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14. ABSTRACT

The molecular mechanisms involved in PTSD are not well characterized; epigenetic factors could offer new insights. Gene expression studies show differences in cytokine expression signatures between PTSD patients and controls. DNA methylation is intrinsically linked to gene expression. We investigated DNA methylation patterns in repetitive DNA elements and in cytokine promoter regions in soldiers prior to OIF/OEF deployment; serum-derived DNA was used. PTSD cases with existing serum samples housed at the Department of Defense Serum Repository were identified via ICD-9 codes; an appropriate control group was identified. For each PTSD case and control, a pre- and post-deployment serum sample was obtained. DNA was extracted and methylation quantified via pyrosequencing in the following promoter regions: IGF2, EDG1, IL-8α, IL-8RA, IL-16, IL-18, p11 and in repetitive elements (LINE-1 and Alu). We made statistical comparisons via t-tests and logistic regressions for patterns of DNA methylation between cases and controls and between pre- and post-deployments of each group. The results of our study indicate that global DNA methylation as measured via Alu is a biomarker of stress incurred during deployment and potentially a marker of greater susceptibility to PTSD. The results for LINE-1 are not as clear. There were no clear patterns of cytokine promoter region hyper- or hypo-methylation. Pre-deployment, people who became PTSD cases had reduced IL-18 methylation compared to controls. Controls post-deployment had lower IGF-2 levels than they did pre-deployment. Since we used serum DNA as the biomarker and not a specific tissue derived DNA (such as brain tissue), our findings could be associated with more of a response in inflammatory tissues, but not specifically in the brain. These findings should be followed up in a larger study.

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Introduction

The underlying molecular mechanisms of PTSD are not known. Epigenetic factors - inherited and acquired modifications of DNA and histones that regulate various genomic functions occurring without a change in nuclear DNA sequence - could offer new insights about PTSD. Profiling during the triggering and development of PTSD using cDNA microarrays has shown differential gene expression signatures in cytokines between PTSD patients and controls. An epigenetic mechanism, DNA methylation may play a significant role in the pathophysiology of PTSD, since the process is intrinsically linked to the regulation of gene expression. We carried out a systematic investigation of DNA methylation patterns in the promoter regions of a group of cytokines and in genomic repetitive elements, in soldiers prior to Operation Iraqi Freedom (OIF) or Operation Enduring Freedom (OEF) deployment/PTSD diagnosis, and post deployment/PTSD diagnosis, and an appropriate group of controls. We carried out this study in DNA extracted from serum samples housed at the Department of Defense Serum Repository (DoDSR). Understanding the differential roles of promoter regional methylation and repetitive element methylation in PTSD will fuel novel therapeutic approaches to PTSD therapy, particularly since modifications in DNA methylation can potentially be reversed. The results of this small molecular epidemiology study will lay the foundation for future epigenetic studies based on the longitudinally collected serum samples (multiple samples per service member) housed at the DoDSR, a vast resource of bio-specimens which can be linked to detailed demographic, deployment, and medical data.

Body

Task 1: Identification of PTSD cases/controls, selection and aliquotting of serum samples

All cases and controls were selected from among the active duty Army and Marines with at least two years of continuous active duty prior to their first OIF/OEF deployment. All subjects carried out their first OIF/OEF deployment between January 01, 2004 and December 31, 2006 and were deployed for a period of 6 – 18 months. Prior to this first OIF/OEF deployment, there was an absence of any mental health diagnosis via ICD-9 codes 290-320, dating back to at least two years prior to deployment. Post-deployment exclusion criteria for both cases and controls was ever having an ICD-9 diagnosis (either inpatient or outpatient) for any of the following mental health diagnoses: schizophrenia (ICD9 code 295), bi-polar disorders (ICD9 code 296), manic phase bipolar disorder (also ICD9 code 296).

Post traumatic stress disorder (PTSD) cases (n=75) with existing serum samples housed at the DoDSR were identified by AFHSC. Cases met all the criteria above and had at least two outpatient records with a primary diagnosis of chronic PTSD, based ICD-9 Code 309.81 in the first diagnostic position. The first outpatient diagnosis was required to be between four and six months after the service member's first deployment. The second outpatient diagnosis was required to be after the first diagnosis but within two years post deployment return. Additional criteria for inclusion as a case to this study was having one serum sample drawn within 12 months prior to first OIF/OEF deployment and one sample drawn within six months after return from first OIF/OEF deployment. We randomly selected from among all cases meeting these

criteria and within the ages of 20 and 35 years on their first day of first OIF/OEF deployment and of black or white race.

We identified an appropriate control group (n=75), who were randomly selected from among those active duty Army and Marines service members who met the same deployment, age, and serum sample criteria as cases, but for whom there was never a diagnosis of PTSD (ICD9 Code=309.81) or TBI ICD9 Codes=800.0-801.9, 803.0-804.9, or 850.0-854.1). As with the cases, controls were required to have one serum sample drawn within 12 months prior to first OIF/OEF deployment and one serum sample drawn within six months post deployment return. Controls were frequency matched to cases based on age group (20-26, 27-35 years), gender, and race (white, black).

For each PTSD case and each control, AFHSC identified a pre-deployment serum sample and a post-deployment serum sample (total $n_{samples} = 300$). PI obtained serum samples directly from AFHSC.

Task 2: DNA extraction from serum samples

The AFHSC permits the utilization of up to 0.5 mL of serum per sample, so DNA was extracted from 0.5mL serum. Genomic DNA was extracted in the PI's lab at USU from serum using charge switch gDNA 0.2ml-1ml serum kit from (Invitrogen Carlsbad, CA 92008). For each sample 500 ul of serum samples were used. The genomic DNA was quantitated by quant-iT ds HS assay kit using a Qubit fluorometer (Invitrogen, Carlsbad CA).

Task 3: Quantification of DNA methylation

The DNA was sent from USU to Epigendx, Inc., where DNA methylation was quantified via bisulfite treatment, PCR, and pyrosequencing. Specifically, percent of 5-methyl cytosine (%5-mC) was measured in repetitive elements, LINE-1 and Alu, IGF-2, EDG1mp, IL-8, IL-8RA, IL-16, IL-18, and p11. For 10% of the samples, we had duplicate aliquots, for the purpose of carrying out quality controls on the sample. Based on the results for the duplicates, we calculated coefficients of variation (CV).

For LINE-1 our overall CV was 5.46% and for Alu it was 7.03%, both very good CVs. Four samples failed the LINE-1 analysis and one sample failed the Alu analysis due to low yield of serum DNA resulting in no or low Pyrosequencing signals. For the promoter region specific assays, the CVs were as such: IGF-2 (6%), EDG1 (28%), IL-8 (29%), IL-8RA (not measured), IL-16 (27%), IL-18 (18%), p11 (4%).

Task 4: Analysis of data

<u>Repetitive Elements (LINE-1 and Alu)</u>

Using t-tests and logistical regressions, we carried out four major comparisons: 1. cases post- versus cases pre-deployment, 2. controls post- versus controls pre-deployment, 3. cases pre-

deployment versus controls pre-deployment, and 4. cases post-deployment versus controls post-deployment. Odds ratios (ORs) and 95% CIs were calculated.

Simple t-tests were used to compare %5-mC levels for LINE-1 and Alu between cases post-deployment and controls post-deployment, while paired t-tests were used to compare %5-mC for cases pre- and post- deployment and for controls pre- and post-deployment.

- Simple t-tests revealed:
    - Pre-deployment LINE-1: no difference in LINE-1 %5-mC between cases and controls
    - Post-deployment LINE-1: no significant difference in LINE-1 %5-mC (p=0.07) for the total population between cases (mean=77.92; sd=1.68) and controls (mean=78.46; sd=1.73), but significantly lower levels for cases vs. controls in the subgroups of service members of younger age, male gender, and white race.
    - Pre-deployment Alu: significantly higher in Alu %5-mC (p=0.01) for cases (mean=28.15; sd=1.45) compared with controls (mean=27.51; sd=1.26).
    - Post-deployment Alu: no difference (p=0.09) between cases (mean=28.36; sd=2.08) and controls (mean=27.72; sd=1.69).
- Paired t-tests revealed:
    - Cases post- vs. pre-deployment: Although both LINE-1 and Alu %5-mC were higher in cases post-deployment (i.e., synonymous with post-diagnosis for this study) than in the same cases pre-deployment, differences were not statistically significant.
    - Controls post- vs. pre-deployment LINE-1: %5-mC was significantly increased for controls post-deployment (mean=78.47; sd=1.74), compared with pre-deployment (mean=77.66; sd=1.81), p=0.01. Statistically significant increased levels of LINE-1 post-deployment among service members of younger age, male gender, and white race, compared with pre-deployment levels, and these differences were statistically significant.
    - Controls post- vs. pre-deployment Alu: No differences between cases and controls

Unconditional logistic regressions were employed to investigate the associations between DNA methylation level and PTSD. Percent 5-mC (%5-mC) was included in models as a continuous variable (the resulting ORs represent the change in estimate per unit (1%) change in DNA methylation); we also carried out the same logistic regressions with %5-mC treated as a categorical variable with three levels. We carried out unconditional logistic regressions for case versus control comparisons, i.e., cases post-deployment vs. controls post-deployment and cases pre-deployment vs. controls post-deployment. Conditional logistic regressions were carried out for all paired case-case and control-control comparisons, i.e., cases post-deployment vs. cases pre-deployment and controls post-deployment vs. controls pre-deployment. All models were adjusted for age (continuous), gender, and race (black, white).

- Unconditional logistic regressions revealed that:
    - For LINE-1, pre-deployment comparisons of cases and controls were relatively null, however post-deployment, a negative association was found for LINE-1 for the total population (OR=0.82; 95%CI, 0.67-1.01) and for younger age (OR=0.71;

95%CI, 0.51-0.98), males (OR=0.71; 95%CI, 0.54-0.93), and whites (OR=0.75; 95%CI, 0.59-0.95). When we further limited the post-deployment analysis to young, white, males, we found a more pronounced statistically significant negative association for LINE-1 (OR=0.63; 95%CI, 0.43-0.94) .

- For Alu, pre-deployment comparisons of cases and controls showed a positive association for the total population (OR=1.46; 95%CI, 1.08-1.97) and for older age (OR=1.62; 95%CI, 1.02-2.57), females (OR=1.97; 95%CI, 1.02-3.79), and black race (OR=2.93; 95%CI, 1.01-8.53). Post-deployment, the elevated ORs for Alu %5-mC persisted between cases and controls in the total population (OR=1.24; 95%CI, 0.96-1.60), however, estimates for the total population and subgroups were not statistically significant. Including %5mC into the models as a categorical variable yielded more pronounced associations (negative for LINE-1 and positive for Alu) in the third tertile vs. first tertile comparisons than in the second vs. first tertile comparisons.

- Conditional logistic regressions revealed that:
  - For LINE-1 no statistically significant associations were found for the case-case comparisons.
  - For Alu ORs were slightly elevated for the total population (OR=1.26; 95%CI, 0.90-1.78) and for various sub-groups, none of the estimates was statistically significant. Controls post-deployment had statistically significant elevated ORs when compared to controls pre-deployment for the total population (OR=1.33; 95%CI, 1.06-1.65), and younger age (OR=2.45; 95%CI, 1.31-4.57), males (OR=1.38; 95%CI, 1.04-1.83), and white race (OR=1.42; 95%CI, 1.10-1.84).
  - We further limited the analysis to young, white, males and found a more pronounced statistically significant elevated OR for LINE-1 (OR=2.05; 95%CI, 1.11-3.82) (data not shown).
  - There were also elevated ORs if time between deployment end and post-deployment serum draw was $\leq$ 7 days (OR=1.47; 95%CI, 1.01-2.14) and if time between pre-deployment serum draw and deployment start was $\leq$ 90 days (OR=1.71; 95%CI, 1.13-2.58).
  - Comparisons of Alu for controls post- versus pre-deployment were relatively null.
  - There were no striking differences for any of the comparisons with respect to length of deployment.
  - Including %5-mC as a categorical variable in the models yielded similar results (data not shown).

<u>Promoter region methylation (IGF-2, EDG1mp, IL-8, IL-8RA, IL-16, IL-18, and p11</u>

We carried out similar analyses for this set of genes as we did for the repetitive elements, i.e., simple and paired t-tests to make case-control and pre-post comparisons, respectively. In addition to carrying out unconditional and unconditional logistic regressions to evaluate the association between methylation and case-control status and pre-post status, respectively, we also used a generalized liner model to evaluate the adjusted means for cases and controls (both pre- and post- deployment) and for pre and post (both case-case and control-control

comparisons). Finally, we also calculated change in methylation (%5-mC) for each case and each control from the pre-deployment sample and the post-deployment sample and carried out an unconditional logistic regression to compare them.

- Simple t-tests revealed:
    - Statistically significant reduced IL-18 %5-mC levels ($p<0.01$) between cases ($u=11.72$, s.d.=4.33) and controls ($u=15.47$, s.d.=8.21), pre-deployment
    - No significant differences in any of the promoter regions between cases and controls, post-deployment

- Paired t-tests revealed:
    - Statistically significant reduced IL8RA %5-mC levels ($p=0.02$) for cases post-deployment ($u=90.58$, s.d.=7.30) compared with pre-deployment ($u=93.94$, s.d.=3.89).
    - Statistically significant reduced IGF2 %5-mC levels ($p<0.01$) for controls post-deployment ($u=54.31$, s.d.=4.16) compared with pre-deployment ($u=56.85$, s.d.=4.78)

- Unconditional logistic regressions revealed:
    - Statistically significant reduced odds ratio (OR) for IL-18 for cases versus controls (OR=0.90; 95%CI, 0.83-0.97). This indicates that for each 1% increase in methylation of the IL-18 promoter region, there is a 10% reduced risk in being a PTSD case.
- Conditional logistic regressions revealed:
    - Statistically significant reduced OR for IGF-2 for post-deployment controls versus the same pre-deployment controls (OR=0.87; 95%CI, 0.78-0.97)

- Generalized Linear Models revealed:
    - Pre-deployment, case/control status adjusted for age, gender, and race, modeled as a predictor for IL-18 %5mC (continuous, dependent variable) resulted in significantly different adjusted means for cases and controls ($p<0.01$)
    - Pre/post status adjusted for age, gender, and race, modeled as a predictor for …..?

Key Research accomplishments

<u>Repetitive Elements (LINE-1 and Alu) methylation</u>

- LINE-1 was hypermethylated in controls post- versus pre-deployment (OR=1.33; 95%CI, 1.06-1.65); cases showed no change post-deployment; LINE-1 was hypomethylated in cases versus controls post-deployment (OR=0.82; 95%CI, 0.67-1.01).
- Alu was hypermethylated for cases versus controls pre-deployment (OR=1.46; 95%CI, 1.08-1.97).
- Patterns of hypermethylation of LINE-1 in controls post-deployment and of Alu in cases post deployment are intriguing and may be suggestive of resilience or vulnerability factors. These findings are preliminary and should be investigated in larger studies.

Promoter region methylation (IGF-2, EDG1mp, IL-8, IL-8RA, IL-16, IL-18, and p11)

- Pre-deployment, cases had lower %5-mC IL-18 levels than controls. This was also the case after adjusting for age, gender, and race.
- Post-deployment, there were no significant differences in methylation between cases and controls with respect to any of the promoter regions we measured
- Cases post-deployment had lower levels of IL-8RA than they did pre-deployment, though once we adjusted for age, gender, and race, this effect was diminished, and the difference was not statistically significant.
- Controls post-deployment had lower levels of IGF-2 than they did pre-deployment, and this difference persisted after adjustment for age, gender, and race.

Reportable outcomes

- We currently have no published or in-press manuscripts from this project. We have the repetitive elements manuscript submitted for review at a peer reviewed journal, Epigenomics.

- We attended the Military Health Research Forum Aug 31- Sep 03, 2009, where we presented both a poster:

P30-3 PTSD and DNA Met hylati on in Serum of OIF and OEF Service Members
Jennifer A. Rusiecki,1 Vasantha Srikantan,1 Andrea Baccarelli,2 Fei Zhang,1 and Stephan C. Messer3
1Uniformed Services University of the Health Sciences, 2Milan University, and 3Walter Reed Army Medical Center

and an oral presentation in a symposium:

PTSD AND DNA METHYLATION IN SERUM OF OIF AND OEF SERVICE MEMBERS (P30-3)
Jennifer A. Rusiecki, *Uniformed Services University of the Health Sciences*

Conclusions

Patterns of hypermethylation of LINE-1 in controls post-deployment and of Alu in cases post deployment are intriguing and may be suggestive of resilience or vulnerability factors. These findings are preliminary and should be investigated in larger studies. DNA methylation as measured using the Alu repetitive element may be a biomarker of stress incurred during deployment and potentially a marker of greater susceptibility to PTSD. The results for LINE-1 repetitive element are not as clear.

Patterns of methylation in cytokines were not what we expected. We expected to see hypermethylation in cases versus controls (post-deployment) and hypermethylation in cases post-deployment versus themselves pre-deployment. In the cytokines promoter region analysis the major findings were for IL-18 and IGF-2. People who eventually became PTSD cases in this study (ie, pre-deployment cases) had reduced IL-18 methylation compared to controls pre-deployment. Reduced IL-18 methylation may be an indicator of susceptibility. Controls post-

deployment had lower IGF-2 levels than they did pre-deployment. It is unclear how to interpret this finding. There were no clear patterns of cytokine promoter region hyper- or hypo-methylation.

Our overall results must be interpreted in light of the fact that we used serum DNA as the biomarker and not a specific tissue derived DNA (such as brain tissue). Thus, our findings could be associated with more of a response in inflammatory tissues, but not specifically in the brain. These findings should be followed up in a study with a larger sample size.

Personnel who received pay from the research effort:

- Dr. Andrea Baccarelli (co-investigator/sub-award)
- Dr. Vasantha Srikantan (research associate at USU)
- Dr. Ligong Chen (research associate at USU)

Appendix. 1. Manuscript of global DNA methylation and PTSD results, which has been submitted for peer review

DNA methylation in repetitive elements and PTSD: a case-control study of U.S. military service members

Jennifer A. Rusiecki, [1] Ligong Chen, [1] Vasantha Srikantan, [1] Lei Zhang, 2 Liying Yan, [3] Matthew L. Polin, [3] Andrea Baccarelli [4]

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ABSTRACT

Background We conducted a case control study to investigate serum DNA methylation patterns in genomic repetitive elements, LINE-1 and Alu, and their potential association with PTSD in U.S. military service members recently deployed to Iraq or Afghanistan. **Methods** Cases (n=75) had a post-deployment diagnosis of PTSD in their medical record (ICD-9-309.81). Controls (n=75) were randomly selected service members with no PTSD diagnosis. Pre- and post-deployment serum samples were accessed, DNA extracted, and DNA methylation (%5-mC) quantified via pyrosequencing. We used conditional logistic regression to calculate odds ratios (OR) and 95% confidence intervals (CIs) comparing 1. controls post- to pre-deployment 2. cases post- to pre-deployment, and unconditional logistic regression comparing 3. cases to controls pre-deployment and 4. cases to controls post-deployment. **Results** LINE-1 was hypermethylated in controls post- versus pre-deployment (OR=1.33; 95%CI, 1.06-1.65); cases showed no change post-deployment. LINE-1 was hypomethylated in cases versus controls post-deployment (OR=0.82; 95%CI, 0.67-1.01). Alu was hypermethylated for cases versus controls pre-deployment (OR=1.46; 95%CI, 1.08-1.97). **Conclusions** Patterns of hypermethylation of LINE-

1 in controls post-deployment and of Alu in cases post deployment are intriguing and may be suggestive of resilience or vulnerability factors. These findings are preliminary and should be investigated in larger studies.

INTRODUCTION

As the conflicts in Iraq and Afghanistan continue, increasing numbers of returning soldiers are suffering from post-traumatic stress disorder (PTSD). A recent study of combat troops following return from deployment found PTSD rates to range from 12.2%-12.9%. (1) The underlying molecular mechanisms of PTSD are largely unknown. Epigenetic factors - inherited and acquired modifications of DNA and histones that regulate various genomic functions occurring without a change in nuclear DNA sequence - could offer new insights into PTSD.

Profiling using cDNA microarrays of peripheral blood during the triggering and development of PTSD in trauma survivors at the emergency room and 4 months later has found differential gene expression signatures in promoter regions of genes which distinguished PTSD patients. (2, 3) An epigenetic mechanism, DNA methylation may play a significant role in the pathophysiology of PTSD, since the process is intrinsically linked to regulation of gene expression - both transcriptional silencing and activation. (4) A recent study applying methylation microarrays to assay CpG sites in peripheral blood from PTSD cases and controls found differential methylation in genes related to immune system functions. Aberrant DNA methylation patterns have also been found in other psychiatric (5, 6) and neurodegenerative (7-11) disorders. Since DNA is inherently stable compared to RNA, development of biomarkers based on DNA provide an attractive potential. Additionally, understanding the role of DNA methylation in PTSD has the

potential to fuel novel therapeutic approaches to PTSD therapy, particularly since modifications in DNA methylation can potentially be reversed.

The typical methylation pattern of mammalian genomes consists of short (<4 kb) unmethylated domains embedded in a matrix of long methylated domains. (12, 13) These long methylated domains reside primarily in interspersed and tandem repetitive elements. (12, 13) Repetitive elements comprise almost 50% of the human genome. (14) In this study we focused on two repetitive elements, the long interspersed nucleotide element (LINE-1) and the interspersed Alu. Since there are about 0.5 million copies of LINE-1 and 1.4 million copies of Alu repetitive elements in the human genome, (15, 16) the methylation status of these sequences is a major contributor of global DNA methylation patterns. (16) In young, healthy mammals, non-CpG island cytosine predominantly located in repetitive genomic regions, such as LINE-1 and Alu, is almost universally methylated. (17, 18) Decreases in global DNA methylation content (i.e., hypomethylation) have been associated with widespread alterations in gene expression and chromatin packaging control, as well as with higher genomic instability. (19)

Since human studies of brain tissue are highly invasive and in many cases impractical, it would be of great value to identify a low-invasive biomarker of epigenetic patterns of PTSD. Serum and cerebrospinal fluid (CSF) have been found to have good correlation with respect to cytokine expression, indicating that serum may be a good biomarker. However, most of the studies which have measured expression signatures or methylation patterns in PTSD have been carried out using whole blood, (2-6) and to date no human serum biomarkers for PTSD have been reported. Additionally, the role of methylation of repetitive elements in PTSD has not been investigated in

humans. We, therefore, carried out a case-control study to investigate DNA methylation patterns in LINE-1 and Alu repetitive elements and their potential association with PTSD in serum DNA from U.S. military soldiers who deployed to Iraq (Operation Iraqi Freedom (OIF)) or Afghanistan (Operation Enduring Freedom (OEF)) between 2004 and 2006. The serum was housed at the Department of Defense Serum Repository (DoDSR), which stores serum remaining following mandatory HIV testing of all active and reserve service members of the U.S. military. We measured LINE-1 and Alu DNA methylation as percentage of 5-methyl cytosine (%5-mC) prior to each participant's first deployment to OIF or OEF and after their deployment. For cases, post-deployment measurement was synonymous with post PTSD diagnosis measurement. Given the significance of PTSD burden in the U.S. military population and availability of serial sera for every service member (1990 to the present), the DoDSR provides a unique opportunity to evaluate epigenetic patterns of this illness.

METHODS AND MATERIALS

Study Population

All cases and controls were selected from among active duty Army and Marine service members with at least two years of continuous active duty prior to their first OIF/OEF deployment. All subjects carried out their first OIF/OEF deployment between January 01, 2004 and December 31, 2006 and were deployed for 6-18 months. Dating back to at least two years prior to first OIF/OEF deployment, there was an absence of any mental health diagnosis, ascertained via query of the International Classification of Diseases, 9th Revision (ICD-9) codes 290-320. To attempt to control for confounding by other psychiatric illnesses, post-deployment exclusion

criteria for both cases and controls was ever having an ICD-9 diagnosis (either inpatient or outpatient) for any of the following mental health diagnoses: schizophrenia (ICD9 code 295), bi-polar disorders (ICD9 code 296), and manic phase bi-polar disorder (also ICD9 code 296).

Post traumatic stress disorder (PTSD) cases (n=75) with existing serum samples housed at the DoDSR were identified by the Armed Forces Health Surveillance Center (AFHSC). Cases met all the criteria above and had at least two outpatient records with a primary diagnosis of chronic PTSD, based ICD-9 Code 309.81 in the first diagnostic position. The first outpatient diagnosis was required to be between four and six months after the service member's return from first deployment. The second outpatient diagnosis was required to be any time after that, but within two years of return from first deployment. Additional criteria for inclusion as a case to this study was having one serum sample drawn within 12 months prior to first OIF/OEF deployment and one sample drawn within six months after return from first OIF/OEF deployment. We randomly selected from among all cases meeting these criteria, within the ages of 20 and 35 years on their first day of first OIF/OEF deployment and of black or white race.

We identified an appropriate control group (n=75), who were randomly selected from among those active duty Army and Marine service members who met the same deployment, age, race and serum sample criteria as cases, but for whom there was never a diagnosis of PTSD (ICD9 Code=309.81) or TBI (ICD9 Codes=800.0-801.9, 803.0-804.9, or 850.0-854.1). Controls were frequency matched to cases based on age group (20-26, 27-35 years), gender, and race.

Sample preparation and laboratory methods

DNA Extraction

For each PTSD case and control, The AFHSC identified a pre-deployment and a post-deployment serum sample (total $n_{samples}$ = 300). The AFHSC permits the utilization of up to 0.5 mL of serum per sample, so genomic DNA was extracted from 0.5mL serum. DNA was extracted using charge switch gDNA 0.2ml-1ml serum kit from (Invitrogen Carlsbad, CA 92008) and quantified via quant-iT ds HS assay kit using a Qubit fluorometer (Invitrogen, Carlsbad, CA).

Quantification of DNA methylation

DNA methylation was quantified via bisulfite treatment, PCR, and pyrosequencing. DNA was bisulfate treated using the Zymo DNA Methylation Kit (Zymo research, Orange, CA). Bisulfate treated DNA was eluted in 20 µl volume and 1 µl of it was used for each PCR. The PCR was performed with one of the PCR primers biotinylated to convert the PCR product to single-stranded DNA templates. The PCR products (each 10 µl) were sequenced by Pyrosequencing PSQ96 HS System (Qiagen Pyrosequencing) following the manufacturer's instructions (Qiagen Pyrosequencing). Pyrosequencing is a real-time sequencing-based on mutation analysis or methylation analysis technology. The methylation status of each locus was analyzed individually as a T/C SNP using QCpG software (Qiagen Pyrosequencing). Loci measured were four positions (-605, -593, -590, -583 from translational start site of ORF1) in LINE-1 (GenBank Accession number: M80343) and 4 positions (nucleotide position at 114, 118, 123,126 based on GenBank Accession number X55933) in Alu. The percentage of methylation was expressed as 5-

mC divided by the sum of methylated and unmethylated cytosine, thus yielding %5-mC. All measurements were run in duplicate and the mean of both runs was calculated for each position in LINE-1 and Alu. For both the LINE-1 and Alu assays %5-mC was measured at four adjacent CpG sites. Four controls (low, medium, high methylated DNA (EpigenDx, Inc.), and a no DNA template) starting from bisulfite modification were included in every pyrosequencing run to ensure completion of bisulfite modification, specificity of PCR amplification, and success of Pyrosequencing reactions.

For 10% of the samples we included duplicates to which laboratory personnel were blinded, for quality control; we calculated coefficients of variation (CV) for the duplicates (sample standard deviation divided by sample mean).

Statistical Methods

Our statistical analyses considered the mean value of CpG methylation in the four LINE-1 and the four Alu CpG sites. This is standard practice and has been used in previous studies. (20-25) We carried out four major comparisons: 1. cases post- versus cases pre-deployment, 2. controls post- versus controls pre-deployment, 3. cases pre-deployment versus controls pre-deployment, and 4. cases post-deployment versus controls post-deployment. Paired t-tests were used to compare %5-mC levels for LINE-1 and Alu for case-case and control-control comparisons, while simple t-tests were used for the case-control comparisons. Likewise, conditional logistic regressions were employed to investigate the potential changes in LINE-1 and Alu methylation level (%5-mC) for cases post- vs. pre-deployment and for controls post- vs. pre-deployment. Unconditional logistic regressions were employed to investigate the potential differences in

LINE-1 and Alu methylation level (%5-mC) between cases and controls pre-deployment and post-deployment. Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated with percent 5-mC included in models as a continuous variable; for these models, the OR represents the per unit (1% methylation) change in estimate. We also carried out logistic regressions with %5-mC treated as a categorical (tertiles) variable, based on controls' pre-deployment levels. All models were adjusted for age (continuous), gender, and race (black, white).

This study was approved by the Institutional Review Board at the Uniformed Services University of the Health Sciences.

RESULTS

Baseline characteristics of the population studied are in Table 1. The age range of the population studied was narrow (20 to 35 years). This study included 100 males (66.7%) and 50 females (33.3%). Racial distribution was 80% white and 20% black. The distribution of these factors did not differ by case-control status, because of the selection and frequency matching criteria. Approximately 72% of cases and controls had deployments of 6 months to less than 12 months, while 25% had deployments of 12 to 18 months. The length of time between deployment and serum draw was not standard for each study member. The number of days between end of deployment and post-deployment serum draw ranged from one to 170 (mean=22.45; sd=38.84; median=7 days), and the number of days between pre-deployment serum draw and start of deployment ranged from 58 to 358 (mean=87.46; sd=84.49; median=58.5 days). We did not find any difference between cases and controls, however, with respect to these time intervals. There were four cases who also had a post-deployment diagnosis of TBI (defined as an ICD-9 code of 800.0-801.9, 803.0-804.9, or 850.0-854.1) (data not shown). We carried out all analyses

including and excluding those four cases to ensure that none of our findings were confounded by TBI and found that results were very similar. We, therefore, present all results for analyses including those four TBI cases.

For the 10% of the samples for which we had duplicates, we calculated coefficients of variation (CV) for %5mC measurements; the overall CV for LINE-1 was 5.46% and for Alu was 7.03%. Four samples failed the LINE-1 analysis and one sample failed the Alu analysis due to low yield of serum DNA resulting in no or low Pyrosequencing signals.

Paired t-test comparisons to investigate mean differences in %5-mC between cases pre- and post-deployment and between controls pre- and post-deployment are presented in Figures 1.a. and 1.b. Figure 1.a. shows that although both LINE-1 and Alu %5-mC were higher in cases post-deployment (i.e., synonymous with post-diagnosis for this study) than in the same cases pre-deployment, differences were not statistically significant. Figure 1.b. shows that LINE-1 %5-mC was significantly increased for controls post-deployment (mean=78.47; sd=1.74), compared with pre-deployment (mean=77.66; sd=1.81), p=0.01. No differences were found for Alu between controls post- and pre-deployment. Simple t-tests comparing %5mC between cases and controls pre-deployment and cases and controls, post-deployment are presented in Figures 2.a. and 2.b. Figure 2.a. shows that there was no difference in LINE-1 %5-mC between cases and controls, pre-deployment, but levels of Alu were significantly higher (p=0.01) for cases (mean=28.15; sd=1.45) compared with controls (mean=27.51; sd=1.26), pre-deployment. However, as shown in Figure 2.b., post-deployment simple t-tests revealed no difference (p=0.09) between cases (mean=28.36; sd=2.08) and controls (mean=27.72; sd=1.69) for Alu. For LINE-1, no significant

difference (p=0.07) was found between cases (mean=77.92; sd=1.68) and controls (mean=78.46; sd=1.73), but significantly lower levels of LINE-1 were found for cases vs. controls in the subgroups of service members of younger age, male gender, and white race (data not shown).

Odds ratios (ORs) and 95% confidence intervals (CIs) from conditional logistic regressions comparing cases post- versus pre-deployment and controls post- versus pre-deployment are presented in Table 2. These ORs represent the change in estimate per unit (1%) change in DNA methylation. For LINE-1 no statistically significant associations were found for the case-case comparisons. For Alu ORs were slightly elevated for the total population (OR=1.26; 95%CI, 0.90-1.78) and for various sub-groups, none of the estimates was statistically significant. Controls post-deployment had statistically significant elevated ORs when compared to controls pre-deployment for the total population (OR=1.33; 95%CI, 1.06-1.65), and younger age (OR=2.45; 95%CI, 1.31-4.57), males (OR=1.38; 95%CI, 1.04-1.83), and white race (OR=1.42; 95%CI, 1.10-1.84). We further limited the analysis to young, white, males and found a more pronounced statistically significant elevated OR for LINE-1 (OR=2.05; 95%CI, 1.11-3.82) (data not shown). There were also elevated ORs if time between deployment end and post-deployment serum draw was $\leq$ 7 days (OR=1.47; 95%CI, 1.01-2.14) and if time between pre-deployment serum draw and deployment start was $\leq$ 90 days (OR=1.71; 95%CI, 1.13-2.58). Comparisons of Alu for controls post- versus pre-deployment were relatively null. There were no striking differences for any of the comparisons with respect to length of deployment. Including %5-mC as a categorical variable in the models yielded similar results (data not shown).

Odds ratios (ORs) and 95% confidence intervals (CIs) from unconditional logistic regressions comparing cases versus controls, pre-deployment and cases versus controls, post-deployment are presented in Table 3. For LINE-1, pre-deployment comparisons of cases and controls were relatively null, however post-deployment, a negative association was found for LINE-1 for the total population (OR=0.82; 95%CI, 0.67-1.01) and for younger age (OR=0.71; 95%CI, 0.51-0.98), males (OR=0.71; 95%CI, 0.54-0.93), and whites (OR=0.75; 95%CI, 0.59-0.95). When we further limited the post-deployment analysis to young, white, males, we found a more pronounced statistically significant negative association for LINE-1 (OR=0.63; 95%CI, 0.43-0.94) (data not shown). For Alu, pre-deployment comparisons of cases and controls showed a positive association for the total population (OR=1.46; 95%CI, 1.08-1.97) and for older age (OR=1.62; 95%CI, 1.02-2.57), females (OR=1.97; 95%CI, 1.02-3.79), and black race (OR=2.93; 95%CI, 1.01-8.53). Post-deployment, the elevated ORs for Alu %5-mC persisted between cases and controls in the total population (OR=1.24; 95%CI, 0.96-1.60), however, estimates for the total population and subgroups were not statistically significant. Including %5mC into the models as a categorical variable yielded more pronounced associations (negative for LINE-1 and positive for Alu) in the third tertile vs. first tertile comparisons than in the second vs. first tertile comparisons (data not shown).

DISCUSSION

We carried out four major comparisons in this study: 1. cases post- versus cases pre-deployment, 2. controls post- versus controls pre-deployment, 3. cases versus controls, pre-deployment, and 4. cases versus controls, post-deployment. As cases pre-deployment had not had any previous diagnosis of PTSD and were equivalent to healthy controls in that respect, we predicted that

comparisons 1 and 2 would give us insight into deployment related stresses potentially associated with DNA methylation, while comparison 2 would additionally provide insight into any potential associations of PTSD and DNA methylation in repetitive elements, as would comparison 4. Comparison 3 would enable us to determine if there were any fundamental underlying differences with respect to DNA methylation between cases and controls prior to deployment, which may indicate vulnerability to stress.

We found that controls post-deployment had higher LINE-1 %5m-C than they did pre-deployment. Interestingly, LINE-1 levels did not significantly change after deployment/diagnosis for cases. Although we found that there was no difference for LINE-1 between cases and controls pre-deployment, post-deployment LINE-1 was hypomethylated in cases versus controls. However, these statistically significant reduced ORs for cases versus controls, post-deployment, cannot be evaluated in isolation of the fact that controls' post-deployment LINE-1 methylation increased (from pre-deployment levels), and this may be largely what drove the negative association between LINE-1 and PTSD. It is unclear why controls' levels increased post-deployment. A possible explanation is that psychological stress incurred during deployment may be associated with a response in LINE-1, and people with that response may be protected against PTSD. For the Alu repetitive element, the case-case comparisons revealed non-statistically significant elevated ORs with increasing Alu %5-mC post-deployment/diagnosis. Additionally, we found that the people who later became cases (i.e., cases pre-deployment), when compared to the controls, pre-deployment, had elevated ORs with increasing Alu %5-mC. Post-deployment ORs were also elevated for Alu, though not statistically significant. These results are suggestive

of a potential protective effect from lower levels of Alu and a potential for vulnerability to stress with higher levels of Alu, even before exposure to a potentially traumatic event.

DNA hypomethylation in repetitive elements is generally associated with chromosomal instability and the expression of genes which would normally be methylated and therefore silenced. (26, 27) The role of global methylation has been implicated in memory formation (8) and plasticity. (28) We found hypomethylation of LINE-1 for cases versus controls, post-deployment, though this finding may have been driven by increases in controls' LINE-1 methylation after deployment. In this study DNA methylation in the Alu repetitive element was hypermethylated for cases post-deployment compared to pre-deployment and for cases compared to controls pre-deployment. A recent epidemiologic study found that night-shift workers had elevated Alu %5-mC in their blood compared with day workers, suggesting that Alu hypermethylation may represent a response to psychological stress (29). Indeed Alu has been reported to have a physiologic role during responses to stress, (30) and it is hypothesized that PTSD develops as a result of an inability to control a normal stress response. (4, 31) Although both LINE-1 and Alu have been implicated in the regulation of cell stress responses of the immune system, the strongest data on regulation of these repetitive elements by cell stress are for Alu. (32)

Although a handful of recent studies have evaluated gene specific DNA methylation and PTSD, (4, 33-35) to our knowledge, this is the first study to investigate the association between DNA methylation in repetitive elements (LINE-1 and Alu) and PTSD in humans. A cross sectional study of PTSD-affected and –unaffected individuals enrolled in a longitudinal study applied methylation microarrays to investigate methylation and immune function profiles in DNA derived from whole blood. (4) The investigators reported that immune system functions were

significantly overrepresented among the genes uniquely unmethylated in those with PTSD. (4) Another recent study in humans found that persons with more traumatic events were at increased risk for PTSD, but only at lower methylation levels of a serotonin transporter gene, SLC6A4. At higher methylation levels, individuals with more traumatic events were protected from PTSD. (36)

Serum has not previously been evaluated as a biomarker for DNA methylation patterns associated with PTSD. The few human studies which have investigated DNA methylation in PTSD have utilized predominantly whole blood-derived DNA.(4, 33-36) How correlated DNA methylation levels are in serum and whole blood to brain and other CNS tissues is not clear. Although relevant to a different class of disease, many of the aberrations that have been detected in the DNA of primary tumor tissue can also be detected in DNA present in serum, (38-49) and cell free DNA in the circulation has increasingly been recognized as a valuable diagnostic tool in various diseases. (50-53) Compared with cultured cells, clinical specimens, such as whole blood, serum, and even brain tissue and other CNS tissues, contain a heterogeneous mixture of cell types, each contributing its own unique methylation profile to the final analysis. We are, therefore, not able to assess serum cell-specific differences in methlyation status.

A limitation of this study is that we do not have detailed data on deployment exposures for either the cases or controls. Deployment was used as a proxy for the potentially traumatic event (PTE), and the exact timing of the PTE is not known. Likewise, the timing of sample collection was not standardized, so there was heterogeneity in the length of time between deployment and serum draw. Although we tried to minimize this time interval, the design of the study was such that we

had to rely on when a service member had serum drawn for HIV testing. We tried to control for differences in deployment experience by ensuring that all cases and controls had not been previously deployed, that they were all active duty Army or Marines, and that they were deployed from between 6 to 18 months, but there is still potential for significant variation among all our subjects with respect to intensity of combat during deployment. We also have no data on other relevant exposures which are known to affect DNA methylation, such as dietary factors (folate, vitamin B_{12} intake), (54, 55) smoking, (56) and alcohol consumption. (25, 56, 57) Ascertainment of PTSD via query of medical encounter coded via the ICD-9 is not ideal. Although we attempted to restrict the definition of PTSD to a scenario which would minimize misclassification of disease, by requiring the ICD-9 code 309.81 be present in the first diagnostic position for two outpatient records spaced at a reasonable calendar time distance, this type of case ascertainment is still prone to misclassification. However, this is the PTSD case definition developed in September 2008 by the Department of Defense Interagency PTSD and Traumatic Brain Injury (TBI) Standardization Committee, and it has been accepted by Military Health Affairs for surveillance. (58) The DNA yields from the sera in this study were small, another potential limitation. Four samples failed the LINE-1 analysis and one sample failed the Alu analysis due to low yield of serum DNA resulting in no or low Pyrosequencing signals.

A major strength of this study is that we had pre-deployment and pre-diagnosis samples. While most case control studies would not be able to infer whether the observed methylation patterns were a consequence of PTSD or whether they indicated vulnerabilities that existed among the cases before the onset of PTSD, our study was able to address both possibilities.

Post-traumatic stress disorder (PTSD) is unique among psychiatric disorders since there is an explicit requirement for a well-defined environmental event, the PTE. This suggests that adaptable molecular processes such as DNA methylation are highly relevant. Indeed, there is growing evidence that the molecular mechanisms that regulate DNA methylation are involved in synaptic plasticity, learning, and memory. (9) Understanding the role of repetitive elements in PTSD is imperative, since they comprise approximately 50% of the human genome. (59) The results of the present study should be considered as preliminary, and future studies are needed to confirm these findings. The Department of Defense Serum Repository, which contains longitudinally collected sera of U.S. military service members will provide a vast resource for carrying out future investigations in larger populations.

CONCLUSIONS

Post-deployment, LINE-1 was hypomethylated in cases versus controls, however this is mainly a function of controls' levels increasing significantly post-deployment. Patterns of hypermethylation of LINE-1 in controls post-deployment and of Alu in cases post deployment are intriguing and may be suggestive of resilience or vulnerability factors. These findings are preliminary and should be investigated in larger studies.

TABLES

Table 1. Baseline Characteristics of Population

Characteristic	Cases		Controls		*p* -value
	N	%	N	%	
Age					
Younger (20-23 years)	40	53.3	39	52.0	0.87
Older (24-35 years)	35	46.7	36	48.0	
Gender					
Male	50	66.7	50	66.7	1.00
Female	25	33.3	25	33.3	
Race					
White	60	80.0	60	80.0	1.00
Black	15	20.0	15	20.0	
Deploy Length					
Short (6 to <12 months)	50	71.4	57	76.0	0.71
Long (12 to 18 months)	20	28.6	18	24.0	
Time between deployment end and post-deployment serum sample draw					
≤7 days	42	56.0	38	50.7	0.51
>7 days	33	44.0	37	49.3	
Time between pre-deployment serum draw and start of deployment					
≤90 days	52	69.3	46	61.3	0.30
>90 days	23	30.7	29	38.7	

Figure 1.a. Distribution of LINE-1 and Alu %5mC for cases pre-deployment and cases post-deployment

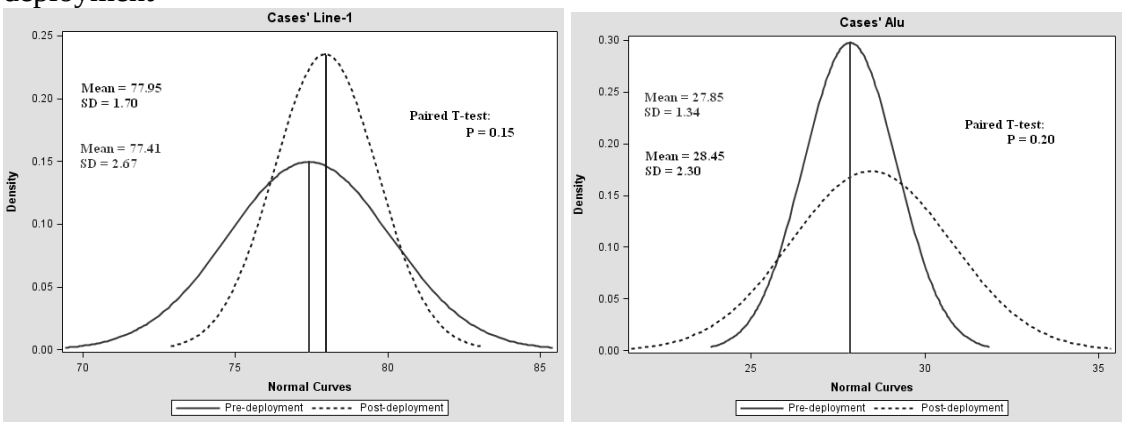

Figure 1.b. Distribution of LINE-1 and Alu %5mC for controls pre-deployment and controls post-deployment

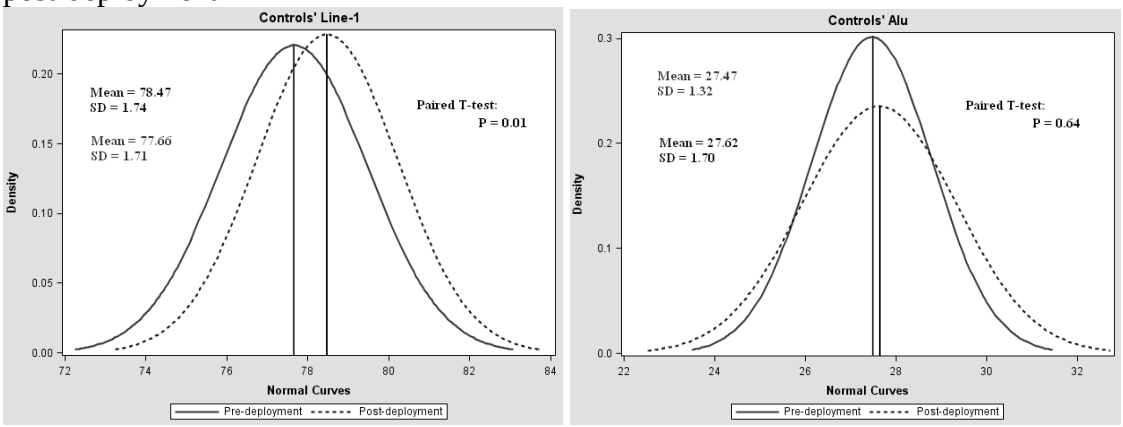

Figure 2.a. Distribution of LINE-1 and Alu %5mC for cases and controls, pre-deployment

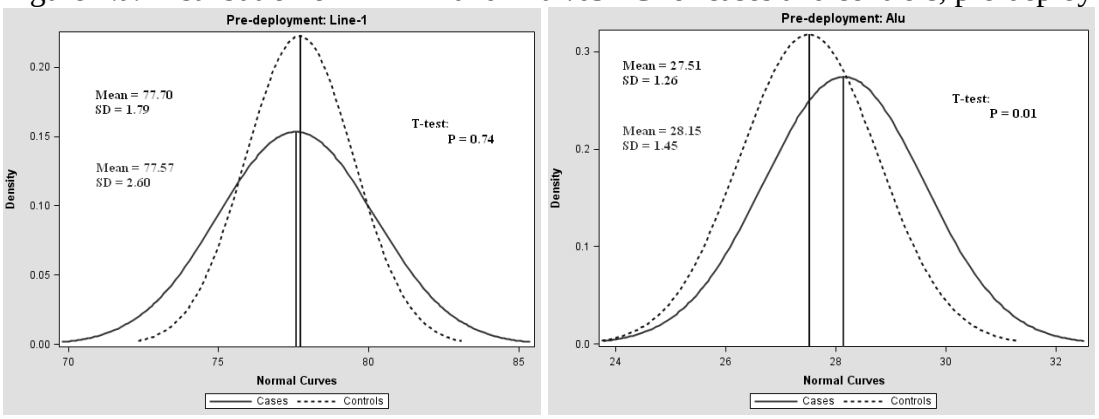

Figure 2.b. Distribution of LINE-1 and Alu %5mC for cases and controls, post-deployment

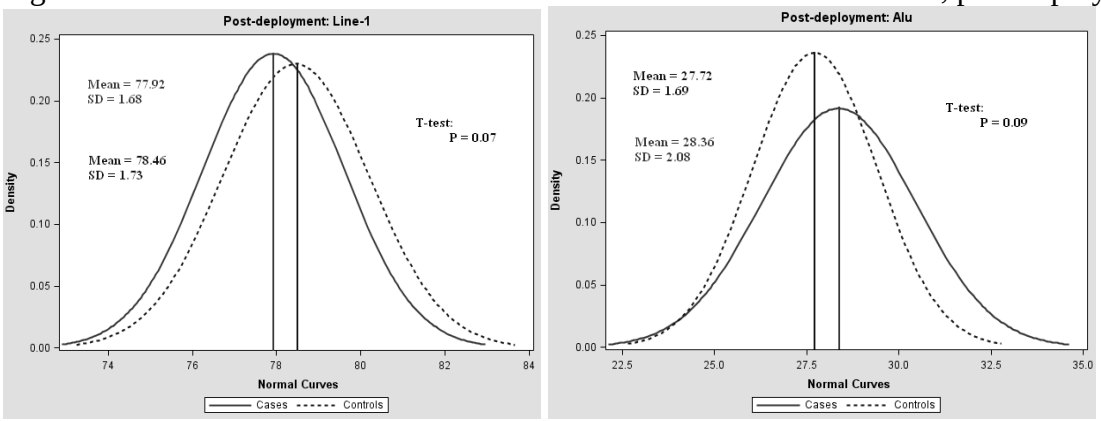

Table 2. Conditional Logistic Regressions comparing cases post- vs. pre-deployment and controls post- vs. pre-deployment: %5-mC LINE-1 and Alu levels

	Cases (post- vs. pre-deployment)						Controls (post- vs. pre-deployment)					
	LINE-1			Alu			LINE-1			Alu		
Population	**$N_{post/pre}$**	**OR§**	**95%CI**	**$N_{post/pre}$**	**OR§**	**95%CI**	**$N_{post/pre}$**	**OR**	**95%CI**	**$N_{post/pre}$**	**OR**	**95%CI**
Total Population	66/71	1.11	0.94-1.31	50/52	1.26	0.90-1.78	70/74	1.33	1.06-1.65 #	51/60	1.1	0.82-1.39
Age groups												
Younger age (20-23)	37/38	1.04	0.79-1.37	28/28	1.35	0.85-2.16	35/39	2.45	1.31-4.57 #	25/33	0.9	0.61-1.42
Older age (24-35)	29/33	1.16	0.92-1.46	22/24	1.12	0.62-2.02	35/35	1.06	0.82-1.36	26/27	1.2	0.82-1.67
Gender groups												
Males	45/48	1.09	0.83-1.43	36/35	1.31	0.87-1.98	45/49	1.38	1.04-1.83 #	32/41	1	0.65-1.42
Females	21/23	1.12	0.90-1.40	14/17	1.14	0.57-2.27	25/25	1.23	0.85-1.78	19/19	1.2	0.80-1.71
Race groups												
Black Race	10/15	1.94	0.82-4.58	7/11	1.29	0.64-2.63	13/15	0.93	0.54-1.60	10/10	1.7	0.62-4.54
White Race	56/56	1.06	0.90-1.25	43/41	1.26	0.85-1.85	57/59	1.42	1.10-1.84 #	41/50	1	0.77-1.34

Table continued on next page

Table 2. Conditional Logistic Regressions comparing cases post- vs. pre-deployment and controls post- vs. pre-deployment: %5-mC LINE-1 and Alu (continued)

Deploy Length												
Short	51/51	1.12	0.94-1.34	39/39	1.16	0.84-1.61	54/57	1.24	0.97-1.57	39/45	1.1	0.76-1.50
Long	15/20	1.04	0.64-1.70	11/13	3.20	0.44-22.97	16/17	1.93	0.94-3.93	12/15	1.1	0.69-1.63
Time between deployment end and post-deployment serum draw												
≤7 days	39/38	1.18	0.91-1.54	31/29	1.66	0.85-3.22	36/37	1.47	1.01-2.14	30/32	1.12	0.81-1.57
>7 days	27/33	1.06	0.87-1.30	19/23	1.13	0.80-1.60	34/37	1.25	0.95-1.63	21/28	0.96	0.61-1.52
Time between pre-deployment serum draw and deployment start												
≤90 days	46/50	1.12	0.94-1.34	36/38	1.26	0.81-1.96	42/45	1.71	1.13-2.58	32/38	1.23	0.83-1.80
>90 days	20/21	1.03	0.65-1.65	14/14	1.27	0.74-2.17	28/29	1.09	0.82-1.46	19/22	0.92	0.62-1.36

§ Odds ratios for the continuous DNA methylation variable represent the per unit (1%) change in estimate.

statistically significant at p≤0.05

Table 3. Unconditional Logistic Regressions* comparing cases vs. controls pre-deployment and cases vs. controls post-deployment: %5mC LINE-1 and Alu

	Pre-Deployment (cases vs. controls)						Post-Deployment (cases vs. controls)					
	LINE-1			Alu			LINE-1			Alu		
Population	**$N_{ca/co}$‡**	**OR**	**(95%CI)**	**$N_{ca/co}$‡**	**OR**	**(95%CI)**	**$N_{ca/co}$‡**	**OR**	**(95%CI)**	**$N_{ca/co}$‡**	**OR**	**(95%CI)**
Total Population	71/74	0.97	0.84-1.13	52/60	1.46	1.08-1.97 #	66/70	0.82	0.67-1.01	50/51	1.24	0.96-1.60
Age groups												
Younger age (20-23)	38/39	1.14	0.88-1.46	28/33	1.36	0.89-2.08	37/35	0.71	0.51-0.98 #	28/25	1.42	0.94-2.15
Older age (24-35)	33/35	0.88	0.71-1.10	24/27	1.62	1.02-2.57 #	29/35	0.92	0.70-1.20	22/26	1.11	0.76-1.62
Gender groups												
Males	48/49	0.98	0.78-1.24	35/41	1.31	0.91-1.86	45/45	0.71	0.54-0.93 #	36/32	1.39	0.95-2.03
Females	23/25	0.96	0.79-1.18	17/19	1.97	1.02-3.79 #	21/25	1.06	0.74-1.50	14/19	1.09	0.74-1.62
Race groups												
Black Race	15/15	0.82	0.51-1.31	11/10	2.93	1.01-8.53 #	10/13	1.26	0.75-2.14	7/10	1.41	0.69-2.86
White Race	56/59	1.00	0.85-1.17	41/50	1.30	0.93-1.81	56/57	0.75	0.59-0.95 #	43/41	1.22	0.93-1.60

Table continued on next page

Table 3. Unconditional Logistic Regressions* comparing cases vs. controls pre-deployment and cases vs. controls post-deployment: %5mC LINE-1 and Alu (continued)

Deploy Length												
Short	51/57	0.92	0.78-1.09	39/45	1.45	1.02-2.07 #	51/54	0.85	0.67-1.08	39/39	1.20	0.90-1.60
Long	20/17	1.83	0.98-3.43	13/15	1.55	0.81-2.94	15/16	0.75	0.46-1.23	11/12	1.11	0.62-1.98
Time between deployment end and post-deployment serum sample draw												
≤7 days	38/37	0.92	0.66-1.29	29/32	1.51	0.98-2.32	39/36	0.87	0.67-1.13	31/31	1.30	0.92-1.86
>7 days	33/37	0.99	0.84-1.17	23/28	1.55	0.99-2.43	27/34	0.71	0.50-1.02	19/21	1.17	0.82-1.67
Time between pre-deployment serum draw and start of deployment												
≤90 days	50/45	1.00	0.85-1.19	38/38	1.45	0.99-2.12	46/42	0.85	0.67-1.09	36/32	1.11	0.77-1.58
>90 days	21/29	0.94	0.67-1.33	14/22	1.45	0.85-2.46	20/28	0.76	0.52-1.11	14/19	1.46	0.91-2.36

* Odds ratios (ORs) for total population adjusted for age (continuous), gender, and race (black, white); ORs for age groups adjusted for gender and race; ORs for gender groups adjusted for age and race; ORs for race groups adjusted for age and gender; ORs

‡ ca = case; co = control

statistically significant at p≤0.05

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