levels of both vitamin B12 and folate were also considered as potential confounders due to their role in maintenance of the one-carbon metabolism pathway, availability of the SAM, and cancer risk, but they did not appreciably change effect estimates of specific pesticides.

Despite the strengths of this study, some limitations must be addressed. First, a large majority of the study sample was white; thus, results may not be generalizable to non-whites. Conversely, due to the homogeneity of the sample, findings were likely not impacted by race. Second, women were excluded from the study due to low counts (<1% of the AHS cohort); therefore, results may not be representative of epigenetic disturbances that occur in women as a result of pesticide exposure.

Third, measures of DNA methylation were derived from PBLs, and cellular heterogeneity could be producing spurious associations; however, a prior study found no difference in LINE-1 DNA methylation between cell types. Furthermore, PBLs are an accessible source of DNA, and DNA methylation levels measured in blood have been found to be associated with cancer risk, and a large number of specific pesticides were evaluated for their associations with LINE-1 DNA methylation changes, and it is possible that some findings were due to chance; however, the majority of these pesticides presenting a relationship with alterations in LINE-1 DNA methylation changes have also presented associations with risk of various cancers in prior studies conducted among applicators in the AHS. However, results generated from the present hypothesis-generating study may guide future studies. Finally, LINE-1 DNA methylation was measured only once, making it difficult to gain a clear understanding of whether or not these changes in methylation persist over time. Future longitudinal studies will be needed to replicate findings, while overcoming limitations of this study.

Conclusion
To date, this is the first population-based study to observe LINE-1 DNA methylation changes in relation to exposure to several pesticides across numerous chemical classes. Considering the non-genotoxic/non-mutagenic designations of some of these pesticides, this study provides some mechanistic insight into the pesticide–cancer relationship, which may be mediated in part by epigenetics.

Material and methods
Study Population
The AHS is a large prospective study of licensed applicators and their spouses enrolled in Iowa and North Carolina between 1993 and 1997 as previously described. In brief, 52 394 private applicators enrolled in the study by completing a self-administered enrollment questionnaire at the time of pesticide licensing and recertification. In addition, a take-home questionnaire was completed within 1 month of enrollment (Phase 1), along with two follow-up phone interviews, which collected data in 1999–2003 and 2005–2010 (Phases 2 and 3, respectively). All questionnaires obtained information on demographics, lifestyle characteristics, pesticide use, personal protective equipment (PPE) use, other farm exposures, and other activities or characteristics that may confound relationships between pesticide exposure and cancer.

A sample of male private applicators who had participated in the enrollment and two follow-up questionnaires and who were recruited for a study on neurobehavioral outcomes was used for this study. Women were not included as they represent a very small proportion of AHS applicators (<1%) of total study participants. A total of 598 out of 701 (85.3%) applicators enrolled into the neurobehavioral outcomes study were cancer-free and provided a whole blood sample. Two participant DNA samples failed laboratory analysis; therefore, the final sample size for this study was 596. The study was approved by the Institutional Review Boards of the National Institutes of Health, University of Iowa, Harvard University, and the Uniformed Services University.

Pesticide Exposure Assessment
Pesticide exposure assessment for the AHS has been described in detail previously. Ever exposure to specific pesticides was evaluated via questionnaire, by asking if a pesticide applicator had ever personally mixed or applied a given pesticide. Lifetime days exposure was then calculated based on the product of years applying it. We utilized data on exposures from Phases 1, 2, and 3 of the AHS to calculate an overall ever/never metric and a lifetime days exposure metric. Lifetime days of specific pesticide use was categorized into three levels: never exposed, low (<median lifetime days), and high (>median lifetime days).

DNA Methylation Assay
One microgram of genomic DNA was used for bisulfite conversion using the EZ DNA Methylation-Gold Kit (Zymo Research,
Plasma Folate and Vitamin B\textsubscript{12}

Since DNAm may be influenced by some micronutrients involved in the 1-carbon metabolism pathway, plasma levels of folate and vitamin B\textsubscript{12} were measured in the same whole blood sample as DNA extraction. Plasma folate levels (ng/ml) were measured using an assay approved by the Food and Drug Administration for clinical use \[53\]. Plasma vitamin B\textsubscript{12} (pg/ml) was measured by a quantitative sandwich enzyme immunoassay technique on the 2010 Elecsys Immunoanalyzer (Roche Diagnostics) \[54\]. Assays of plasma folate and B\textsubscript{12} measurements were carried out at the Clinical & Epidemiologic Research Laboratory, which is part of Laboratory Medicine, Children’s Hospital in Boston.

Statistical Analysis

Methylation levels of the four adjacent CpG sites measured were averaged to yield a mean % LINE-1 DNAm. The coefficient of variation, based on blinded duplicates, was 3%. Linear regression was used to examine the association of LINE-1 DNAm with pesticide exposure for both ever versus never use and for lifetime days of individual pesticides (categorized into three levels). For each pesticide evaluated, we did not use that pesticide as the reference. We evaluated only pesticides used by at least 20 applicators.

We evaluated a common set of covariates, potentially associated with LINE-1 DNAm, using unadjusted linear regression. Covariates associated with LINE-1 DNAm based on a P-value of <0.10 were subsequently selected for inclusion into an initial full multiple linear regression base model. Age at blood draw was forced into the base model, based on a priori understanding of its influence on DNAm levels. Using a backwards regression, we then removed covariates from the initial full base model if they had a P-value of >0.20. Based on our backwards regression models, the following variables were included in final models: age at blood draw (30–39, 40–49, 50–59, 60–69, and 70 years of age or more), the number of drinks per week in the 12 months prior to enrollment (none, one to three drinks per week, and four or more drinks per week), and race (white, black, and other). Addition of folate and vitamin B\textsubscript{12} plasma levels to models did not appreciably change estimates for any pesticide exposure (<20%), so they were not included in final models. Because pesticide applicators may apply multiple pesticides, we also considered potential confounding by other pesticides when evaluating the relationship between use of a specific pesticide and LINE-1 DNAm. Spearman correlations (\(r\)) were calculated between pairs of pesticides for lifetime days of use. For a specific pesticide being evaluated, all other pesticides modestly correlated with it (correlation coefficient (\(r\)) of \(>0.50\) and P-value \(<0.10\)) were considered for inclusion into the final model, along with the other confounders - age at blood draw, number of drinks per week, and race. Parameter estimates of models with adjustment for other pesticides were compared with models not including the other pesticides. If estimates for the specific pesticide being evaluated using the former model changed by \(>20\%), then the model estimates that included the confounding pesticides were presented. Supplementary Table 3 lists the individual pesticides of interest which were adjusted for other pesticides and lists those corresponding other pesticides that were included into final models. To test for trend, median values for the lifetime days categories defined a continuous variable.

Because we found differences in the association between LINE-1 DNAm and HPEEs among individuals with high versus low plasma folate levels \[22\], we conducted stratified analyses by the median cut-off of plasma folate (16.56 ng/ml) to examine potential effect modification.

To examine associations of more recent pesticide exposure with LINE-1 DNAm, analyses were limited to include participants’ pesticide exposures closer to the time of blood collection, based on data from the last follow-up interview in this study (Phase 3), with additional adjustment for historical pesticide exposures occurring before this time.

All analyses were performed using SAS software, version 9.3 (SAS Institute, Cary, NC, USA). Associations were considered statistically significant at P \(<0.05\).

Disclaimer: The content of this publication is the sole responsibility of the authors and does not necessarily reflect the views or policies of the Uniformed Services University of the Health Sciences (USUHS), the Department of Defense (DoD), or the Departments of the Army, Navy, or Air Force. Mention of trade names, commercial products, or organizations does not imply endorsement by the U.S. Government.

Supplementary data

Supplementary data are available at EnvEpig online.

Acknowledgements

We thank Drs. Fred Gerr and Sarah Starks for the collection of biological samples, Dr. Jane Hoppin for providing biological samples/data and Mr. Stuart Long for the preparation of and detailed assistance with AHS data. The P1RE1071201, P2RE1071202, and P3RE10901 releases of the AHS dataset were used.

Funding

This study was supported through funding from the following grants: National Institutes of Health R21 CA131934, and,
Conflict of interest statement. None declared.

References

1. Feinberg Ap, Tycko B. The history of cancer epigenetics. Nat Rev Cancer 2004;4:143–53.
2. Chen RZ, Pettersson U, Beard C, Jackson-Grusby L, Jaenisch R. DNA hypomethylation leads to elevated mutation rates. Nature 1998;395:89–93.
3. Kazazian Hh, Jr, Goodier JL. LINE drive. retrotransposition and genome instability. Cell 2002;110:277–80.
4. Barchitta M, Quattrocchi A, Maugeri A, Vinciguerra M, Agodi A. LINE-1 hypomethylation in blood and tissue samples as an epigenetic marker for cancer risk: a systematic review and meta-analysis. PLoS One 2014;9: e109478.
5. Brennan K, Flanagan JM. Is there a link between genome-wide hypomethylation in blood and cancer risk?. Cancer Prev Res 2012;5:1345–57.
6. Andreotti G, Karami S, Pfeifer RM, Hurwitz L, Liao LM, Weinstein SJ, Albanes D, Virtamo J, Silverman DT, Rothman N, et al. LINE1 methylation levels associated with increased bladder cancer risk in pre-diagnostic blood DNA among US (PLCO) and European (ATBC) cohort study participants. Epigenetics 2014;9:404–15.
7. Karami S, Andreotti G, Liao LM, Pfeifer RM, Weinstein SJ, Purdue MP, Hofmann JN, Albanes D, Mannisto S, Moore LE. LINE1 methylation levels in pre-diagnostic leukocyte DNA and future renal cell carcinoma risk. Epigenetics 2015;10:282–92.
8. van Bemmelen D, Lenz P, Liao LM, Baris D, Sternberg LR, Warner A, Johnson A, Jones M, Kida M, Schwenn M. Correlation of LINE-1 methylation levels in patient-matched buffy coat, serum, buccal cell, and bladder tumor tissue DNA samples. Cancer Epidemiol Biomarkers Prev 2012;21:1143–8.
9. Fraga MF, Ballestar E, Paz MF, Ropero S, Setien F, Ballestar ML, Heine-Sueter D, Cigudosa JC, Urioste M, Benitez J, et al. Epigenetic differences arise during the lifetime of monozygotic twins. Proc Natl Acad Sci U S A 2005;102:10604–9.
10. Alavanja MC, Ross MK, Bonner MR. Increased cancer burden among pesticide applicators and others due to pesticide exposure. CA Cancer J Clin 2013;63:120–42.
11. De Coster S, van Laerebeke N. Endocrine-disrupting chemicals: associated disorders and mechanisms of action. J Environ Public Health 2012;2012:713696.
12. Sesti F, Tsitsilonis OE, Kotsinas A, Trougakos IP. Oxidative stress-mediated biomolecular damage and inflammation in tumorigenesis. In Vivo 2012;26:395–402.
13. Zhang X, Wallace AD, Du P, Kibbe WA, Jafari N, Xie H, Lin S, Baccarelli A, Soares MB, Hou L. DNA methylation alterations in response to pesticide exposure in vitro. Environ Mol Mutagen 2012;53:542–9.
14. Anway MD, Skinner MK. Epigenetic transgenerational actions of endocrine disruptors. Endocrinology 2006;147: S43–9.
15. Tao L, Yang S, Xie M, Kramer PM, Pereira MA. Effect of trichloroethylene and its metabolites, dichloroacetic acid and trichloroacetic acid, on the methylation and expression of c-Jun and c-Myc protooncogenes in mouse liver: prevention by methionine. Toxicol Sci 2000;54:399–407.
16. Zhang X, Wallace AD, Du P, Lin S, Baccarelli AA, Jiang H, Jafari N, Zheng Y, Xie H, Soares MB, et al. Genome-wide study of DNA methylation alterations in response to diazinon exposure in vitro. Environ Toxicol Pharmacol 2012;34:959–68.
17. Itoh H, Iwasaki M, Kasuga Y, Yokoyama S, Onuma H, Nishimura H, Kusama R, Yoshida T, Yokoyama K, Tsgane S. Association between serum organochlorines and global methylation level of leukocyte DNA among Japanese women: a cross-sectional study. Sci Total Environ 2014;490:603–9.
18. Rusiecki JA, Baccarelli A, Bollati V, Tarantini L, Moore LE, Bonefeld-Jorgensen EC. Global DNA hypomethylation is associated with high serum-persistent organic pollutants in Greenlandic Inuit. Environ Health Perspect 2008;116:1547–52.
19. Lind L, Penell J, Luttropp K, Nordfors L, Svanen AC, Axelsson T, Salihovic S, van Bavel B, Fall T, Ingelsson E. Global DNA hypermethylation is associated with high serum levels of persistent organic pollutants in an elderly population. Environ Int 2013;59:456–61.
20. Kim Ky, Kim DS, Lee SK, Lee IK, Kang JH, Chang YS, Jacobs DR, Steffes M, Lee DH. Association of low-dose exposure to persistent organic pollutants with global DNA hypomethylation in healthy Koreans. Environ Health Perspect 2010;118:370–4.
21. Huen K, Yousefi P, Bradman A, Yan L, Harley KG, Kogut K, Ekenazi B, Holland N. Effects of age, sex, and persistent organic pollutants on DNA methylation in children. Environ Mol Mutagen 2014;55:209–22.
22. Rusiecki JA, Beane Freeman LE, Bonner MR, Alexander M, Chen L, Andreotti G, Barry KH, Moore LE, Byun HM, Kamel P, et al. High pesticide exposure events and DNA methylation among pesticide applicators in the agricultural health study. Environ Mol Mutagen 2017;58:19–29.
23. Weichenthal S, Mosea C, Chan P. A review of pesticide exposure and cancer incidence in the Agricultural Health Study cohort. Environ Health Perspect 2010;118:1117–25.
24. Koutros S, Silverman DT, Alavanja MC, Andreotti G, Lerro CC, Heltshue S, Lynch CF, Sandler DP, Blair A, Beane Freeman LE, et al. Occupational exposure to pesticides and bladder cancer risk. Int J Epidemiol 2016;45:792–805.
25. Alavanja MC, Hofmann JN, Lynch CF, Hines CJ, Barry KH, Barker J, Buckman DW, Thomas K, Sandler DP, Hoppin JA, et al. Non-hodgkin lymphoma risk and insecticide, fungicide and fungimutant in the agricultural health study. PLoS One 2014;9:e109332.
26. Koutros S, Beane Freeman LE, Lubin JH, Heltshue SL, Andreotti G, Barry KH, DellaValle CT, Hoppin JA, Sandler DP, Lynch CF, et al. Risk of total and aggressive prostate cancer and pesticide use in the Agricultural Health Study. Am J Epidemiol 2013;177:59–74.
27. Bonner MR, Williams BA, Rusiecki JA, Blair A, Beane Freeman LE, Hoppin JA, Dosemeci M, Lubin J, Sandler DP, Alavanja MC. Occupational exposure to terbufos and the incidence of cancer in the Agricultural Health Study. Cancer Causes Control 2010;21:871–7.
28. Bailey KA, Fry RC. Arsenic-associated changes to the epigenome: what are the functional consequences? Curr Environ Health Rep 2014;1:22–34.
29. Hoffman AM, Cairns P. Epigenetics of kidney cancer and blader cancer. Epigenomics 2011;3:19–34.
30. Lench SM, Mahajan R, Beane Freeman LE, Hopkin JA, Alavanja MC. Cancer incidence among pesticide applicators exposed to butylate in the Agricultural Health Study (AHS). Environ Res 2009;109:860–8.
31. Purdue MP, Hoppin JA, Blair A, Dosemeci M, Alavanja MC. Occupational exposure to organochlorine insecticides and cancer incidence in the Agricultural Health Study. Int J Cancer 2007;120: 642–9.

32. Collotta M, Bertazzi PA, Bollati V. Epigenetics and pesticides. Toxicology 2013;307: 35–41.

33. Mnif W, Hassine AI, Bouaziz A, Bartegi A, Thomas O, Roig B. Effect of endocrine disruptor pesticides: a review. Int J Environ Res Public Health 2011;8: 2265–303.

34. Ogut S, Gultekin F, Kisioglu AN, Kucukoner E. Oxidative stress. Int J Environ Res Public Health 2011;8: 2265–303.

35. Ranjbar A, Pasalar P, Abdollahi M. Induction of oxidative stress and acetylcholinesterase inhibition in organophosphorous pesticide manufacturing workers. Hum Exp Toxicol 2002;21: 179–82.

36. Valinluck V, Tsai HH, Rogstad DK, Burdzy A, Bird A, Sowers LC. Oxidative damage to methyl-CpG sequences inhibits the binding of the methyl-CpG binding domain (MBD) of methyl-CpG binding protein 2 (MeCP2). Nucleic Acids Res 2004;32: 4100–8.

37. Shepherd KR, Lee ES, Schmued L, Jiao Y, Ali SF, Oriaku ET, Lamargo NS, Soliman KN, Charlton CG. The potentiating effects of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) on paraquat-induced neurochemical and behavioral changes in mice. Pharmacol Biochem Behav 2006;82: 349–59.

38. Reichard JF, Puga A. Effects of arsenic exposure on DNA methylation and epigenetic gene regulation. Epigenomics 2010;2: 87–104.

39. Hoppin JA, Yucel F, Dosemeci M, Sandler DP. Accuracy of self-reported pesticide use duration information from licensed pesticide applicators in the Agricultural Health Study. J Expo Anal Environ Epidemiol 2002;12: 313–8.

40. Blair A, Tarone R, Sandler D, Lynch C, Rowland A, Wintersteen W, Steen W, Dosemeci M, Alavanja M. Reliability of reporting on lifestyle and agricultural factors by a sample of participants in the agricultural health study from Iowa. Ann Epidemiol 2000;10: 478.

41. Coppede F. Epigenetic biomarkers of colorectal cancer: focus on DNA methylation. Cancer Lett 2014;342: 238–47.

42. El-Maarri O, Becker T, Junen J, Manzoor SS, Diaz-Lacava A, Schwaab R, Wienker T, Oldenburg J. Gender specific differences in levels of DNA methylation at selected loci from human total blood: a tendency toward higher methylation levels in males. Hum Genet 2007;122: 505–14.

43. Tajuddin SM, Amaral AF, Fernández AF, Chanock S, Silverman DT, Tardón A, Carrato A, García-Closas M, Jackson BP, Tornán EG, Márquez M, et al. LINE-1 methylation in leukocyte DNA, interaction with phosphatidylethanolamine N-methyltransferase variants and bladder cancer risk. Br J Cancer 2014;110: 2123–30.

44. Woo HD, Kim J. Global DNA hypomethylation in peripheral blood leukocytes as a biomarker for cancer risk: a meta-analysis. PLoS One 2012;7: e34615.

45. Rakitsky VN, Koblyakov VA, Turusov VS. Nongenotoxic (epigenetic) carcinogens: pesticides as an example. A critical review. Teratog Carcinog Mutagen 2000;20: 229–40.

46. Starks SE, Gerr F, Kamef M, Lynch CF, Alavanja MC, Sandler DP, Hoppin JA. High pesticide exposure events and central nervous system function among pesticide applicators in the Agricultural Health Study. Int Arch Occup Environ Health 2012;85: 505–15.

47. Alavanja MC, Sandler DP, McMaster SB, Zahm SH, McDonnell CJ, Lynch CF, Pennybacker M, Rothman N, Dosemeci M, Bond AE, et al. The Agricultural Health Study. Environ Health Perspect 1996;104: 362–9.

48. Alavanja MC, Samanic C, Dosemeci M, Lubin J, Tarone R, Lynch CF, Knott C, Thomas K, Hoppin JA, Barker J. Use of agricultural pesticides and prostate cancer risk in the Agricultural Health Study cohort. Am J Epidemiol 2003;157: 800–14.

49. Dosemeci M, Alavanja MC, Rowland AS, Mage D, Zahm SH, Rothman N, Lubin JH, Hoppin JA, Sandler DP, Blair A. A quantitative approach for estimating exposure to pesticides in the Agricultural Health Study. Ann Occup Hyg 2002;46: 245–60.

50. Tabish AM, Baccarelli AA, Godderis L, Barrow TM, Hoet P, Byun HM. Assessment of changes in global DNA methylation levels by pyrosequencing(R) of repetitive elements. Methods Mol Biol 2015;1315: 201–7.

51. Yang AS, Doshi KD, Choi SW, Mason JR, Mannari RK, Gharybian V, Luna R, Rashid A, Shen L, Estecio MR, et al. DNA methylation changes after 5-aza-2’-deoxycytidine therapy in patients with leukemia. Cancer Res 2006;66: 5495–503.

52. Yang AS, Estécio MR, Doshi K, Kondo Y, Tajara EH, Issa JP. A simple method for estimating global DNA methylation using bisulfite PCR of repetitive DNA elements. Nucleic Acids Res 2004;32: e38.

53. Rothenberg SP, DaCosta M, Rosenberg Z. A radioassay for serum folate: use of a two-phase sequential-incubation, ligand-binding system. N Engl J Med 1972;286: 1335–9.

54. Rothenberg SP. Assay of serum vitamin B12 concentration using Co57-B12 and intrinsic factor. Proc Soc Exp Biol Med 1961;108:45–8.