AD_____

Award Number: W81XWH-08-2-0654

TITLE: Epigenetic Patterns of TBI: DNA Methylation in Serum of OIF/OEF Servicemembers.

PRINCIPAL INVESTIGATOR: Jennifer A. Rusiecki, Ph.D.

CONTRACTING ORGANIZATION: Henry M. Jackson Foundation
Rockville, MD 20850

REPORT DATE: September 2012

TYPE OF REPORT: Revised Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
September 2012	Revised Final	15 September 2008 – 31 August 2012

4. TITLE AND SUBTITLE

Epigenetic Patterns of TBI: DNA Methylation in Serum of OIF/OEF Servicemembers.

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-08-2-0654

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Jennifer A. Rusiecki, Louis French, Zygmunt Galdzicki, Celia Byrne, Ligong Chen, Liying Yan, Matthew Polin

E-Mail: jennifer.rusiecki@usuhs.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Henry M. Jackson Foundation
Rockville, MD 20850

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

In a case-control study of OEF/OIF service members with both pre- and post-deployment/injury serum samples, we investigated DNA methylation patterns and serum cytokine expression for mild, moderate, and severe TBI. We found differential methylation (%5-mC) of IL1ƃ for mild cases, TNFƃ for moderate cases, and IL8 for severe cases, as well as differential serum cytokine expression of IL6 in mild and moderate cases, IL8 in mild, moderate, and severe cases, and MMP3 in severe cases. This is the first study, to our knowledge, of chronic epigenetic and protein-based alterations in TBI. These patterns are based on a small sample size, should be interpreted with caution, and should be examined in larger studies.

15. SUBJECT TERMS
TBI, epigenetics, DNA methylation, trauma, deployment, combat, serum, cytokines, repetitive elements

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	36	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

Page

Introduction

The molecular mechanisms involved in traumatic brain injury (TBI) are not well characterized. From both human and animal studies, we know that a profound inflammatory response is initiated immediately following TBI and is characterized by the expression of several cytokines with both pro- and anti-inflammatory functions. [1-5] An epigenetic mechanism, DNA methylation is intrinsically linked to the regulation of gene expression. Thus, the purpose of our investigation is to elucidate how patterns of DNA methylation may vary by TBI diagnosis and by severity of the disease. TBI cases with existing serum samples housed at the Department of Defense Serum Repository (DoDSR) were identified by query of the clinical database of TBI patients at Walter Reed Army Medical Center (WRAMC) for those cases who were diagnosed with a non-penetrating blast TBI (mild, moderate, severe), from 01 February, 2003 to 27 August, 2010. Social security numbers, names, date of diagnosis, classification of mild, moderate, or severe TBI, and some clinical variables for each TBI patient identified were transferred from WRAMC to the Armed Forces Health Surveillance Center (AFHSC). Personnel at AFHSC queried the Defense Medical Surveillance System (DMSS) database for those members with at least one serum sample taken before their first deployment and one serum sample taken after their first deployment. We identified an appropriate control group consisting of randomly selected service members who were never diagnosed with a TBI at WRAMC and who were frequency matched on various demographics of cases. For each TBI case and each control, a serum sample drawn prior to first OIF/OEF deployment and a sample drawn after that deployment was identified, pulled from the Repository and given to the PI. DNA was extracted from each serum sample, and percent methylated cytosine (%5-mC) were quantified in the promoter regions of the following cytokines, β-NGF, IL-1α, IL-1β, IL-6, IL-8, IL-10, IL-13, TGFβ1, and TNFβ and in two repetitive elements, LINE-1 and Alu. Comparisons were made for patterns of DNA methylation between cases (stratified into mild, moderate, and severe) and controls and between pre- and post-deployments for both cases and controls. We also measured serum levels of cytokines (IL-1α, IL-1β, IL-6, IL-8, IL-10, IL-13, β-NGF, TGF- β1, TNF β, IL-4, IL-17, MCP-1, MMP-3, MMP-9, and TNFα) in residual serum not needed for DNA extraction and comparisons made for patterns of DNA methylation between cases (stratified into mild, moderate, and severe) and controls and between pre- and post-deployments for both cases and controls. This study will help to elucidate the molecular sequelae of brain injury and will fuel novel therapeutic approaches to TBI therapy, particularly since modifications in DNA methylation can potentially be reversed.

Body:

From the time that this grant was awarded in September, 2008 until August, 2010, the PI had tried to obtain IRB approval for the protocol. During that period, the PI also had many discussions with TBI experts at USU and Walter Reed Army Medical Center (WRAMC), who advised her to revise the methodology of identifying cases. In the original narrative, PI proposed that the Armed Forces Health Surveillance Center (AFHSC) would identify cases with specific ICD-9 codes indicating a TBI. This was considered less than optimal, according to many TBI experts at USU and WRAMC (since there is no specific ICD-9 code for TBI, and combining various other trauma codes would run the risk of lacking specificity), who advised PI to try to identify cases via the clinical database of TBI patients at WRAMC. PI contacted Dr. Louis French at WRAMC, who is the Director, Traumatic Brain Injury Service at the Department of Orthopaedics and Rehabilitation at WRAMC to inquire about the possibility of identifiers being sent from the database he maintains directly to AFHSC, so that personnel at AFHSC could pull

serum samples on cases identified via this database. The PI would never have access to any personal identifiers, and all transfer of identifying data between WRAMC and AFHSC would be carried out via double password protected, encrypted methods.

Dr. French agreed to this, and PI contacted Army Contracting Officer Representative, Dr. Tammy Crowder to get permission to proceed with this change in methodology of identifying cases. Since this would significantly improve the quality of the study and since it was not outside the scope of the original grant protocol, Dr. Crowder informed the PI that this would be acceptable (telephone communications with Dr. Tamara Crowder on November 21, 2008). PI subsequently commenced IRB submission to WRAMC. USU IRB informed PI that she would need to get WRAMC IRB approval first and then an expedited review would be carried out after that at USU to update the USU approval. PI submitted protocol to WRAMC on 31 January, 2009 as an exempt study request. WRAMC Department of Clinical Investigation (DCI) then informed PI that the review would have to be expedited, as opposed to exempt, so PI prepared another submission, which was finally submitted on 23 April, 2009 (this submission required a lot of additional work and was very lengthy). The protocol was reviewed by the Clinical Investigation Committee on 02 June, 2009. It was then tabled at this meeting, due to extensive comments by the committee on the protocol. By the end of August, 2009 the PI had addressed each comment, revised the protocol accordingly, and re-submitted the protocol for a second CIC review; this occurred in September, 2009. The protocol was then approved by the CIC and forwarded to the Human Use Committee (HUC) at WRAMC (the equivalent of the IRB for that institution). The HUC held a meeting in October, 2009, at which PI was present to clarify some additional concerns. One outstanding issue was the establishment of a CRADA between the Henry Jackson Foundation for the Advancement of Military Medicine and Walter Reed Army Medical Center. The PI inquired with the Jackson Foundation in December, 2009 as to the status of the CRADA and found that work had not been initiated to establish it. The representative from the Jackson Foundation ensured the PI that it would be taken care of within weeks of that discussion. PI was on maternity leave from late December, 2009 through late March, 2010 and did not push the Jackson Foundation to resolve the CRADA issues. There were further delays throughout the Spring and Summer of 2010 because of questions posed by the U.S. Army Medical Research and Materiel Command (USAMRMC) regarding the funding of the project, particularly concerns about Dr. French's personnel.

On 27 August, 2010 PI finally received approval to begin work on the protocol from the Chief, Research Review Service Department of Clinical Investigation, Walter Reed Army Medical Center.

The following is a description of research accomplishments associated with each task outlined in the approved Statement of Work.

Task 1: Identification of TBI cases and controls, selection of serum samples, and aliquotting of serum samples.

On 17 September, 2010, Dr. French at WRAMC forwarded the list of potential cases queried from the WRAMC clinical database to AFHSC so that we could begin our case selection. He searched for all non-penetrating, blast TBI cases identified at Walter Reed Army Medical Center from the clinical database of TBI patients seen at WRAMC from 01 February 2003 to 27 August, 2010. Individuals who were still receiving treatment at WRAMC were not included in this study

because of HIPAA concerns. Dr. French extracted the following data to be sent to AFHSC for each TBI case: Social security number, Name, TBI diagnosis (mild, moderate, or severe), Date of TBI diagnosis (month, year), Extracranial injuries (yes/no), Extracranial injuries (type, if applicable).

The AFHSC then applied all the following criteria to the population and identified cases and controls for this study:

Cases:

For the mild and moderate cases, the following inclusion/exclusion criteria were used to select cases:

1. Cases had to be <40 years of age at the start of their first OEF/OIF deployment.
2. Only cases with an injury date occurring after the start of their first OEF/OIF deployment, but before the start of any subsequent deployments were kept as cases.
3. Cases were excluded if they ever had at least 2 outpatient or 1 inpatient encounters for any cancer as per ICD-9 codes for Neoplasms (codes 140-239), with the exception of ICD-9 codes: 209.4-209.6, 210-229, or 173.0-173.9
4. Cases were excluded if they had at least 2 outpatient or 1 inpatient encounters for 295.xx , 296.0, 296.4-296.8 any time after the end date of their 1st OIF/OEF deployment.

Case serum requirements (all severities):

Only keep cases that have the following serum specimens available:

a. A serum specimen collected within the 1 year prior to the start of their first OIF/OEF deployment. If multiple specimens were available, then the specimen closest to the start of the deployment was selected.

b. A serum specimen collected after the end date of the first OEF/OIF deployment. If multiple specimens were available, then the specimen closest to the end date of the deployment was selected.

All severe cases were kept in the study regardless of whether they met the inclusion/exclusion criteria with the exception that they had to have a deployment and pre- and post-deployment serum specimens available in the appropriate windows. A total of 19 severe cases met these criteria.

Of the mild and moderate cases that meet all of the criteria listed above, all moderate (38) cases were selected and 93 mild cases were selected. Since more than 93 mild cases were eligible, selection was based on preferentially selecting subjects with the least amount of time between end date of the first OIF/OEF deployment and the date of the post serum specimen.

Controls:

Eligible controls had to meet the following criteria:

1. They must have had at least one OEF/OIF deployment.
2. They must be <40 years of age at the start of their first OEF/OIF deployment.
3. Potential controls were excluded if they were listed in the original case file or the blast injured file. Additionally, they were excluded if they had any diagnosis of ICD-9=800.00-854.19 ever.

4. Potential controls were excluded if they ever had at least 2 outpatient or 1 inpatient encounters for any cancer as per ICD-9 codes for Neoplasms (codes 140-239), with the exception of ICD-9 codes: 209.4-209.6, 210-229, or 173.0-173.9.
5. Potential controls were excluded if they had at least 2 outpatient or 1 inpatient encounters for 295.xx , 296.0, 296.4-296.8 anytime after the end date of their 1st OIF/OEF deployment.

Control serum requirements:
Only potential controls that had the following serum specimens available were kept:

a. A serum specimen collected within the 1 year prior to the start of their first OIF/OEF deployment. If multiple specimens were available, then the specimen closest to the start of the deployment was selected.

b. A serum specimen collected after the end date of the first OEF/OIF deployment. If multiple specimens were available, then the specimen closest to the end date of the deployment was selected.

Of the controls who met the criteria above, 50 were randomly selected for the study based on frequency matching to the case population by sex and race (coded as black, white, or other). A flowchart of the selection of cases and controls is presented in Appendix 1.

Serum samples for each case and control included in the study were pulled, 0.5 mL aliquots were prepared for the PI at the Dept. of Defense Serum Repository (DoDSR), Silver Spring, MD. PI picked up the samples at the on 18 November, 2010 at the DoDSR.

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). DNA was extracted from a total of 412 samples – 400 study samples and an additional 12 quality control (QC) samples, which were duplicates of 12 of the study samples.

Task 3: Quantification of DNA methylation and serum cytokine measurements

We had DNA methylation quantified at a commercial laboratory, Epigendx, Inc, Worcester, MA. Assays were run for the following cytokine promoter regions in duplicate: IL-1α, IL-1β, IL-6, IL-8, IL-10, IL-13, NGFβ, TGFβ1, and TNFβ. We also had DNA methylation quantified at two repetitive elements, LINE-1 and Alu. An additional approximately 30 duplicate samples were sent to the lab, to which lab technicians were blinded, for the purpose of quality control (QC).

Regarding the serum cytokine measurements, the traditional method for cytokine detection and quantification has been through the use of an enzyme-linked immunosorbent array (ELISA). In this method, target protein is first immobilized to a solid support. The immobilized protein is then complexed with an antibody that is linked to an enzyme. Detection of the enzyme-complex can then be visualized through the use of a substrate that produces a detectable signal. While the traditional method works well for a single protein, the overall procedure is time consuming and

requires a lot of sample. With little sample to work with, conservation of precious small quantities becomes a risky task. Advancement in microarray technology over the last decade has helped to overcome this disadvantage traditional Elisa Assay. We used the Quantibody array platform for this study, which uses multiplexed sandwich ELISA-based technology and enables the accurate determination of the concentration of multiple cytokines simultaneously. It combines the advantages of the high detection sensitivity/specificity of ELISA and the high throughput of an array. Like a traditional sandwich-based ELISA, it uses a pair of cytokine specific antibodies for detection. A capture antibody is first bound to the glass surface. After incubation with the sample, the target cytokine is trapped on the solid surface. A second biotin-labeled detection antibody is then added, which can recognize a different isotope of the target cytokine. The cytokine-antibody-biotin complex can then be visualized through the addition of the streptavidin-labeled Cy3 equivalent dye using a laser scanner. Unlike the traditional ELISA, Quantibody products use array format. By arraying multiple cytokine specific capture antibodies onto a glass support, multiplex detection of cytokines in one experiment is made possible.

In detail, one standard glass slide is spotted with 16 wells of identical cytokine antibody arrays. Each antibody, together with the positive controls is arrayed in quadruplicate. The slide comes with a 16-well removable gasket which allows for the process of 16 samples in one slide. For cytokine quantification, the array specific cytokine standards, whose concentration has been predetermined, are provided to generate a standard curve for each cytokine. In a real experiment, standard cytokines and samples will be assayed in each array simultaneously through a sandwich ELISA procedure. By comparing signals from unknown samples to the standard curve, the cytokine concentration in the samples will be determined.

The cytokines we measured using our custom-designed Quantibody (SeraCare Life Sciences, Milford, MA) were: IL-1α, IL-1β, IL-6, IL-8, IL-10, IL-13, β-NGF, TGF- β1, TNF β, IL-4, IL-17, MCP-1, MMP-3, MMP-9, and TNFα. The expression of each cytokine in each sample was obtained as pictograms /ml (pg/ml).

Task 4: Analyses

We carried out statistical analyses to make comparisons of serum cytokine levels and DNA methylation levels. We stratified cases into mild, moderate, and severe. For the mild (N=93) and moderate cases (N=38), there were sufficient numbers to calculate adjusted means via generalized linear models, -adjusting for age, gender and race. Since there were only 19 severe cases, we were limited in statistical analyses we could carry out, so we made comparisons via simple t-tests and paired t-tests. There were four comparisons we made: (1) cases vs. controls, pre-deployment, (2) cases vs. controls, post-deployment, (3) cases pre- vs. cases post-deployment, and (4) controls pre- vs. controls post-deployment. Paired analyses were carried out for the case-case and control-control comparisons. We carried out the same analyses for each serum cytokine separately (IL-1α, IL-1β, IL-6, IL-8, IL-10, IL-13, β-NGF, TGF- β1, TNF β, IL-4, IL-17, MCP-1, MMP-3, MMP-9, and TNFα). For each methylation marker (LINE-1, Alu, IL-1α, IL-1β, IL-6, IL-8, IL-10, IL-13, NGFβ, TGFβ1, and TNFβ) we carried out the same analyses for each specific position (CpG site) measured. For example, for the IL-1 β promoter, we measured DNA methylation (%5-mC) at four positions (CpGs), so we carried out an analysis for each comparison for each position in IL-1 β and also for the mean %5-mC across those four positions in IL-1 β.

After carrying out generalized linear models to compare cases to controls, pre- and post-deployment and to make paired comparisons of cases pre- and post-deployment and controls pre- and post-deployment, we used a multivariate analysis of variance (ANOVA) to compare adjusted means of pre-post methylation differences for cases versus controls.

Our QC analyses based on duplicates for the DNA methylation assays yielded excellent results for the 12 pairs of duplicates for all loci measured, with the exception of IL-1α and TGFβ1, so we dropped those assays from our study, as their results were deemed unreliable.

Results of Analyses:

Baseline characteristics of the study population are in Table 1. Although the study was designed with 150 cases and 50 controls, we excluded 7 post-deployment samples (6 severe and 1 mild) because their injury dates were after the post-serum sample was taken. The final count of study subjects included in the study did include all 200, which included 50 controls, 93 mild cases, 38 moderate cases, and 19 severe cases, but for six of the severe and one of the mild cases, post-deployment samples were dropped from the study for the reason mentioned above.

The study population did not differ by case-control status for age, gender, and race because of the selection and frequency matching criteria. There were only 8 women included in the study (6 cases and 2 controls), and there were only 13 people of other than white race included. The mean number of days of deployment for cases was u=248 (s.d.=122) and for controls u=219 (s.d. =126), but this difference was not statistically significant (p=0.21). The mean number of days between the pre-deployment serum sample and deployment start was 99 (sd=79) for cases and 118 (sd=94) for controls, but the difference was not statistically significant (p=0.16). This study is characterized by a relatively long period of time between injury and post-injury serum sample. The mean number of days from injury to post-injury serum among the cases was 316 days (sd=261), the range was 1 to 1186 days, and the median was 250 days. This distribution did not differ much by case severity. Twenty five percent (25%) of the cases had comorbid PTSD, distribution by severity was 24.6% for mild, 29% for moderate, and 10.5% for severe cases. Most cases (90%) also had an extracranial injury, distribution by severity was 86% for mild, 97.4% for moderate, and 94.7% for severe. All of the injuries were blast injuries, of which 77.3% were blast only, and the remainder included blast and some combination of fragment, vehicular, and/or fall.

Table 2a. presents the results from the case-control comparisons for DNA methylation. We found that post-deployment, mild TBI cases had lower IL8 %5-mC than controls at position 1 (cases: u=3.86%, controls: u=7.37%; p=0.05) and position 2 (cases: u=3.95%, controls: u=7.81%; p=0.04). For the mean of the three IL8 positions, there was a similar, non-statistically significant (p=0.06) pattern (cases: u=3.55%, controls: u=6.72%). Post-deployment, moderate TBI cases also had significantly lower IL8 %5-mC at position 1 (cases: u=1.96%, controls: u=7.37%; p=0.03). The same patterns, albeit non-statistically significant were found at position 2 and the mean of the three positions in IL8. Moderate TBI cases also had higher TNFβ %5-mC compared with controls post-deployment for positions 1,2,3,4,5,6, and 9 and at the mean of the nine positions measured (cases: u=79.12%, controls: u=70.68%; p=0.01). Pre-deployment, there was also a pattern of moderate cases having higher IL8 %5-mC compared with controls; only the differences at position 6 and the mean of the nine positions were statistically significant. There were other pre-deployment differences found for the mild cases: IL1β positions 14 and 15, IL13 position 3, and NGF positions 1 and 11, but there were no consistent patterns found in those

promoters. For severe cases, an adjusted analysis proved to be too unstable for the low number of cases, so we carried out simple t-tests on all severe cases versus controls. We found that severe cases had reduced IL8 %5-mC post-deployment (3.54%) compared with controls post-deployment (6.72%); p=0.03.

Table 2b. presents the results from the case-case and the control-control comparisons, pre-deployment to post-deployment. For mild cases there was a pattern of increased IL1β %5-mC post-deployment, compared with pre-deployment, particularly at positions 14, 15, and 16 and at for the mean of the four positions measured (pre: u=8.70%, post: u=17.30%; p=0.01). There was only one significant control-control difference, at NGF position 15 (pre: u=2.09%, post: u=0.45%; p=0.04). For moderate cases there was significantly lower NGF %5-mC at one out of the 17 positions measured (NGF position 2). There were no significant changes in IL1β methylation for cases from pre- to post-deployment. Likewise, there were no significant changes in IL1β methylation for controls from pre- to post-deployment. The only significant change found in controls from pre- to post-deployment was reduced NGF %5-mC post-deployment at position 15.

Table 2c. presents the results from the multivariate ANOVA, comparing adjusted mean of pre-post methylation difference for cases versus controls. The adjusted mean of the difference was adjusted for age, gender, and race. From this analysis we see that IL1β %5-mC for the mean of the four positions measured increased in cases, pre- to post-deployment (+6.75%), while it decreased in controls (-6.56%), pre- to post-deployment, and the difference was statistically significant (p=0.02). We also found that NGF%5-mC change was significantly different for cases and controls at positions 1, 3, 13, and 15 (ie, at 4 positions out of the 17 measured). However the direction of these differences was not consistent across the positions with a significant difference. We did not carry out this analysis on severe cases, due to lack of power in that small group of subjects.

Table 3a presents the results from the case-control comparisons for serum cytokines for mild and moderate cases vs. controls, pre- and post-deployment. We found that post-deployment, mild TBI cases had lower serum protein expression compared with controls for IL6 (cases: μ=24.78 pg/ml, controls: μ=44.88 pg/ml; p=0.02) and IL8 (cases: u=82.52 pg/ml, controls: u=158.85 pg/ml; p=0.03). Post-deployment, moderate TBI cases had lower serum protein expression compared with controls IL6 (cases: u=16.48 pg/ml, controls u=44.88 pg/ml; p=0.01), IL8 (cases: u=60.09 pg/ml, controls: u=158.85 pg/ml; p=0.02). Although not a statistically significant difference, moderate cases also had lower IL1β levels than controls, post-deployment (cases: u=3.68, controls: u=13.25; p=0.08). Pre-deployment, however, moderate cases had significantly elevated IL8 levels compared with controls (cases: u=162.98, controls: u=77.29; p=0.04). Pre-deployment, mild cases had lower levels of TNFa compared with controls (cases: u=30.79, controls: u=41.48; p=0.02). Pre-deployment, moderate cases had higher levels of IL8 than controls (cases: u=32.18, controls: u=31.12; p=0.04), and lower levels than controls for IL10 (cases: u=25.17, controls: u=26.76; p=0.01), IL17 (cases: u=22.48, controls: u=23.39; p=0.01), and MMP9 (cases: u=37.67, controls: u=48.02; p=0.05). For severe cases, an adjusted analysis proved to be too unstable for the low number of cases, so we carried out simple t-tests on all severe cases versus controls. We found that severe cases had reduced MMP3 cytokine levels post-deployment (14,486.17 pg/ml) compared with controls post-deployment (22,591.80 pg/ml); p=0.01.

Table 3b. presents the results from the case-case and the control-control comparisons, pre-deployment to post-deployment. The paired analyses of case-case comparisons and control-control comparisons from pre- to post-deployment indicated no changes of controls' protein levels from pre- to post-deployment time periods. However, mild cases had significantly decreased levels post-deployment compared with pre-deployment for IL8 (post: u=8.21, pre: u=78.57; $p<0.01$), as did moderate cases (post: u=87.15, pre: u=222.36; $p<0.01$). Mild cases also had significantly reduced MMP3 protein levels post-deployment, compared with pre-deployment (post: u=16,734.76, pre: u=19,766.71; p=0.05). Moderate cases had the same pattern for MMP3, though the difference between pre- and post- levels was not statistically significant (cases: u=18,874.00, controls: u=22,088.43; p=0.10). For severe cases, an adjusted analysis proved to be too unstable for the low number of paired cases, so we carried out unadjusted paired t-tests on all severe cases. Severe cases post-injury/deployment had significantly lower IL8 serum cytokine levels than pre-deployment (post: u=100.49 pg/ml, pre: u=151.91 pg/ml; p=<0.01). The same pattern of reduced serum cytokines in post-injury severe cases was found for MMP3 (post: u=20,449.80, pre: u=22,423.39; p=0.04).

Table 3c. presents the results from the multivariate ANOVA, comparing adjusted mean of pre-post cytokine difference for cases versus controls. The adjusted mean of the difference was adjusted for age, gender, and race. From this analysis we see that IL8 decreased in mild cases, pre- to post-deployment (-50.73), while it increased in controls (+48.64), pre- to post-deployment, and the difference was statistically significant (p=0.03). For moderate cases, levels decreased (-196.81) significantly more than controls' levels (-16.44); $p<0.01$. For moderate cases, levels of MMP3 decreased (-4,328.85), while levels in controls increased (+1,200.28); p=0.05. We did not carry out this analysis on severe cases, due to lack of power in that small group of subjects.

Key Research Accomplishments:

We investigated potential epigenetic and protein biomarkers for TBI in serum. The results from this exploratory, small study are intriguing, such as differential methylation of IL1β for mild cases, TNFβ for moderate cases, and IL8 for severe cases, as well as differential serum cytokine expression of IL6 in mild and moderate cases, IL8 in mild, moderate, and severe cases, and MMP3 in severe cases. This is the first study, to our knowledge, of chronic epigenetic and protein-based alterations in TBI, which we were able to investigate based on the long period (mean was approximately one year) between injury and serum sample draw.

Reportable Outcomes:

This research has been presented at three conferences. At the time of the first two conferences, we did not have any scientific results. The results of this study were recently presented at the 10th Annual Society for Brain Mapping and Therapeutics Conference in a session of the epigenetics of TBI. All three conferences are listed below.

1. Military Health Research Forum, September, 2009, Kansas City, MO: "Epigenetic Patterns of TBI: DNA methylation in serum of OIF/OEF Service Members."
2. Department of Defense TBI Biomarkers In-Progress Review, July, 2011, Fort Lauderdale, FL: "DNA methylation and cytokine markers in TBI: a case-control study."

3. Society for Brain Mapping and Therapeutics, 14 May, 2013, Baltimore, MD "Epigenetic Patterns of TBI: DNA Methylation in Serum of OEF/OIF Service Members"

Conclusions:

Our study found a consistent pattern of reduced IL8 %5-mC in both mild and moderate cases, compared with controls, post-deployment. However, levels in people who experienced a TBI during their deployment did not change significantly from the pre-deployment sample to the post-injury/deployment sample, while levels in controls increased, though not significantly, across deployment. We found no difference between cases and controls in the change in IL8 %5-mC from pre- to post-deployment. Thus, while we see a significantly lower level in cases compared with controls post-deployment, it appears to stem from a non-statistically significant increase in controls' levels from pre- to post-deployment. Cytokine levels of IL8, however were significantly lower in both mild and moderate cases compared with controls, post-deployment, and there was a significant difference in the change of cytokines levels from pre- to post-deployment between cases (- change) and controls (+ change in mild and a slight – change in moderate). We also found a reduction in IL-8 serum cytokines post-deployment among severe cases, compared with controls, and cases had reduced IL8 %5mC post-deployment compared with pre-deployment. This change in IL8 among cases of mild, moderate, and severe TBI suggests a chronic reduction of IL8 in serum after a TBI, despite the severity.

IL-1β %5-mC was significantly lower in mild cases compared with controls, pre-deployment. Levels in cases increased significantly in cases after injury/deployment. Although levels in controls did not significantly decrease post-deployment, our analysis of the change in methylation from pre- to post-deployment, comparing cases to controls indicates a significant difference in the increase among cases and the decrease among controls. Cytokine levels, however of IL-1β were not altered significantly in any of our comparisons.

TNFβ %5-mC levels pre-deployment were significantly higher in moderate cases compared to controls, and a similar pattern persisted post-injury/deployment. This was evident at most of the 9 positions measured and for the mean %5-mC of the 9 positions. We found no difference between cases and controls in the change in TNFβ %5-mC from pre- to post-deployment. Thus, while we see a significantly higher level in cases compared with controls post-deployment, this was also the pattern found pre-deployment, and there was no appreciable change in cases from pre- to post-injury/deployment.

IL6 serum cytokine expression was reduced in both mild and moderate cases compared with controls, post-deployment. However, we found no difference between cases and controls in the change in IL6 serum cytokines from pre- to post-deployment.

For MMP3, our analysis of the change in cytokine levels from pre- to post-deployment, comparing moderate cases to controls indicates a significant difference in the decrease among moderate cases and the increase among controls. We also found significantly reduced serum cytokine MMP3 levels in cases compared with controls, post-injury/deployment as well as reduced levels in cases post- vs. cases pre-.

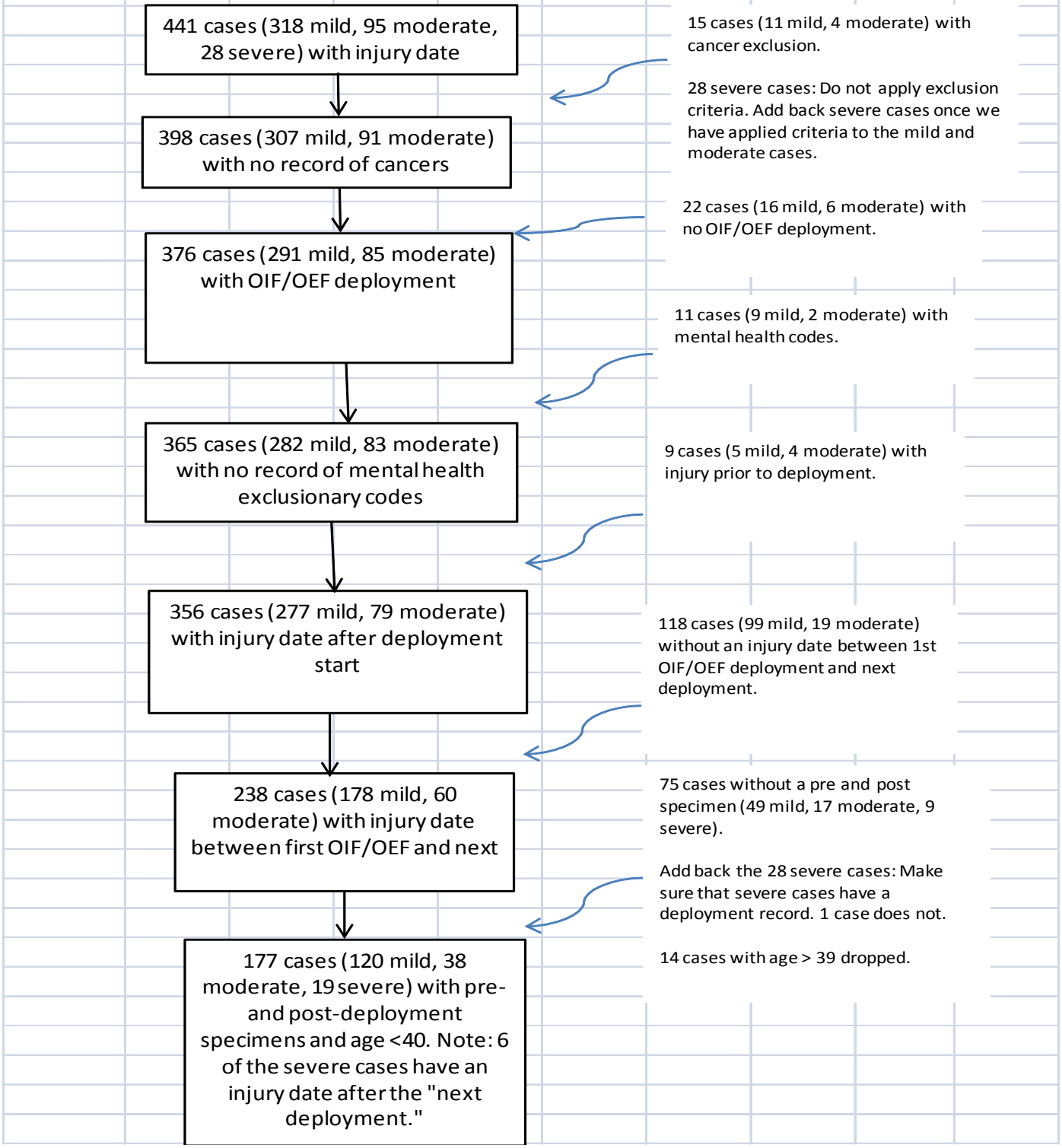
These findings may represent possible biomarkers for TBI, particularly the change in IL8 among cases of mild, moderate, and severe TBI suggests a chronic reduction of IL8 in serum after a

TBI, despite the severity. However, these results should be confirmed in larger studies and should be interpreted with caution.

References:

1. Dash P, Kobori N, Moore A: **A Molecular Description of Brain Trauma Pathophysiology Using Microarray Technology: An Overview.** *Neurochemical Research* 2004, **29** 1275-1286.
2. P. G-H: **Microarrays and Expression Profiling in Microglia Research and in Inflammatory Brain Disorders.** *Journal of Neuroscience Research* 2005, **81:**327-341.
3. Marciano P, Eberwine J, Raghupathi R, McIntosh T: **The assessment of genomic alterations using DNA arrays following traumatic brain injury: a review.** *Restor Neurol Neurosci* 2001, **18:**105-113.
4. Marciano P, Eberwine J, Ragupathi R, Saatman K, Meaney D, McIntosh T: **Expression Profiling following Traumatic Brain Injury: A Review.** *Neurochemical Research* 2002, **27:**1147-1155.
5. Morganti-Kossman M, Lenzlinger P, Hans V, Stahel P, Csuka E, Ammann E, Stocker R, Trentz O, Kossmann T: **Production of cytokines following brain injury: beneficial and deleterious for the damaged tissue.** *Molecular Psychiatry* 1997, **2:**133-136.

Appendix 1. Rusiecki TBI case-control serum study comparing DNA methylation in serum



Supporting Data

Table 1. Basic Characteristics of Study Population

	Case		Controls		*p*-value
Categorical Characteristic	**N**	**%**	**N**	**%**	
Age	150		50		
<20	20	13.3	3	6.0	
20~24	82	54.7	27	54.0	0.19
25~29	33	22.0	10	20.0	
>=30	15	10.0	10	20.0	
Gender	150		50		
Male	144	96.0	48	96.0	1.00
Female	6	4.0	2	4.0	
Race	150		50		
White	129	86.0	43	86.0	
Black	10	6.7	3	6.0	0.98
Other	11	7.3	4	8.0	
Deployment Length	150		50		
Shorter	122	81.3	42	84.0	0.67
Longer	28	18.7	8	16.0	
PTSD	148		42		
No	111	75.0	42	100.0	0.00
Yes	37	25.0	0	0.0	
Extra Cranial Injury	150		50		
No	15	10.0	0	0.0	N/A
Yes	135	90.0	0	0.0	
Blast Injuries	150		50		
Blast only	116	77.3	0	0.0	
Blast and fragment	5	3.3	0	0.0	
Blast and vehicular	21	14.0	0	0.0	
Blast and fall	3	2.0	0	0.0	N/A
Blast, fragment and vehicular	3	2.0	0	0.0	
Blast, vehicular and fall	1	0.7	0	0.0	
Blast and other	1	0.7	0	0.0	

Continuous Characteristic	N	Mean (SD)	N	Mean (SD)	*p*-value
Length of Deployment (days)	150	248 (172)	50	219 (126)	0.21
Time from Start Deployment to Pre-Deployment serum sample draw	150	-99 (79)	50	-118 (94)	0.16
Time from Injury to Post-Injury serum sample - cases only	143	316 (261)			N/A
Time from End Deployment to Post-Deployment serum sample - controls only			50	192 (280)	N/A

Table 2a. Generalized Linear Models investigating the adjusted means and differences between

Gene/locus	Comparison	Mild								Moderate							
		Pre-deployment				Post-deployment				Pre-deployment				Post-deployment			
		N	mean*	*s.e.*	p-value	N	mean*	*s.e.*	p-value	N	mean*	*s.e.*	p-value	N	mean*	*s.e.*	p-value
Line1_Pos1	case	93	81.11	0.29	0.84	92	80.90	0.31	0.78	38	80.24	0.53	0.28	37	80.55	0.50	0.76
	control	50	81.01	0.40		50	80.75	0.42		50	81.01	0.46		50	80.75	0.43	
Line1_Pos2	case	93	76.48	0.28	0.07	92	76.19	0.28	0.40	38	75.10	0.46	0.38	37	75.78	0.45	0.99
	control	50	75.63	0.38		50	75.79	0.38		50	75.63	0.40		50	75.79	0.39	
Line1_Pos3	case	93	73.00	0.26	0.17	92	72.83	0.29	0.18	38	72.08	0.46	0.61	37	72.26	0.47	0.91
	control	50	72.39	0.36		50	72.18	0.39		50	72.39	0.40		50	72.18	0.40	
Line1_Pos4	case	93	69.95	0.31	0.22	92	69.96	0.33	0.28	38	68.67	0.50	0.35	37	69.40	0.58	0.96
	control	50	69.30	0.42		50	69.36	0.44		50	69.30	0.44		50	69.36	0.50	
Line1_Mean	case	93	75.13	0.25	0.19	92	74.97	0.27	0.33	38	74.02	0.44	0.34	37	74.50	0.45	0.97
	control	50	74.58	0.34		50	74.52	0.37		50	74.58	0.38		50	74.52	0.39	
Alu_Pos1	case	82	31.00	0.22	0.99	77	31.10	0.23	0.51	34	31.69	0.32	0.11	28	31.45	0.37	0.83
	control	40	31.00	0.31		44	31.35	0.30		40	31.00	0.29		44	31.35	0.29	
Alu_Pos2	case	82	25.98	0.25	0.19	77	25.96	0.26	0.14	34	25.59	0.42	0.09	28	26.21	0.45	0.50
	control	40	26.57	0.36		44	26.59	0.34		40	26.57	0.39		44	26.59	0.36	
Alu_Pos3	case	82	15.31	0.18	0.61	77	15.56	0.18	0.88	34	16.06	0.29	0.14	28	15.61	0.26	1.00
	control	40	15.47	0.26		44	15.61	0.24		40	15.47	0.27		44	15.61	0.20	
Alu_Pos4	case	82	22.87	0.16	0.21	77	22.63	0.19	0.31	34	22.55	0.26	0.07	28	23.06	0.27	0.73
	control	40	23.21	0.22		44	22.94	0.24		40	23.21	0.24		44	22.94	0.21	
Alu_Mean	case	82	23.79	0.12	0.19	77	23.81	0.13	0.14	34	23.97	0.16	0.68	28	24.08	0.19	0.86
	control	40	24.06	0.17		44	24.12	0.17		40	24.06	0.15		44	24.12	0.15	
IL1B_Pos14	case	69	6.55	1.95	0.03	66	14.74	2.43	0.25	31	12.41	3.59	0.67	25	15.79	3.99	0.27
	control	29	14.59	3.00		34	9.94	3.38		29	14.59	3.71		34	9.94	3.42	
IL1B_Pos15	case	69	8.99	2.09	0.02	66	18.42	2.73	0.49	31	13.90	3.62	0.45	25	15.59	3.83	0.93
	control	29	17.89	3.22		34	15.17	3.81		29	17.89	3.74		34	15.17	3.28	
IL1B_Pos16	case	66	22.04	2.12	0.49	57	28.80	2.62	0.25	31	29.00	3.29	0.40	22	29.36	4.75	0.37
	control	25	24.83	3.45		33	23.77	3.44		25	24.83	3.66		33	23.77	3.88	
IL1B_Pos17	case	66	15.90	1.64	0.86	57	18.07	2.01	0.24	31	21.90	2.62	0.17	22	19.93	3.18	0.17
	control	25	16.44	2.67		33	14.18	2.64		25	16.44	2.92		33	14.18	2.59	
IL1B_Mean	case	65	13.27	1.62	0.10	56	20.35	2.13	0.20	29	19.92	2.72	0.70	22	20.78	3.51	0.28
	control	25	18.35	2.62		32	15.76	2.82		25	18.35	2.93		32	15.76	2.91	
IL6_Pos20	case	93	1.81	0.13	0.56	92	2.12	0.16	0.61	38	1.85	0.22	0.76	38	2.03	0.27	0.89
	control	50	1.94	0.18		50	1.98	0.21		50	1.94	0.19		50	1.98	0.24	
IL6_Pos21	case	93	2.17	0.15	0.98	92	2.54	0.18	0.49	38	2.11	0.22	0.85	38	2.04	0.23	0.33
	control	50	2.17	0.20		50	2.33	0.25		50	2.17	0.19		50	2.33	0.20	
IL6_Pos22	case	93	1.73	0.20	0.37	92	1.84	0.17	0.96	38	1.82	0.29	0.59	38	1.78	0.31	0.86
	control	50	2.03	0.27		50	1.86	0.23		50	2.03	0.25		50	1.86	0.27	
IL6_Mean	case	93	1.90	0.13	0.53	92	2.17	0.13	0.61	38	1.93	0.22	0.68	38	1.95	0.20	0.69
	control	50	2.04	0.18		50	2.06	0.17		50	2.04	0.19		50	2.06	0.18	
IL8_Pos1	case	90	3.15	0.42	1.00	86	3.86	1.04	0.05	37	4.09	0.80	0.39	36	1.96	1.89	0.03
	control	44	3.15	0.61		46	7.37	1.42		44	3.15	0.74		46	7.37	1.67	
IL8_Pos2	case	89	3.51	0.49	0.27	86	3.95	1.08	0.04	37	5.34	1.05	0.54	36	2.70	2.00	0.06
	control	42	4.46	0.71		44	7.81	1.51		42	4.46	0.98		44	7.81	1.81	
IL8_Pos3	case	86	2.25	0.31	0.55	86	2.84	0.97	0.29	35	2.06	0.44	0.39	37	2.68	1.83	0.44
	control	40	2.59	0.46		46	4.60	1.33		40	2.59	0.41		46	4.60	1.65	
IL8_Mean	case	84	3.01	0.32	0.44	85	3.55	0.98	0.06	35	4.01	0.62	0.51	35	2.50	1.89	0.10
	control	40	3.45	0.46		43	6.72	1.37		40	3.45	0.58		43	6.72	1.70	
IL10_Pos1	case	91	79.18	1.40	0.48	92	79.99	1.31	0.43	38	80.39	2.00	0.28	36	76.50	2.15	0.56
	control	47	77.49	1.95		46	78.17	1.86		47	77.49	1.80		46	78.17	1.90	
IL10_Pos2	case	91	82.76	1.12	0.49	92	83.02	1.04	0.51	38	80.67	1.62	0.12	36	80.24	1.79	0.10
	control	47	84.11	1.56		46	84.20	1.46		47	84.11	1.46		46	84.20	1.59	
IL10_Mean	case	91	80.97	1.01	0.92	92	81.51	0.90	0.84	38	80.53	1.48	0.89	36	78.37	1.41	0.14
	control	47	80.80	1.41		46	81.19	1.27		47	80.80	1.33		46	81.19	1.25	
IL13_CpG2	case	82	90.68	0.50	0.80	80	90.98	0.60	0.43	36	90.99	0.80	0.63	29	89.61	1.21	0.71
	control	43	90.46	0.70		44	90.19	0.81		43	90.46	0.73		44	90.19	0.98	
IL13_CpG3	case	82	91.25	0.66	0.05	80	90.89	0.44	0.10	36	91.61	1.14	0.10	29	91.53	0.74	0.05
	control	43	89.01	0.91		44	89.66	0.59		43	89.01	1.05		44	89.66	0.60	
IL13_CpG4	case	85	90.97	0.48	0.68	86	91.17	0.64	0.28	38	91.07	0.63	0.61	34	91.51	1.22	0.35
	control	43	90.63	0.68		44	89.98	0.90		43	90.63	0.59		44	89.98	1.07	
IL13_Mean	case	78	91.02	0.37	0.14	76	90.94	0.35	0.70	36	91.17	0.55	0.15	28	91.09	0.79	0.71
	control	40	90.08	0.51		41	90.71	0.48		40	90.08	0.52		41	90.71	0.66	
NGF_Pos1	case	86	0.69	0.66	0.03	81	1.04	0.20	0.64	37	0.53	1.27	0.14	37	0.97	0.30	0.85
	control	42	3.16	0.95		45	0.89	0.26		42	3.16	1.19		45	0.89	0.27	
NGF_Pos2	case	86	1.19	0.17	0.39	81	1.22	0.21	0.55	37	2.08	0.42	0.05	37	0.72	0.22	0.32
	control	41	0.94	0.24		45	1.02	0.28		41	0.94	0.40		45	1.02	0.20	
NGF_Pos3	case	86	1.73	0.50	0.19	81	2.21	0.40	0.15	37	1.27	0.86	0.18	37	1.34	0.39	0.84
	control	42	2.88	0.71		45	1.24	0.54		42	2.88	0.81		45	1.24	0.35	
NGF_Pos4	case	86	2.28	0.38	0.10	81	2.55	0.43	0.97	37	1.95	0.67	0.12	37	3.65	0.72	0.28
	control	42	3.38	0.54		45	2.58	0.58		42	3.38	0.63		45	2.58	0.65	
NGF_Pos5	case	86	3.24	0.68	0.20	81	3.32	0.52	0.92	37	3.91	1.28	0.62	37	2.98	0.63	0.77
	control	42	4.78	0.98		45	3.23	0.70		42	4.78	1.20		45	3.23	0.57	
NGF_Pos6	case	86	4.70	0.48	0.75	81	4.58	0.58	0.84	37	5.54	0.91	0.66	37	5.02	0.85	0.57
	control	41	4.98	0.69		45	4.38	0.78		41	4.98	0.87		45	4.38	0.77	
NGF_Pos7	case	86	2.85	0.45	0.64	81	2.79	0.41	0.08	37	2.54	0.78	0.53	37	2.82	0.47	0.05
	control	41	3.23	0.66		45	1.57	0.55		41	3.23	0.74		45	1.57	0.43	
NGF_Pos8	case	86	1.47	0.36	0.44	83	2.15	0.39	0.14	38	2.05	0.57	0.90	37	2.38	0.59	0.14
	control	42	1.95	0.51		46	1.19	0.52		42	1.95	0.54		46	1.19	0.53	
NGF_Pos9	case	86	1.60	0.40	0.49	83	2.13	0.41	0.05	38	1.46	0.71	0.52	37	1.66	0.33	0.06
	control	42	2.08	0.58		46	0.81	0.55		42	2.08	0.67		46	0.81	0.29	
NGF_Pos10	case	86	1.27	0.52	0.09	83	1.14	0.30	0.68	38	1.30	0.97	0.26	37	1.19	0.39	0.75
	control	42	2.84	0.75		46	1.35	0.41		42	2.84	0.93		46	1.35	0.35	
NGF_Pos11	case	86	2.39	0.50	0.02	83	3.32	0.68	0.64	38	2.81	0.78	0.15	37	3.49	1.13	0.81
	control	41	4.40	0.72		46	3.86	0.92		41	4.40	0.75		46	3.86	1.01	
NGF_Pos12	case	86	2.21	0.47	0.36	83	2.82	0.71	0.86	38	1.96	0.83	0.39	37	1.15	0.78	0.08
	control	42	2.96	0.67		46	3.04	0.96		42	2.96	0.79		46	3.04	0.70	
NGF_Pos13	case	86	1.34	0.26	0.17	83	1.34	0.54	0.38	38	1.41	0.42	0.22	37	0.99	0.96	0.38
	control	41	0.70	0.38		46	2.13	0.72		41	0.70	0.40		46	2.13	0.86	
NGF_Pos14	case	86	1.18	0.31	0.21	83	1.76	0.35	0.22	38	2.70	0.72	0.40	37	1.56	0.55	0.47
	control	42	1.86	0.44		46	1.03	0.47		42	1.86	0.68		46	1.03	0.49	
NGF_Pos15	case	86	1.15	0.33	0.07	83	1.13	0.30	0.27	38	1.49	0.66	0.43	37	1.55	0.43	0.10
	control	41	2.21	0.48		46	0.58	0.40		41	2.21	0.63		46	0.58	0.39	
NGF_Pos16	case	86	2.45	0.29	0.99	83	2.12	0.30	0.98	38	2.30	0.34	0.74	37	2.55	0.38	0.42
	control	42	2.46	0.41		46	2.14	0.40		42	2.46	0.33		46	2.14	0.34	
NGF_Pos17	case	86	0.42	0.14	0.52	83	0.57	0.16	0.73	38	0.99	0.31	0.33	37	0.48	0.24	0.98
	control	42	0.58	0.19		46	0.47	0.22		42	0.58	0.29		46	0.47	0.22	
NGF_Mean	case	86	1.89	0.22	0.15	79	2.13	0.21	0.43	37	2.14	0.43	0.58	37	2.03	0.20	0.53
	control	41	2.47	0.32		45	1.86	0.28		41	2.47	0.41		45	1.86	0.19	
TNFb_Pos1	case	66	74.07	1.86	0.18	52	73.85	2.31	0.21	30	77.23	2.75	0.05	22	78.30	2.82	0.02
	control	30	69.56	2.76		30	69.07	3.04		30	69.56	2.75		30	69.07	2.41	
TNFb_Pos2	case	66	67.56	2.04	0.33	52	69.43	2.32	0.08	30	71.28	2.89	0.08	22	71.92	3.13	0.03
	control	30	64.02	3.03		30	62.56	3.05		30	64.02	2.89		30	62.56	2.68	
TNFb_Pos3	case	66	67.65	1.91	0.38	52	68.99	2.28	0.08	30	70.58	2.82	0.14	22	71.31	3.04	0.03
	control	30	64.64	2.83		30	62.29	3.00		30	64.64	2.82		30	62.29	2.61	
TNFb_Pos4	case	66	71.97	1.91	0.27	52	72.81	2.28	0.06	30	73.91	2.69	0.13	22	75.45	3.16	0.02

	control	30	68.15	2.83		30	65.53	3.00		30	68.15	2.69		30	65.53	2.71	
TNFb_Pos5	case	66	68.37	1.83	0.51	52	71.11	2.28	0.06	30	72.83	2.87	0.11	22	73.80	2.92	0.01
	control	30	66.19	2.71		30	63.98	3.00		30	66.19	2.87		30	63.98	2.50	
TNFb_Pos6	case	66	79.79	1.93	0.20	52	79.62	2.33	0.36	30	83.89	2.84	0.04	22	84.89	2.63	0.01
	control	30	75.29	2.87		30	76.05	3.07		30	75.29	2.84		30	76.05	2.25	
TNFb_Pos7	case	80	83.53	1.60	0.22	77	83.22	1.83	0.82	34	84.84	2.46	0.16	30	86.96	2.23	0.32
	control	37	80.02	2.35		39	83.96	2.58		37	80.02	2.36		39	83.96	1.96	
TNFb_Pos8	case	80	82.83	1.63	0.14	77	82.69	1.79	0.94	34	81.74	2.63	0.37	30	88.08	2.11	0.07
	control	37	78.47	2.40		39	82.91	2.51		37	78.47	2.52		39	82.91	1.85	
TNFb_Pos9	case	80	75.99	1.74	0.50	77	75.29	2.10	0.63	34	78.27	2.58	0.23	30	83.39	2.80	0.01
	control	37	73.91	2.56		39	73.53	2.94		37	73.91	2.48		39	73.53	2.46	
TNFb_Mean	case	65	74.90	1.64	0.14	52	75.21	2.13	0.20	30	77.56	2.43	0.05	22	79.12	2.41	0.01
	control	30	70.55	2.42		30	70.68	2.81		30	70.55	2.43		30	70.68	2.07	

Table 2b. Generalized Linear Models for investigating the adjusted means and differences between controls pre- and post-deployment and cases pre- and post-deployment for DNA methylation

Gene/locus	Comparison	Control				Mild				Moderate			
		N	mean*	*s.e.*	p-value	N	mean*	*s.e.*	p-value	N	mean*	*s.e.*	p-value
Line1_Pos1	Pre	50	81.59	0.96	0.6820	92	81.29	0.61	0.6243	37	80.49	1.51	0.6175
	Post		81.33	0.96			81.09	0.61			80.87	1.51	
Line1_Pos2	Pre	50	75.86	0.78	0.7540	92	76.71	0.61	0.4894	37	75.75	1.37	0.2791
	Post		76.02	0.78			76.43	0.61			76.50	1.37	
Line1_Pos3	Pre	50	71.99	0.83	0.7041	92	72.89	0.57	0.6667	37	71.47	1.41	0.7375
	Post		71.78	0.83			72.73	0.57			71.70	1.41	
Line1_Pos4	Pre	50	69.44	0.94	0.9280	92	70.30	0.68	0.9734	37	68.55	1.68	0.3595
	Post		69.49	0.94			70.31	0.68			69.33	1.68	
Line1_Mean	Pre	50	74.72	0.78	0.9025	92	75.30	0.55	0.6683	37	74.06	1.36	0.4367
	Post		74.65	0.78			75.14	0.55			74.60	1.36	
Alu_Pos1	Pre	44	31.71	0.76	0.3304	87	31.03	0.54	0.6154	35	31.25	1.16	0.3710
	Post		32.21	0.76			30.85	0.54			30.71	1.16	
Alu_Pos2	Pre	44	25.98	0.92	0.7230	87	25.80	0.61	0.3769	35	25.59	1.28	0.2510
	Post		26.20	0.92			26.16	0.61			26.36	1.28	
Alu_Pos3	Pre	44	15.45	0.62	0.6277	87	15.69	0.50	0.9641	35	16.03	1.00	0.8676
	Post		15.65	0.62			15.71	0.50			15.95	1.00	
Alu_Pos4	Pre	44	23.15	0.55	0.5636	87	22.72	0.46	0.8931	35	22.47	0.74	0.0814
	Post		22.94	0.55			22.76	0.46			23.15	0.74	
Alu_Mean	Pre	44	24.07	0.35	0.4597	87	23.81	0.31	0.7736	35	23.84	0.47	0.4009
	Post		24.25	0.35			23.87	0.31		35	24.04	0.47	
IL1B_Pos14	Pre	21	15.11	10.52	0.3332	49	4.03	3.93	0.0171	21	10.17	9.67	0.5095
	Post		8.58	10.52			12.05	3.93			15.18	9.67	
IL1B_Pos15	Pre	21	22.66	10.56	0.6855	49	5.10	4.19	0.0081	21	9.51	8.52	0.7056
	Post		19.94	10.56			14.62	4.19			12.03	8.52	
IL1B_Pos16	Pre	17	24.17	5.28	0.6293	40	7.00	7.88	0.0235	19	16.02	8.85	0.6674
	Post		26.91	5.28			16.43	7.88			19.13	8.85	
IL1B_Pos17	Pre	17	14.56	4.54	0.5621	40	12.35	5.88	0.3511	19	12.28	6.64	0.8304
	Post		11.73	4.54			15.20	5.88			13.44	6.64	
IL1B_Mean	Pre	17	20.91	4.35	0.4138	39	8.70	3.43	0.0079	18	11.14	7.76	0.4615
	Post		17.07	4.35			17.30	3.43			15.91	7.76	
IL6_Pos20	Pre	50	1.49	0.46	0.8824	92	2.40	0.29	0.1148	38	3.88	0.65	0.5679
	Post		1.53	0.46			2.71	0.29			4.07	0.65	
IL6_Pos21	Pre	50	1.90	0.47	0.5880	92	2.41	0.36	0.1262	38	1.58	0.55	0.7842
	Post		2.07	0.47			2.77	0.36			1.51	0.55	
IL6_Pos22	Pre	50	1.73	0.52	0.6082	92	2.30	0.39	0.6284	38	4.20	0.87	0.9256
	Post		1.55	0.52			2.42	0.39			4.16	0.87	
IL6_Mean	Pre	50	1.71	0.38	0.9614	92	2.37	0.27	0.1402	38	3.22	0.58	0.9372
	Post		1.72	0.38			2.63	0.27			3.24	0.58	
IL8_Pos1	Pre	42	8.56	4.28	0.0541	83	2.18	1.05	0.2675	35	1.80	2.24	0.0908
	Post		13.08	4.28			2.98	1.05			-0.19	2.24	
IL8_Pos2	Pre	38	8.87	2.98	0.1633	82	2.58	1.05	0.3657	35	3.52	2.61	0.1123
	Post		12.76	2.98			3.23	1.05			1.35	2.61	
IL8_Pos3	Pre	39	6.71	2.61	0.3788	79	1.86	0.72	0.3081	34	0.76	1.46	0.3316

	Post		8.83	2.61			2.37	0.72			1.51	1.46	
IL8_Mean	Pre	36	7.36	2.73	0.1475	76	2.06	0.74	0.1769	32	2.08	1.64	0.1714
	Post		11.14	2.73			2.77	0.74			0.86	1.64	
IL10_Pos1	Pre	44	84.06	5.61	0.4126	90	80.66	2.82	0.6330	36	79.39	5.02	0.0756
	Post		86.45	5.61			81.56	2.82			74.78	5.02	
IL10_Pos2	Pre	44	88.04	3.99	0.8201	90	80.03	2.34	0.8250	36	85.98	5.11	0.8070
	Post		88.51	3.99			80.37	2.34			85.34	5.11	
IL10_Mean	Pre	44	86.05	3.61	0.4460	90	80.34	2.07	0.6531	36	82.69	3.98	0.1993
	Post		87.48	3.61			80.97	2.07			80.06	3.98	
IL13_CpG2	Pre	38	88.02	2.06	0.7688	69	88.97	1.15	0.8838	27	89.47	2.18	0.2110
	Post		88.37	2.06			89.08	1.15			87.43	2.18	
IL13_CpG3	Pre	38	89.68	1.56	0.6967	69	90.90	0.73	0.1736	27	91.94	0.95	0.2396
	Post		89.33	1.56			90.22	0.73			91.11	0.95	
IL13_CpG4	Pre	39	89.04	1.12	0.7542	78	89.15	1.43	0.5929	34	90.38	2.25	0.7187
	Post		88.71	1.12			89.56	1.43			90.81	2.25	
IL13_Mean	Pre	35	89.09	0.79	0.6541	61	89.02	0.78	0.5033	26	89.49	1.33	0.7010
	Post		89.43	0.79			88.72	0.78			89.11	1.33	
NGF_Pos1	Pre	39	17.99	2.59	0.0954	75	0.50	0.39	0.2586	37	0.75	0.66	0.1975
	Post		15.60	2.59			0.80	0.39			1.18	0.66	
NGF_Pos2	Pre	38	0.84	0.36	0.9557	75	0.85	0.43	0.6921	37	1.83	1.19	0.0257
	Post		0.83	0.36			0.97	0.43			0.47	1.19	
NGF_Pos3	Pre	39	1.45	2.16	0.1068	75	1.65	0.86	0.6831	37	0.27	1.16	0.8991
	Post		-0.47	2.16			1.89	0.86			0.35	1.16	
NGF_Pos4	Pre	39	1.91	2.00	0.5447	75	2.21	0.74	0.5240	37	2.69	1.77	0.0609
	Post		1.25	2.00			2.54	0.74			4.38	1.77	
NGF_Pos5	Pre	39	18.62	2.71	0.2470	75	2.61	1.04	0.8777	37	3.92	1.70	0.2783
	Post		16.89	2.71			2.72	1.04			2.99	1.70	
NGF_Pos6	Pre	38	6.52	1.18	0.6522	75	4.06	1.19	0.8845	37	7.03	2.76	0.7100
	Post		6.02	1.18			3.94	1.19			6.51	2.76	
NGF_Pos7	Pre	38	3.71	1.17	0.1378	75	2.57	0.91	0.7893	37	1.58	1.51	0.7219
	Post		2.08	1.17			2.74	0.91			1.86	1.51	
NGF_Pos8	Pre	40	1.56	1.38	0.3188	77	0.85	0.84	0.2066	37	0.62	1.86	0.6294
	Post		0.81	1.38			1.60	0.84			1.07	1.86	
NGF_Pos9	Pre	40	1.27	1.65	0.1465	77	2.16	0.89	0.5293	37	3.42	1.21	0.7959
	Post		-0.04	1.65			2.56	0.89			3.57	1.21	
NGF_Pos10	Pre	40	12.77	2.17	0.1771	77	0.96	0.59	0.9195	37	2.07	0.90	0.7538
	Post		11.17	2.17			0.92	0.59			1.93	0.90	
NGF_Pos11	Pre	39	3.62	1.73	0.7742	77	3.63	1.09	0.2660	37	7.22	1.69	0.3163
	Post		4.07	1.73			4.48	1.09			8.08	1.69	
NGF_Pos12	Pre	40	1.03	2.15	0.5092	77	1.84	1.21	0.2939	37	1.17	1.36	0.2172
	Post		0.26	2.15			2.73	1.21			0.31	1.36	
NGF_Pos13	Pre	39	0.61	1.44	0.1899	77	1.90	0.65	0.9670	37	1.92	1.46	0.5341
	Post		2.37	1.44			1.88	0.65			1.46	1.46	
NGF_Pos14	Pre	40	0.13	1.51	0.3420	77	1.21	0.58	0.3071	37	3.93	1.98	0.2263
	Post		-0.65	1.51			1.63	0.58			2.71	1.98	
NGF_Pos15	Pre	39	2.09	0.86	0.0419	77	0.73	0.64	0.9418	37	1.14	1.68	0.9816
	Post		0.45	0.86			0.76	0.64			1.16	1.68	

NGF_Pos16	Pre	40	0.89	0.91	0.4972	77	2.37	0.68	0.5353	37	2.26	1.00	0.7099
	Post		0.56	0.91			2.08	0.68			2.45	1.00	
NGF_Pos17	Pre	40	-0.16	0.60	0.7752	77	0.63	0.31	0.6397	37	0.83	0.91	0.2479
	Post		-0.25	0.60			0.73	0.31			0.29	0.91	
NGF_Mean	Pre	38	2.66	0.64	0.3333	73	1.82	0.42	0.4538	37	2.51	0.63	0.7293
	Post		2.09	0.64			2.04	0.42			2.40	0.63	
TNFb_Pos1	Pre	22	70.54	4.52	0.9176	36	80.64	5.63	0.9700	16	84.38	5.43	0.9910
	Post		70.08	4.52			80.76	5.63			84.33	5.43	
TNFb_Pos2	Pre	22	64.72	4.62	0.8910	36	78.40	6.36	0.7881	16	80.53	5.91	0.8818
	Post		64.09	4.62			79.35	6.36			79.76	5.91	
TNFb_Pos3	Pre	22	65.32	4.41	0.7906	36	78.23	5.93	0.9183	16	78.73	5.60	0.9211
	Post		64.15	4.41			78.57	5.93			79.21	5.60	
TNFb_Pos4	Pre	22	68.95	4.72	0.8789	36	81.80	5.72	0.9234	16	80.04	5.42	0.7855
	Post		68.24	4.72			82.10	5.72			81.32	5.42	
TNFb_Pos5	Pre	22	67.00	4.59	0.8449	36	73.82	5.91	0.4444	16	80.45	5.19	0.9033
	Post		67.90	4.59			76.33	5.91			81.00	5.19	
TNFb_Pos6	Pre	22	72.44	4.53	0.5582	36	84.35	6.32	0.7967	16	89.21	5.97	0.9403
	Post		75.09	4.53			85.25	6.32			89.60	5.97	
TNFb_Pos7	Pre	32	78.31	3.84	0.3068	66	79.84	3.93	0.7283	26	89.03	4.95	0.7669
	Post		81.97	3.84			80.79	3.93			90.08	4.95	
TNFb_Pos8	Pre	32	78.10	3.58	0.1764	66	83.01	3.94	0.7415	26	86.58	5.36	0.1413
	Post		82.63	3.58			83.91	3.94			92.30	5.36	
TNFb_Pos9	Pre	32	73.43	3.95	0.2317	66	71.06	4.31	0.6530	26	84.49	5.21	0.3767
	Post		77.84	3.95			72.40	4.31			87.80	5.21	
TNFb_Mean	Pre	22	69.47	4.04	0.6746	35	81.62	5.05	0.7772	16	83.41	4.95	0.8022
	Post		71.16	4.04			82.43	5.05			84.49	4.95	

Table 2c. Multivariate ANOVA comparing adjusted* mean of pre-post methylation difference for cases versus controls

Gene promoter	Comparison	Mild				Moderate			
		N_{pairs}	mean *	*(s.e.)*	p-value	N_{pairs}	mean *	*(s.e.)*	p-value
Line1_Pos1	Case	92	-0.26	1.07	0.8002	37	-0.06	1.72	0.5412
	Control	50	-0.46	1.16		50	-0.70	1.61	
Line1_Pos2	Case	92	0.32	0.89	0.3977	37	-0.12	1.47	0.5317
	Control	50	0.88	0.97		50	-0.67	1.37	
Line1_Pos3	Case	92	0.63	0.93	0.7708	37	-0.42	1.58	0.6444
	Control	50	0.43	1.01		50	-0.85	1.48	
Line1_Pos4	Case	92	0.80	1.06	0.8989	37	0.99	1.77	0.4960
	Control	50	0.90	1.15		50	0.27	1.66	
Line1_Mean	Case	92	0.37	0.89	0.9209	37	0.10	1.49	0.5130
	Control	50	0.44	0.96		50	-0.49	1.39	
Alu_Pos1	Case	68	-0.83	0.86	0.3152	24	-0.96	1.26	0.2497
	Control	37	-0.16	0.96		37	-0.06	1.21	
Alu_Pos2	Case	68	1.46	0.96	0.8101	24	0.68	1.59	0.5699
	Control	37	1.64	1.08		37	0.12	1.52	
Alu_Pos3	Case	68	-0.15	0.70	0.9478	24	-0.11	1.02	0.2879
	Control	37	-0.11	0.79		37	0.55	0.98	
Alu_Pos4	Case	68	0.41	0.65	0.7936	24	0.56	0.95	0.0859
	Control	37	0.27	0.73		37	-0.46	0.91	
Alu_Mean	Case	68	0.22	0.43	0.5747	24	0.04	0.58	0.9965
	Control	37	0.41	0.49		37	0.04	0.55	
IL1B_Pos14	Case	49	14.39	14.14	0.0715	21	2.85	22.70	0.4196
	Control	21	1.88	13.57		21	-6.08	20.17	
IL1B_Pos15	Case	49	8.04	14.67	0.1308	21	-7.34	20.48	0.8225
	Control	21	-2.81	14.08		21	-9.57	18.20	
IL1B_Pos16	Case	40	-2.67	13.41	0.1317	19	7.76	11.66	0.6122
	Control	17	-14.00	14.60		17	3.11	11.44	
IL1B_Pos17	Case	40	-8.97	11.17	0.1538	19	0.18	11.12	0.5472
	Control	17	-17.89	12.15		17	-5.08	10.90	
IL1B_Mean	Case	39	6.75	5.18	0.0222	18	-0.16	11.25	0.3290
	Control	17	-6.56	6.52		17	-8.87	11.03	
IL6_Pos20	Case	92	-0.43	0.46	0.7358	38	1.81	0.73	0.7206
	Control	50	-0.55	0.50		50	1.66	0.68	
IL6_Pos21	Case	92	-0.65	0.56	0.5828	38	0.30	0.72	0.6063
	Control	50	-0.87	0.61		50	0.51	0.67	
IL6_Pos22	Case	92	-0.20	0.58	0.5695	38	1.38	0.91	0.7315
	Control	50	-0.44	0.63		50	1.19	0.85	
IL6_Mean	Case	92	-0.42	0.40	0.5101	38	1.16	0.62	0.9125
	Control	50	-0.62	0.44		50	1.12	0.58	
IL8_Pos1	Case	83	1.82	2.73	0.0785	35	-0.14	5.02	0.0503
	Control	42	5.47	3.05		42	5.28	4.92	
IL8_Pos2	Case	82	4.41	3.16	0.1339	35	4.00	7.14	0.1235
	Control	38	7.81	3.63		38	8.86	7.37	
IL8_Pos3	Case	79	4.19	2.61	0.4384	34	6.83	5.93	0.9060
	Control	39	5.63	2.99		39	7.14	6.12	
IL8_Mean	Case	76	4.26	2.80	0.1438	32	4.65	6.31	0.1953
	Control	36	7.28	3.22		36	8.38	6.51	
IL10_Pos1	Case	90	-1.13	4.91	0.8866	36	-7.23	7.74	0.0891
	Control	44	-0.62	5.53		44	-0.25	7.67	
IL10_Pos2	Case	90	6.46	3.65	0.8403	36	8.88	6.37	0.7409
	Control	44	7.00	4.11		44	9.99	6.32	
IL10_Mean	Case	90	2.66	3.44	0.8345	36	0.82	5.31	0.1502
	Control	44	3.19	3.87		44	4.87	5.27	
IL13_CpG2	Case	69	-0.66	1.97	0.5129	27	-7.05	4.27	0.1165
	Control	38	0.33	2.18		38	-3.99	4.00	
IL13_CpG3	Case	69	-2.29	1.36	0.4827	27	-5.37	2.99	0.4232
	Control	38	-1.56	1.51		38	-4.28	2.80	
IL13_CpG4	Case	78	0.27	2.47	0.7386	34	-1.33	3.54	0.7494
	Control	39	-0.17	2.72		39	-1.82	3.68	
IL13_Mean	Case	61	0.85	1.53	0.1261	26	-1.44	1.44	0.3096
	Control	35	2.23	1.70		35	-0.20	1.26	
NGF_Pos1	Case	75	-5.78	1.71	0.0313	37	-14.37	2.85	0.0693
	Control	39	-8.59	1.91		39	-17.19	2.83	
NGF_Pos2	Case	75	0.12	0.65	0.9247	37	-1.11	1.67	0.0598
	Control	38	0.16	0.74		38	0.24	1.73	
NGF_Pos3	Case	75	1.48	1.62	0.0372	37	0.73	2.58	0.1421
	Control	39	-1.08	1.80		39	-1.32	2.56	
NGF_Pos4	Case	75	-0.20	1.49	0.3829	37	3.07	2.69	0.1003
	Control	39	-1.18	1.66		39	0.66	2.67	
NGF_Pos5	Case	75	-5.55	2.08	0.2602	37	-16.04	3.11	0.6945
	Control	39	-7.33	2.32		39	-16.70	3.09	
NGF_Pos6	Case	75	-0.48	2.14	0.8084	37	-3.25	3.89	0.9962
	Control	38	-0.84	2.44		38	-3.26	4.04	
NGF_Pos7	Case	75	0.89	1.70	0.1374	37	-1.96	3.22	0.1297
	Control	38	-0.89	1.94		38	-4.06	3.35	
NGF_Pos8	Case	77	0.06	1.33	0.0844	37	-0.81	2.33	0.3326
	Control	40	-1.70	1.51		40	-2.03	2.31	
NGF_Pos9	Case	77	1.51	1.51	0.2031	37	-0.92	2.18	0.2156
	Control	40	0.04	1.70		40	-2.38	2.17	

NGF_Pos10	Case	77	-4.49	1.52	0.1376	37	-10.53	2.43	0.1932
	Control	40	-6.22	1.72		40	-12.24	2.42	
NGF_Pos11	Case	77	-4.15	2.20	0.5874	37	2.49	4.49	0.8671
	Control	39	-5.00	2.54		39	2.17	4.66	
NGF_Pos12	Case	77	0.56	1.90	0.1867	37	-0.45	2.43	0.9323
	Control	40	-1.36	2.15		40	-0.56	2.42	
NGF_Pos13	Case	77	-0.14	1.64	0.0397	37	-0.74	3.75	0.1075
	Control	39	2.26	1.89		39	1.84	3.90	
NGF_Pos14	Case	77	2.73	1.03	0.1032	37	-3.45	2.47	0.8842
	Control	40	1.44	1.16		40	-3.26	2.46	
NGF_Pos15	Case	77	0.02	1.26	0.0398	37	0.19	2.59	0.1111
	Control	39	-1.82	1.45		39	-1.58	2.70	
NGF_Pos16	Case	77	0.86	1.08	0.8733	37	-0.40	1.41	0.5306
	Control	40	0.99	1.22		40	-0.87	1.40	
NGF_Pos17	Case	77	0.24	0.52	0.3393	37	-0.41	0.93	0.5040
	Control	40	-0.14	0.58		40	-0.08	0.92	
NGF_Mean	Case	73	0.24	0.88	0.1838	37	-0.77	1.60	0.4449
	Control	38	-0.59	1.00		38	-1.29	1.66	
TNFb_Pos1	Case	36	-0.08	10.39	0.5645	16	6.25	8.02	0.7057
	Control	22	-3.04	10.79		22	3.93	8.02	
TNFb_Pos2	Case	36	0.05	10.71	0.4222	16	4.43	8.12	0.7650
	Control	22	-4.21	11.12		22	2.57	8.12	
TNFb_Pos3	Case	36	-0.82	10.07	0.3744	16	5.69	8.03	0.5705
	Control	22	-5.25	10.46		22	2.20	8.02	
TNFb_Pos4	Case	36	-4.15	9.83	0.5077	16	6.95	7.92	0.5134
	Control	22	-7.37	10.20		22	2.97	7.92	
TNFb_Pos5	Case	36	2.90	10.96	0.4649	16	2.66	8.01	0.7369
	Control	22	-1.06	11.37		22	0.60	8.01	
TNFb_Pos6	Case	36	-5.38	12.43	0.8450	16	5.17	8.92	0.9799
	Control	22	-6.58	12.90		22	5.35	8.92	
TNFb_Pos7	Case	66	8.80	6.65	0.4458	26	7.44	9.94	0.6960
	Control	32	12.37	7.67		32	9.27	10.13	
TNFb_Pos8	Case	66	9.72	6.62	0.3059	26	9.60	10.67	0.6569
	Control	32	14.50	7.64		32	7.37	10.87	
TNFb_Pos9	Case	66	4.88	7.04	0.3190	26	8.86	11.20	0.9439
	Control	32	9.82	8.13		32	9.23	11.41	
TNFb_Mean	Case	35	0.62	9.64	0.8124	16	5.70	7.54	0.8034
	Control	22	-0.52	10.01		22	4.26	7.53	

* Adjusted mean of the difference from pre- to post-deployment for cases and for controls

Table 3a. Generalized Linear Models investigating the adjusted means and differences between cases and controls, pre-deployment and post-deployment for serum cytokines

Cytokines	Comparison	Mild								Moderate							
		Pre-deployment				Post-deployment				Pre-deployment				Post-deployment			
		N	mean*	*s.e.*	p-value	N	mean*	*s.e.*	p-value	N	mean*	*s.e.*	p-value	N	mean*	*s.e.*	p-value
b_NGF	Case	92	69.39	17.24	0.5451	91	64.77	13.32	0.5296	37	72.70	20.57	0.5992	38	67.18	14.19	0.3813
	Control	50	87.01	23.38		47	50.38	18.54		50	87.01	17.69		47	50.38	12.76	
IL1a	Case	92	28.56	4.87	0.1258	91	20.51	4.07	0.7133	37	30.84	6.18	0.0706	38	22.40	4.96	0.9187
	Control	50	15.91	6.61		47	23.08	5.66		50	15.91	5.32		47	23.08	4.46	
IL1b	Case	92	15.33	5.06	0.6194	91	5.91	2.36	0.0723	37	13.05	5.41	0.7836	38	3.68	3.98	0.0771
	Control	50	11.09	6.86		47	13.25	3.29		50	11.09	4.65		47	13.25	3.58	
IL4	Case	93	69.95	0.31	0.2171	91	4.52	1.04	0.1182	38	68.67	0.50	0.3485	38	4.76	1.55	0.2246
	Control	50	69.30	0.42		47	7.31	1.44		50	69.30	0.44		47	7.31	1.40	
IL6	Case	93	75.13	0.25	0.1946	91	24.78	5.15	0.0242	38	74.02	0.44	0.3361	38	16.48	8.35	0.0134
	Control	50	74.58	0.34		47	44.88	7.16		50	74.58	0.38		47	44.88	7.51	
IL8	Case	91	31.25	0.25	0.7679	91	82.52	19.71	0.0254	38	32.18	0.37	0.0355	38	60.09	30.98	0.0201
	Control	47	31.12	0.35		47	158.85	27.43		47	31.12	0.33		47	158.85	27.86	
IL10	Case	91	26.00	0.28	0.1142	91	9.90	1.64	0.8462	38	25.17	0.44	0.0096	38	10.58	2.04	0.6564
	Control	47	26.76	0.39		47	9.35	2.28		47	26.76	0.40		47	9.35	1.84	
IL13	Case	91	15.74	0.23	0.9374	91	55.83	11.55	0.9350	38	16.35	0.32	0.1865	38	56.31	12.09	0.8975
	Control	47	15.77	0.33		47	54.21	16.07		47	15.77	0.29		47	54.21	10.87	
IL17	Case	91	22.92	0.19	0.1454	91	79.11	15.23	0.9953	38	22.48	0.27	0.0136	38	68.30	18.05	0.6621
	Control	47	23.39	0.26		47	78.95	21.19		47	23.39	0.24		47	78.95	16.23	
MCP1	Case	91	23.98	0.14	0.2396	91	127.77	8.69	0.4375	38	24.04	0.18	0.3827	38	100.77	9.49	0.2307
	Control	47	24.26	0.19		47	116.17	12.09		47	24.26	0.16		47	116.17	8.53	
MMP3	Case	61	49.10	2.55	0.9451	91	20407.64	994.69	0.2022	27	42.46	3.54	0.2130	38	20011.29	1467.90	0.1947
	Control	27	48.78	3.83		47	22591.80	1384.08		27	48.78	3.54		47	22591.80	1319.89	
MMP9	Case	74	39.12	2.48	0.0535	91	28781.32	1212.17	0.2893	30	37.67	3.69	0.0500	38	25267.68	1957.63	0.6217
	Control	31	48.02	3.83		47	26571.67	1686.69		31	48.02	3.63		47	26571.67	1760.25	
TGFb1	Case	74	23.76	1.90	0.1473	91	4558.05	1070.39	0.9583	30	23.86	3.07	0.2492	38	3515.26	1188.96	0.4783
	Control	31	28.87	2.94		47	4654.22	1489.41		31	28.87	3.02		47	4654.22	1069.08	
TNFa	Case	74	30.79	2.46	0.0203	91	696.00	89.41	0.9891	30	36.47	4.00	0.3756	38	598.44	125.06	0.5551
	Control	31	41.48	3.81		47	698.10	124.42		31	41.48	3.94		47	698.10	112.45	
TNFb	Case	74	38.00	2.12	0.1217	91	0.00	0.00		30	37.72	3.47	0.1972	38	0.00	0.00	
	Control	31	44.08	3.27		47	0.00	0.00		31	44.08	3.42		47	0.00	0.00	

Table 3b. Generalized Linear Models for investigating the adjusted means and differences between controls pre- and post-deployment and cases pre- and post-deployment for serum cytokines

Cytokines	Comparison	Control				Mild				Moderate			
		N	mean*	*s.e.*	p-value	N	mean*	*s.e.*	p-value	N	mean*	*s.e.*	p-value
b_NGF	Pre	47	117.99	36.21	0.1136	90	115.56	35.92	0.8302	37	100.68	35.08	0.2830
	Post	47	78.81	36.21		90	110.41	35.92		37	81.57	35.08	
IL1a	Pre	47	20.78	8.78	0.2023	90	36.81	10.97	0.2568	37	38.06	18.95	0.4141
	Post	47	28.42	8.78		90	28.48	10.97		37	30.22	18.95	
IL1b	Pre	47	13.92	9.88	0.8131	90	11.13	9.25	0.1175	37	22.21	11.36	0.0973
	Post	47	15.51	9.88		90	1.42	9.25		37	12.59	11.36	
IL4	Pre	47	7.85	2.67	0.1416	90	6.61	2.07	0.6802	37	3.00	4.32	0.4307
	Post	47	10.53	2.67		90	6.04	2.07		37	1.28	4.32	
IL6	Pre	47	57.06	26.27	0.7779	90	29.05	14.94	0.3143	37	52.79	44.58	0.0784
	Post	47	62.09	26.27		90	18.98	14.94		37	12.68	44.58	
IL8	Pre	47	102.15	68.72	0.3396	90	78.57	36.48	0.0044	37	222.36	74.41	0.0006
	Post	47	146.83	68.72		90	8.21	36.48		37	87.15	74.41	
IL10	Pre	47	13.69	3.82	0.6267	90	15.79	3.66	0.9442	37	15.59	4.96	0.8561
	Post	47	12.43	3.82		90	15.62	3.66		37	15.13	4.96	
IL13	Pre	47	94.68	25.30	0.5110	90	106.02	26.17	0.6251	37	145.05	38.54	0.0851
	Post	47	83.38	25.30		90	97.47	26.17		37	111.15	38.54	
IL17	Pre	47	110.99	32.17	0.4992	90	91.84	27.70	0.4967	37	107.81	37.85	0.4038
	Post	47	125.77	32.17		90	104.43	27.70		37	91.80	37.85	
MCP1	Pre	47	98.44	17.98	0.3817	90	101.50	17.98	0.5765	37	101.60	23.56	0.5855
	Post	47	109.14	17.98		90	108.22	17.98		37	95.10	23.56	
MMP3	Pre	47	19706.91	2837.43	0.3358	90	19766.71	2312.97	0.0519	37	22088.43	3800.60	0.0976
	Post	47	21566.52	2837.43		90	16743.76	2312.97		37	18874.00	3800.60	
MMP9	Pre	47	28117.76	3716.46	0.7807	90	29179.37	2659.90	0.4950	37	34487.29	5071.61	0.3596
	Post	47	27415.08	3716.46		90	30393.57	2659.90		37	32131.98	5071.61	
TGFb1	Pre	47	5874.74	2449.03	0.7866	90	5483.01	2131.58	0.6027	37	10584.54	2208.41	0.1440
	Post	47	5424.42	2449.03		90	4741.04	2131.58		37	8940.97	2208.41	
TNFa	Pre	47	1117.71	220.98	0.9257	90	711.78	182.18	0.6707	37	1313.82	327.11	0.2917
	Post	47	1131.71	220.98		90	763.57	182.18		37	1138.80	327.11	
TNFb	Pre	47	0.00	0.00		90	0.00	0.00		37	0.00	0.00	
	Post	47	0.00	0.00		90	0.00	0.00		37	0.00	0.00	

Table 3c. Multivariate ANOVA comparing adjusted mean of pre-post serum cytokines difference for cases versus controls

Cytokines	Comparison	Mild				Moderate			
		N_{pairs}	mean *	*(s.e.)*	p-value	N_{pairs}	mean *	*(s.e.)*	p-value
b_NGF	Case	90	9.30	48.42	0.3862	37	-70.22	52.75	0.6322
	Control	47	-22.72	52.83		47	-85.53	49.20	
IL1a	Case	90	6.94	12.97	0.1079	37	-1.52	16.61	0.1034
	Control	47	22.92	14.15		47	15.01	15.49	
IL1b	Case	90	-6.57	13.33	0.4993	37	-31.11	14.59	0.1600
	Control	47	0.30	14.54		47	-18.61	13.61	
IL4	Case	90	0.92	2.66	0.1411	37	-1.96	4.15	0.0639
	Control	47	3.92	2.91		47	2.74	3.87	
IL6	Case	90	-7.46	25.63	0.6390	37	-61.71	46.76	0.1028
	Control	47	1.71	27.97		47	-15.10	43.61	
IL8	Case	90	-50.73	59.37	0.0295	37	-196.81	98.62	0.0033
	Control	47	48.64	64.78		47	-16.44	91.97	
IL10	Case	90	-1.30	3.48	0.7994	37	-4.07	5.25	0.9694
	Control	47	-1.97	3.80		47	-4.20	4.89	
IL13	Case	90	8.95	22.82	0.6681	37	-18.75	37.89	0.2703
	Control	47	1.49	24.90		47	6.66	35.34	
IL17	Case	90	46.73	31.60	0.9760	37	-56.26	43.70	0.1834
	Control	47	46.01	34.48		47	-20.85	40.75	
MCP1	Case	90	37.19	27.21	0.9113	37	5.09	28.88	0.3296
	Control	47	34.88	29.69		47	22.19	26.93	
MMP3	Case	90	186.14	3225.32	0.0675	37	-4328.85	4629.62	0.0515
	Control	47	4709.54	3519.25		47	1200.28	4317.68	
MMP9	Case	90	7932.04	4320.41	0.5438	37	-778.35	5657.53	0.5426
	Control	47	5931.43	4714.13		47	1310.87	5276.32	
TGFb1	Case	90	686.79	2535.88	0.5728	37	-3577.49	3406.38	0.3905
	Control	47	1777.34	2766.97		47	-1801.46	3176.86	
TNFa	Case	90	108.53	265.43	0.9669	37	-330.55	381.58	0.3327
	Control	47	100.12	289.62		47	-105.96	355.87	
TNFb	Case	90	0.00	0.00		37	0.00	0.00	

Control	47	0.00	0.00		47	0.00	0.00

* Adjusted mean of the difference from pre- to post-deployment for cases and for controls

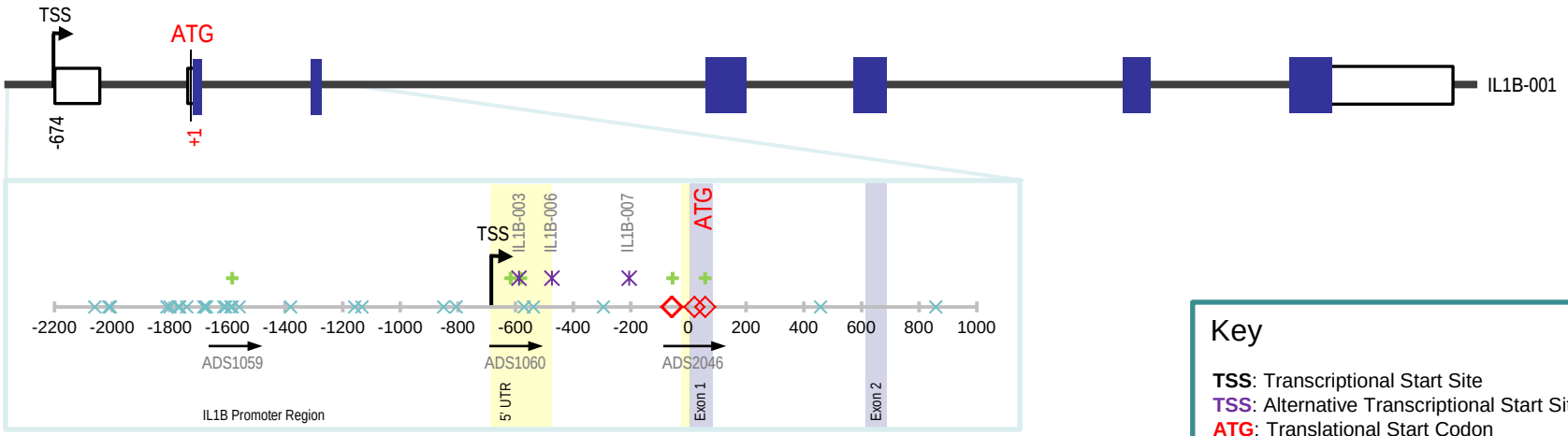
Figure 1. Gene Maps for DNA Methylation assays

EPIGENDX
Solutions for Genomic and Epigenomic Research

Human IL1B Gene
(Ensembl ID: ENSG00000125538)

Transcript ID: IL1B-001, ENST00000263341
Transcript Length: 1631 bp, 269 aa
CCDS: CCDS2102
Location: Chromosome 2: 113,587,328-113,594,480 reverse strand

Alternative Transcripts (7):
Major Alternative Transcripts (Protein-Coding):
IL1B-003 ENST00000418817 **IL1B-007** ENST00000432018
IL1B-006 ENST00000416750
Other transcripts available at ENSG00000125538



Key

TSS: Transcriptional Start Site
TSS: Alternative Transcriptional Start Site
ATG: Translational Start Codon

: Alternative TSS
: CpG site analyzed
: CpG site not analyzed
: SnP analyzed
: Exon region
: 5' or 3' UTR

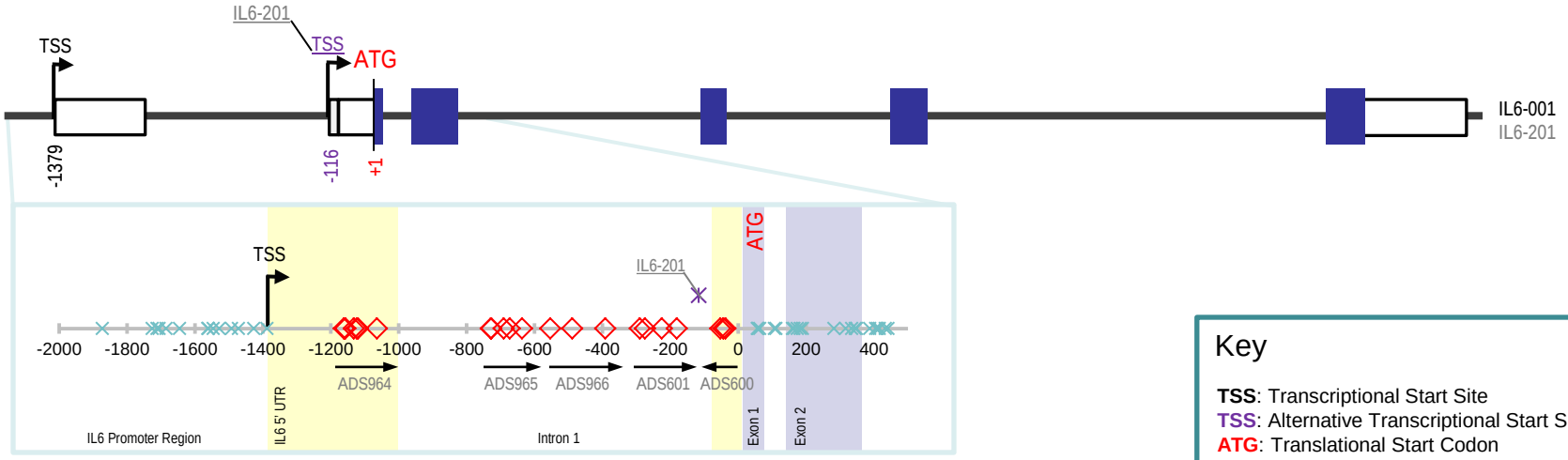
EPIGENDX
Solutions for Genomic and Epigenomic Research

Human IL6 Gene
(Ensembl ID: ENSG00000136244)

Transcript ID: IL6-001, ENST00000404625
Transcript Length: 1527 bp, 212 aa
CCDS: CCDS5375
Location: Chromosome 7: 22,765,503-22,771,621 forward strand

Alternative Transcripts (9):

Major Alternative Transcripts (CCDS-Referenced):
IL6-201 ENST00000258743 – CCDS5375
Other transcripts available at ENSG00000136244



Key

TSS: Transcriptional Start Site
TSS: Alternative Transcriptional Start Site
ATG: Translational Start Codon

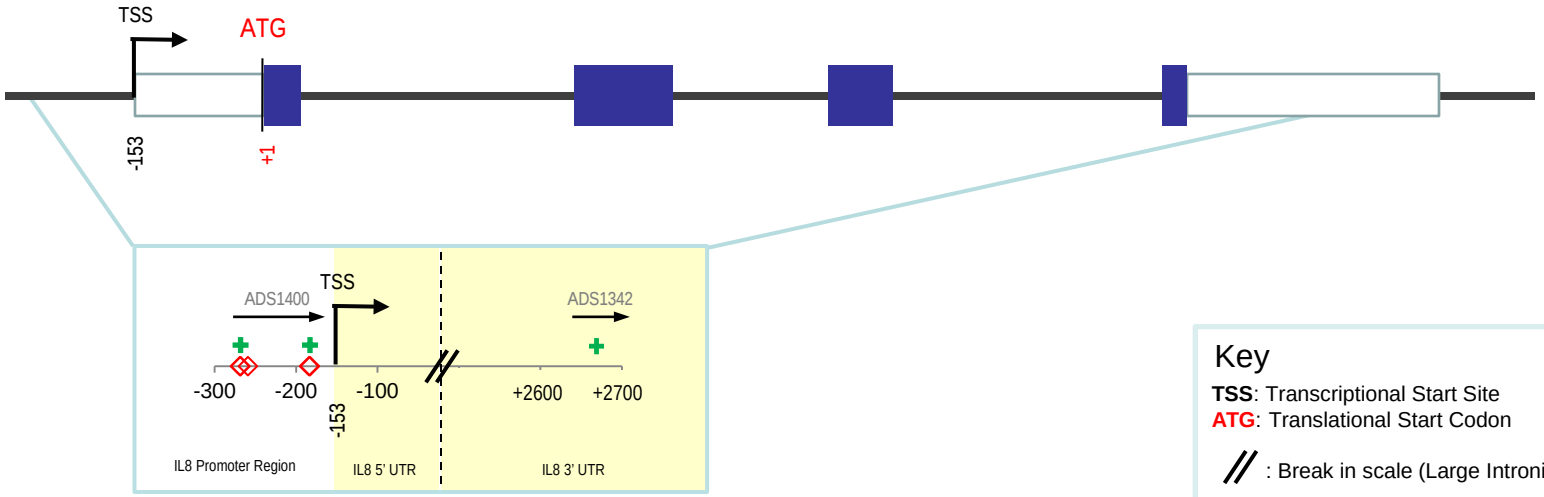
: Alternative TSS
: CpG site analyzed
: CpG site not analyzed
: SnP analyzed
: Exon region
: 5' or 3' UTR

EPIGENDX
Solutions for Genomic and Epigenomic Research

Human IL-8 Gene (Ensembl ID: ENSG00000169429)

Transcript ID IL8-001, ENST00000307407
Transcript Length: 1705 bp, 99 aa
Location: Chromosome 4: 74,606,223-74,609,433

Alternative Transcripts (2):
IL8-002 ENST00000401931
IL8-003 ENST00000483500



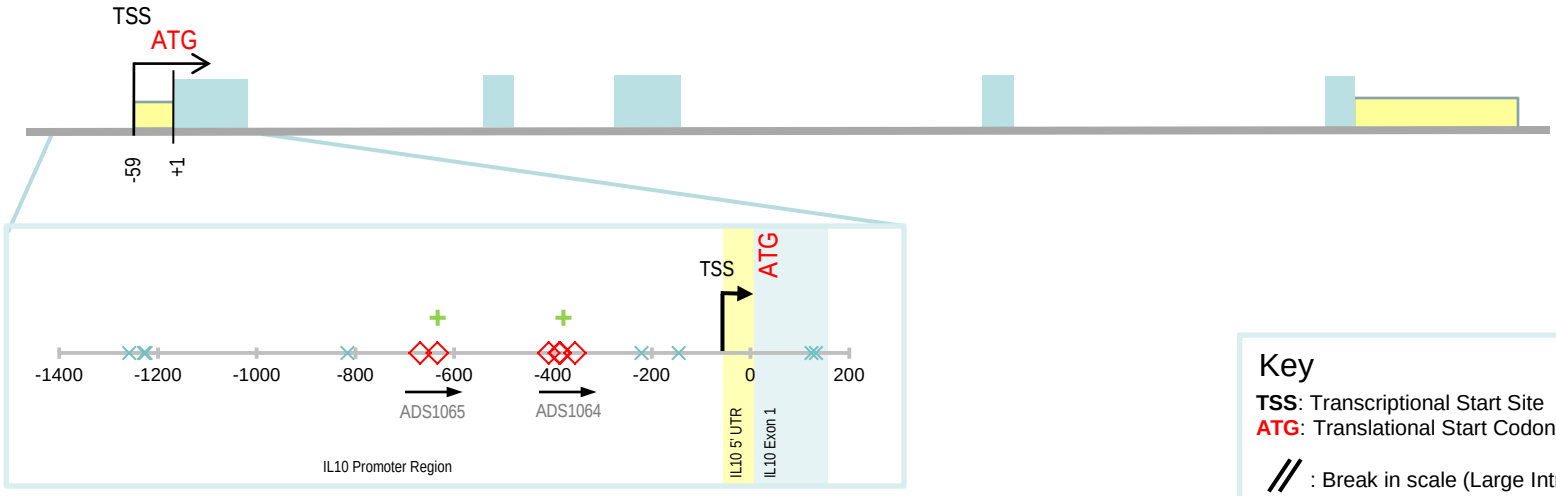
Key
TSS: Transcriptional Start Site
ATG: Translational Start Codon
: Break in scale (Large Intronic region)
: Analyzed CpG site
: Non-analyzed CpG site
: Analyzed SnP
: Exon region
: 5' or 3' UTR

EPIGENDX
Solutions for Genomic and Epigenomic Research

Human IL-10 Gene (Ensembl ID:ENSG00000136634)

Transcript ID IL10-001, ENST00000423557
Transcript Length: 1630 bp; 178 aa
Location: Chromosome 1: 206,940,947-206,945,839

Alternative Transcripts (2):
IL10-002 ENST00000471071
IL10-003 ENST00000367099



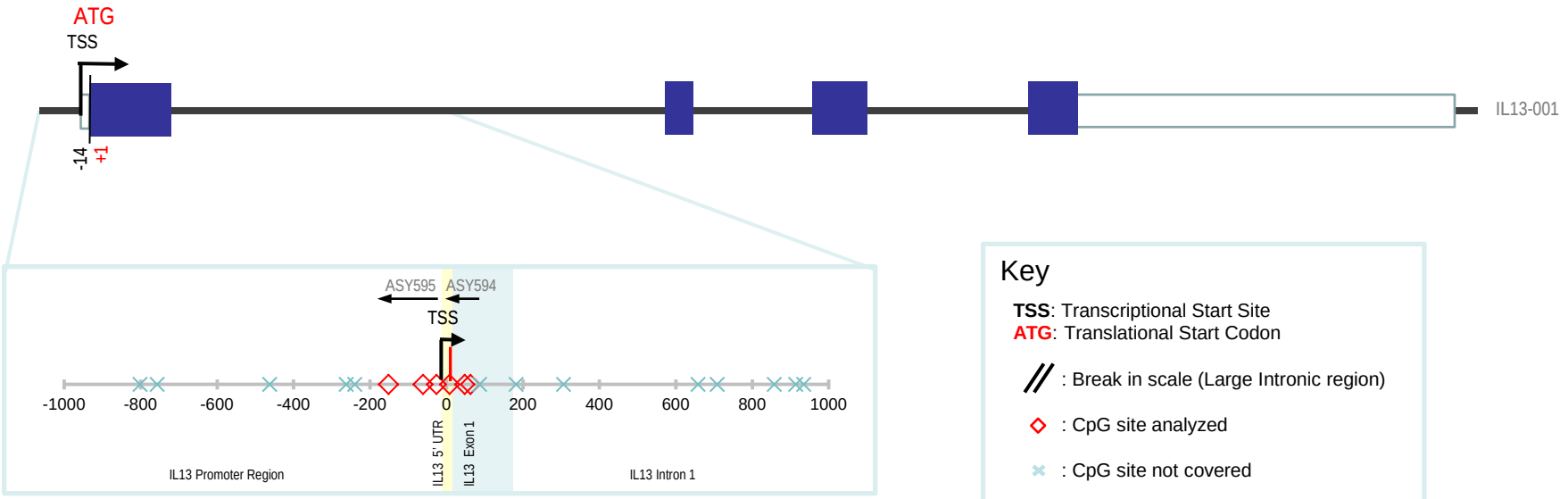
Key
TSS: Transcriptional Start Site
ATG: Translational Start Codon
: Break in scale (Large Intronic region)
: Analyzed CpG site
: Non-analyzed CpG site
: Analyzed SnP
: Exon region
: 5' or 3' UTR

EPIGENDX
Solutions for Genomic and Epigenomic Research

Human IL13 Gene
(Ensembl ID: ENSG00000169194)

Transcript ID IL13-001, ENST00000304506
Transcript Length: 1283 bp, 146 aa
Location Chromosome 5: 131,991,955-131,996,802 forward strand

Alternative Transcripts (4):
IL13-004 ENST00000459878
IL13-005 ENST00000462480
IL13-002 ENST00000468334
IL13-003 ENST00000487267



Key

TSS: Transcriptional Start Site
ATG: Translational Start Codon

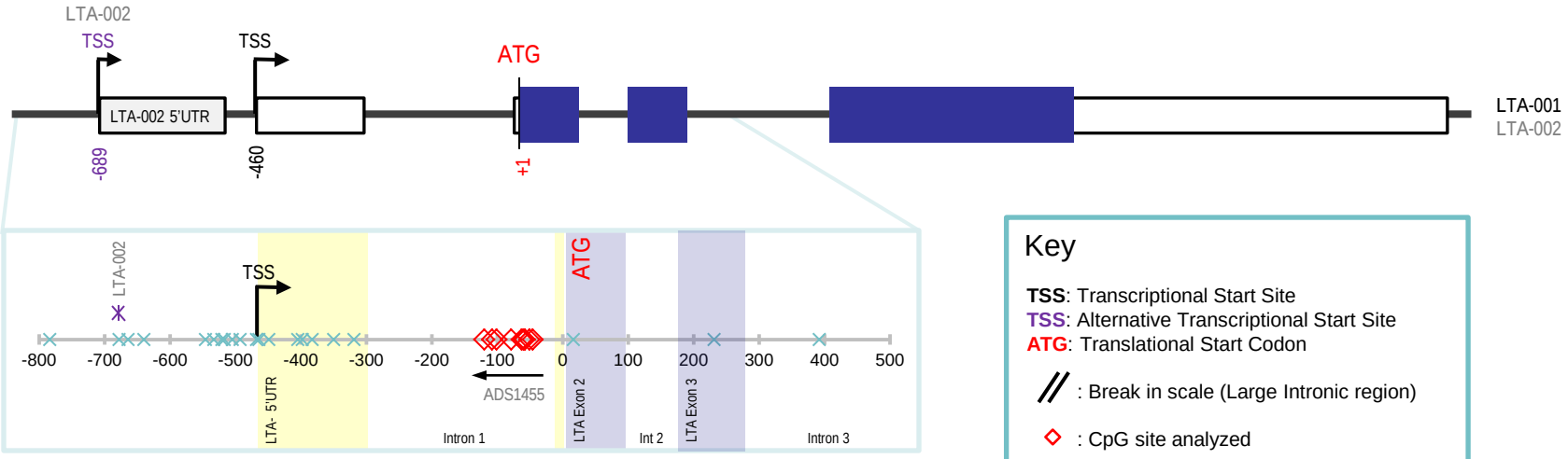
: Break in scale (Large Intronic region)
: CpG site analyzed
: CpG site not covered
: SNP analyzed
: Exon region
: 5' or 3' UTR

EPIGENDX
Solutions for Genomic and Epigenomic Research

Human LTA Gene
(Ensembl ID: ENSG00000226979)

Transcript ID: LTA-001, ENST00000418386
Transcript Length: 1422 bp, 205 aa
CCDS: CCDS4701
Location: Chromosome 6: 31,539,831-31,542,101 forward strand

Alternative Transcripts (4):
Major Alternative Transcripts (CCDS Referenced):
LTA-002 ENST00000454783 – CCDS4701
Other transcripts available at ENSG00000226979



Key

TSS: Transcriptional Start Site
TSS: Alternative Transcriptional Start Site
ATG: Translational Start Codon

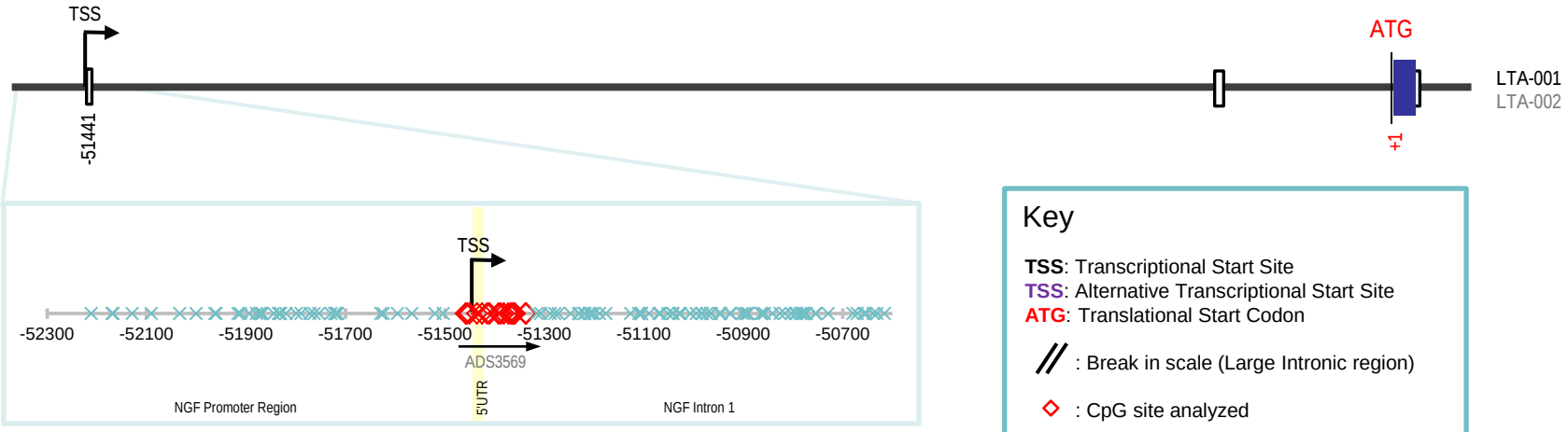
: Break in scale (Large Intronic region)
: CpG site analyzed
: CpG site not analyzed
: Alternative TSS
: Exon region
: 5' or 3' UTR

EPIGENDX
Solutions for Genomic and Epigenomic Research

Human NGF Gene
(Ensembl ID: ENSG00000134259)

Transcript ID: NGF-001, ENST00000369512
Transcript Length: 1047 bp, 241 aa
CCDS: CCDS882
Location: Chromosome 1: 115,828,539-115,880,857 reverse strand

Alternative Transcripts (0):
This gene has no alternative transcripts

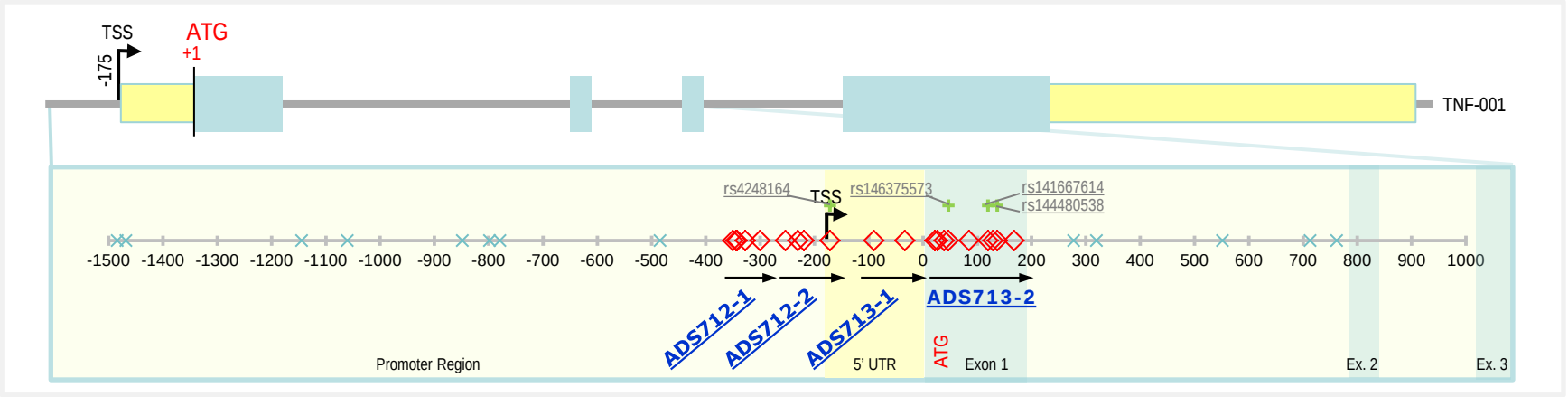


EPIGENDX
Solutions for Genomic and Epigenomic Research

Human TNF Gene
(Ensembl ID: ENSG00000134086)

Transcript ID: TNF-001, ENST00000449264
Transcript Length: 1676 bp, 233 aa
CCDS: CCDS4702
Location: Chromosome 6: 31,543,344-31,546,113 forward strand

Alternative Transcripts (0):
This gene has no alternative transcripts



Key

TSS: Transcriptional Start Site
TSS: Alternative Transcriptional Start Site
ATG: Translational Start Codon

: Break in scale (Large Intronic region)

: CpG site analyzed

:

: CpG site not analyzed

: SnP analyzed

: Exon region

: 5' or 3' (UTR)

: Alternative TSS (Protein Coding)

EPIGENDX
Solutions for Genomic and Epigenomic Research