Research Article

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Epigenomics

Epigenetic meta-analysis across three civilian cohorts identifies *NRG1* and *HGS* as blood-based biomarkers for post-traumatic stress disorder

Monica Uddin*[,1,2], Andrew Ratanatharathorn[3], Don Armstrong[1], Pei-Fen Kuan[4], Allison E Aiello[5], Evelyn J Bromet[6], Sandro Galea[7], Karestan C Koenen[8,9,10], Benjamin Luft[11], Kerry J Ressler[12], Derek E Wildman[1,13], Caroline M Nievergelt[14,15] & Alicia Smith[16]

[1]Carl R Woese Institute for Genomic Biology, University of Illinois at Urbana-Champaign, 1206 West Gregory Drive, Urbana, IL 61801, USA

[2]Department of Psychology, University of Illinois Urbana-Champaign, 603 East Daniel St, Champaign, IL 61820, USA

[3]Department of Epidemiology, Mailman School of Public Health, Columbia University, 722 West 168th St, NY 10032, USA

[4]Department of Applied Mathematics & Statistics, Stony Brook University, John S Toll Drive, Stony Brook, NY 11794, USA

[5]Department of Epidemiology, Gillings School of Public Health, University of North Carolina, 135 Dauer Drive, Chapel Hill, NC 27599, USA

[6]Department of Psychiatry, Stony Brook University School of Medicine, 101 Nicolls Rd, Stony Brook, NY 11794, USA

[7]Boston University School of Public Health, 715 Albany St, Boston, MA 02118, USA

[8]Department of Epidemiology, Harvard TH Chan School of Public Health, 677 Huntington Avenue, Boston, MA 02115, USA

[9]Psychiatric & Neurodevelopmental Genetics Unit & Department of Psychiatry, Massachusetts General Hospital, Simches Research Building, 185 Cambridge Street, Boston, MA 02114, USA

[10]Stanley Center for Psychiatric Research, Broad Institute, Cambridge, MA 02142, USA

[11]Department of Medicine, Stony Brook University School of Medicine, 101 Nicolls Road, Stony Brook, NY 11794, USA

[12]Department of Psychiatry, McLean Hospital, Harvard Medical School, 115 Mill Street, Belmont, MA 02478, USA

[13]Department of Molecular & Integrative Physiology, University of Illinois at Urbana-Champaign, 407 South Goodwin Avenue, Urbana, IL 61801, USA

[14]Department of Psychiatry, University of California San Diego School of Medicine, 9500 Gilman Dr, La Jolla, CA 92093, USA

[15]VA Center of Excellence for Stress & Mental Health, VA San Diego Healthcare System, 3350 La Jolla Village Drive, San Diego, CA 92161, USA

[16]Department of Psychiatry & Behavioral Sciences & Department of Obstetrics & Gynecology, Emory University School of Medicine, 100 Woodruff Circle, Atlanta, GA 30322, USA

*Author for correspondence: muddin@illinois.edu

Aim: Trauma exposure is a necessary, but not deterministic, contributor to post-traumatic stress disorder (PTSD). Epigenetic factors may distinguish between trauma-exposed individuals with versus without PTSD. **Materials & methods:** We conducted a meta-analysis of PTSD epigenome-wide association studies in trauma-exposed cohorts drawn from civilian contexts. Whole blood-derived DNA methylation levels were analyzed in 545 study participants, drawn from the three civilian cohorts participating in the PTSD working group of the Psychiatric Genomics Consortium. **Results:** Two CpG sites significantly associated with current PTSD in *NRG1* (cg23637605) and in *HGS* (cg19577098). **Conclusion:** PTSD is associated with differential methylation, measured in blood, within *HGS* and *NRG1* across three civilian cohorts.

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Post-traumatic stress disorder (PTSD) is a debilitating mental disorder that occurs following exposure to a life-threatening event. Symptoms include: intrusive recollections and persistent avoidance of stimuli associated with the traumatic event; negative alterations in cognition and mood; and notable changes in arousal and reactivity, all of which must persist for at least 1 month following trauma and be accompanied by a high degree of social and/or occupational impairment [1]. PTSD confers a high public health burden, with lifetime prevalence rates estimated at 6.8% in the USA [2] and 3.9% globally [3]. These estimates, however, are eclipsed by the prevalence of trauma exposure, which ranges from 60 to 90% among US adults, depending on the population being considered [4,5],

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and 70% globally [6]. The marked difference in prevalence between trauma exposure and PTSD onset suggests that distinct characteristics may distinguish between individuals with versus without the disorder.

Epigenetic factors – mechanisms that regulate gene function without altering underlying DNA sequence – have gained prominence as potential markers of PTSD risk, in part because of their ability to change in relation to lived experiences [7], including stressful and traumatic exposures [8]. Many of these findings have centered on loci involved in neurogenesis and/or neuronal plasticity and hypothalamic–pituitary–adrenal (HPA) axis signaling [9]. For example, work in animal models has shown that a PTSD-like phenotype is associated with increased hippocampal DNA methylation in *Bdnf* [10]. Similarly, exposure to early life adversity – a key risk factor for PTSD during adulthood [11] – has been associated with increased hippocampal DNA methylation levels in the glucocorticoid receptor in both rodent [12] and human *post mortem* studies [13]. Although brain-based work examining global epigenetic processes such as histone deacetylase activity is possible in living humans via the use of PET tracers [14], examination of DNA methylation at specific CpG sites of interest is not yet tractable; nevertheless, studies performed in blood have also shown alterations in DNA methylation at these loci among individuals with PTSD (reviewed in [9]), albeit with directions of effect which sometimes differ from brain-based findings in rodents.

Although previous locus-specific studies have helped to demonstrate that traumatic exposures are associated with changes in DNA methylation, such studies can miss identifying changes in additional genomic sites relevant to PTSD that are not captured by *a priori* hypotheses. A handful of studies have demonstrated PTSD-associated DNA methylation profiles in immune-related gene clusters using genome-scale, epigenome-wide association study (EWAS) approaches [15,16], although these studies have been modest in size and relied on early versions of available genome-scale DNA methylation arrays. A few EWAS studies have reported results based on newer versions of such genome-scale technologies in the context of PTSD; yet they, too, have been quite modest in size [17], have focused more explicitly on the integration of gene expression and DNA methylation data [18], or have included only male first responders and military personnel [19,20]. Moreover, given the potential heterogeneity within and among cohorts in relation to type, timing and duration of trauma exposures, which may increase variability within smaller samples and thus dilute potential signals of PTSD-associated differential methylation, it is critical to employ approaches that maximize power by combining data from single, smaller scale studies. Limited work comparing military versus civilian contexts more broadly indicates that military-related PTSD is accompanied by more severe symptomatology [21,22] but that civilian-related PTSD is associated with distinct patterns of brain activity in the default mode network [23], suggesting distinct clinical and brain network profiles may characterize PTSD onset due to these diverging social contexts. Finally, extant EWAS studies have not typically accounted for childhood maltreatment or smoking, both of which are associated with PTSD [11,24] and related to epigenetic differences [25].

To address this, here we use meta-analysis to test whether current PTSD is associated with differential methylation in civilian cohorts participating in the PTSD working group of the Psychiatric Genomics Consortium (PGC) [26]. We focus specifically on cohorts comprised of individuals recruited from civilian contexts in order to maximize our potential to discern PTSD-associated differential methylation in noncombat situations. We hypothesize that DNA methylation differences will distinguish individuals with current PTSD from trauma-exposed controls in three civilian cohorts; and a subset of these differences will remain following the control for important potential confounders, including gender and smoking status, as well as potential moderators, specifically exposure to childhood maltreatment [18]. To provide context to these findings, we also identify the likely transcript variants expressed in PTSD-relevant tissues associated with our topmost hits, and conduct exploratory secondary analyses of the relation between PTSD-associated differential methylation and mRNA expression, in order to assess the potential functional significance of observed differential methylation.

Methods

PGC-PTSD workgroup

The PGC-PTSD workgroup was established in 2013 in order to facilitate the sharing of genomic data across multiple cohorts to support large-scale studies of PTSD genetics [26]. Several subgroups within this workgroup have been established, including a PCG-PTSD Epigenetics Workgroup, which provided the data used for the work reported in this study. Within this workgroup, epigenomic data were available from a total of three civilian cohorts. These cohorts are described in more detail below.

Cohort descriptions

Detroit Neighborhood Health Study

See reference for details [15]. Briefly, the Detroit Neighborhood Health Study (DNHS) is an epidemiologic, community-based sample of adult (18 years or older) participants recruited as part of a representative sample of adult Detroit residents. DNHS participants (n = 1547 at the baseline wave) were assessed for PTSD symptoms using the PTSD checklist (PCL-C), a 17-item self-report measure of Diagnostic and Statistical Manual of Mental Disorders (DSM-IV) symptoms, and additional questions about duration, timing and impairment or disability due to the symptoms [27]. Participants were initially asked to identify potentially traumatic events that they experienced in the past from a list of 19 events. PTSD symptoms were then assessed by referencing two traumatic events that the respondent may have experienced: one that the participant regarded as the worst and one randomly selected event from the remaining potentially traumatic events a respondent may have experienced. Respondents were considered affected by lifetime PTSD if all six DSM-IV criteria were met in reference to either the worst or the random event. Current PTSD is defined in the DNHS as meeting the criteria for lifetime PTSD, with symptoms reported during the past month. Controls for this study had a history of trauma exposure but did not meet criteria for lifetime or current PTSD.

Childhood maltreatment was assessed in the DNHS using questions from the Conflict Tactics Scale [28], the Childhood Trauma Questionnaire [29], and Wyatt's eight-item interview guide as implemented in the Nurse's Health Study II [30]. Conflict Tactics Scale items assessed physical and emotional abuse before age 11, and the Childhood Trauma Questionnaire assessed physical and sexual abuse before age 18. Response options were rated on a five-point scale that ranged from 'never true' to 'very often true'. In total, sexual and emotional abuse were assessed with two questions each and physical abuse with seven questions. Scores were summed to create a continuous measure for each abuse type. Physical abuse was dichotomized at a score of four or greater, emotional abuse at a score of three or greater, and sexual abuse at a score of one or greater. The DNHS was approved by the institutional review board at the University of Michigan and University of North Carolina at Chapel Hill.

Grady Trauma Project

See reference for details [31]. In brief, the Grady Trauma Project (GTP) is comprised of participants recruited through waiting rooms of primary care or obstetric-gynecological clinics at Grady Memorial Hospital in Atlanta. Current and lifetime PTSD diagnosis was assessed by clinical psychologists using the Clinician-Administered PTSD Scale for DSM IV (CAPS-4) [32] or the Mini International Neuropsychiatric Interview DSM IV (MINI), an instrument designed to assess major Axis 1 disorders with high validity and reliability [33,34]. For this study, cases were specified as having current PTSD, and controls had no current or lifetime history of the disorder. Demographic variables including age, sex and race were assessed through self report. DNA methylation data used in this study is available at (GSE72680). The institutional review boards of Emory University School of Medicine and Grady Memorial Hospital approved this study.

In GTP, childhood maltreatment was assessed using the Childhood Trauma Questionnaire [29]. Items for each type of abuse added together and transformed into categories of 'none to minimal', 'low to moderate', 'moderate to severe' and 'severe to extreme'. Each abuse type was dichotomized by the 'severe to extreme' criteria. In both DNHS and GTP, a dichotomous childhood maltreatment variable was defined as experiencing two or more abuse types.

World Trade Center 9/11 first responders

See reference for details [35]. In brief, the World Trade Center (WTC) cohort was established based on a screening of treatment-seeking first responders who served as rescue and recovery workers during and after the WTC disaster. First responders were administered the Structured Clinical Interview for DSM-IV (SCID) PTSD module with interval instructions (i.e., worst episode of symptoms since 9/11/2001) and SCID items were modified to assess PTSD symptoms in relation to traumatic WTC exposures. The Committees on Research Involving Human Subjects at Stony Brook University approved the study.

DNA methylation quality control

Each cohort's Illumina HumanMethylation450 data were processed through a previously described pipeline that was developed to optimize performance of association testing in cohorts contributing to the EWAS arm of the PTSD PGC [36]. Briefly, following an inspection of control probes to confirm each step of the Infinium protocol, background normalized β-values along with the methylated and unmethylated signals and detection p-values were

imported into R for processing and analysis [37]. Samples with probe detection call rates <90% and those with an average intensity value of either <50% of the experiment-wide sample mean or <2000 arbitrary units (AU) were removed using the R package CpGassoc [38]. Probes that cross hybridized between autosomes and sex chromosomes were removed and probes with detection p-values >0.001 or those based on less than three beads had their methylation values set to missing [39]. CpGs with missing data for >10% of samples were also excluded from analysis. Beta Mixture Quantile Normalization was used to normalize the distribution of types I and II probes [40]. Following normalization, the ComBat procedure in the R package SVA was used to remove chip and positional effects controlling for gender and PTSD status [41]. Since β-values have been shown to be heteroskedastic, β-values were logit transformed into M-values prior to analysis [42].

Ancestry

For the DNHS and WTC cohorts, ancestry principal components (PCs) were estimated using probes within one base pair of SNPs. The second, third and fourth PCs, which have been shown to control for variation in methylation by ancestry [36], were included as covariates in all statistical models to control for potential differential methylation by ancestry [43]. In GTP, the first, second and third PCs from genome-wide genetic data were estimated as previously described and included in all analyses to control for ancestry [31].

Cell type estimation

DNA methylation varies by cell type, which can confound analyses of whole blood if the cell type proportions in whole blood are also impacted by the disease of interest. All three cohorts estimated the proportions of CD8, CD4, natural killer, B cells, monocytes and granulocytes in each individuals' blood sample using publicly available reference data (GSE36069) and the method implemented in the R package minfi [44,45]. Estimated proportions of CD8, CD4, natural killer, B cells, and monocytes were included as covariates in all statistical analyses.

Statistical analysis

Meta analysis

Within each cohort, M-values were modeled as a linear function of current PTSD (vs trauma-exposed controls without lifetime PTSD), controlling for age, sex, estimated cell proportions and ancestry PCs. Using the empirical Bayes method in the R package limma [46], moderated t-statistics were calculated for each CpG site, converted into one-sided p-values to capture the direction of effects, and then transformed into z-scores. Next, weights for each cohort's z-score were calculated based on square root of the sample size of the cohort relative to the total sample size across cohorts. Finally, the weighted z-scores were combined and two-sided p-values calculated. In order to account for multiple hypothesis testing, p-values were adjusted using the false discovery rate (FDR) procedure with the type I error rate level set to 0.05 based on the number of CpG sites included in the analysis after quality-control procedures were applied across all cohorts [47]. Cochran's Q test and I^2 were calculated to assess heterogeneity of results across studies [48,49].

Power analysis

We performed a *post hoc* power analysis [50] using DNHS data as a reference for CpG site variation to assess the power to detect an effect size equivalent to that reported in our top hit. The power to detect a differentially methylated CpG site depends on the percent difference in methylation between cases and controls, the pooled variation in methylation across CpG sites, and the number of cases and controls [51]. A mean difference between PTSD cases and controls of 3% (i.e., the difference reported in our top hit) requires that we assume a maximum pooled standard deviation of 0.09; this assumption appears reasonable for our reference dataset, in which 96% of CpG sites have a standard deviation of 0.09 or less. Assuming these parameters, our EWAS of n = 545 would have 80% power to detect a 3% difference in methylation between cases and controls if the sample included 180 cases. Our current sample exceeds this (n cases = 198), thus we are well powered to detect an effect size comparable to our top hit.

Sensitivity analyses

A number of sensitivity analyses were conducted for PTSD-associated CpG sites. First, we conducted a gender-stratified meta-analysis to explore whether results from the meta-analysis were driven by a specific gender. Second, since DNA methylation is known to vary with tobacco smoking [25], we conducted a second EWAS including

a dichotomous variable to control for whether a participant was a current smoker. Third, we hypothesized that childhood maltreatment may moderate the relationship between PTSD and DNA methylation, as suggested by previous work [18]. In the two cohorts (DNHS and GTP) with information on childhood maltreatment, we therefore conducted two additional analyses: one including childhood maltreatment as a covariate and another including an additional interaction term with PTSD to assess whether childhood trauma had an effect on the relationship between DNA methylation and adult PTSD status. Finally, to assess whether participant ancestry was driving the observed association between PTSD and DNA methylation at our topmost sites, we evaluated our FDR-significant findings within self-reported African–Americans only (while continuing to control for ancestry-related PCs); this represented the largest race/ethnic group within our civilian cohorts (63%).

Methylation-expression correlation

Correlation of blood-derived DNA methylation and mRNA expression in PTSD-associated CpG sites was examined in a subset of samples with available data from the DNHS [52] and GTP [53], respectively, for expression probes passing quality control (QC) cutoffs. In the DNHS, raw gene expression was quantified using the Illumina HT-12V4 Expression BeadChip (Illumina, San Diego, CA, USA) and normalized using quantile normalization. For *NRG1*, a subset of 71 trauma-exposed DNHS participants (16 with current PTSD) with both gene expression and methylation data was available; correlation between *NRG1* expression (probe ILMN_1737252) in arbitrary florescence units and DNA methylation at probe cg23637605 was tested in these 71 individuals. For *HGS*, correlation between expression (probe ILMN_1715994) and DNA methylation at cg19577098 was assessed in the same subset of 71 DNHS participants analyzed for *NRG1*; in addition, *HGS* expression in 133 trauma-exposed GTP study participants (32 with current PTSD) was assessed using the Illumina HT12v4 expression beadchip (dataset GSE58137) and normalized using quantile normalization. The probe interrogating *NRG1* expression (ILMN_1737252) did not pass QC in the GTP cohort and, thus, could not be included in the analyses.

Expression of *NRG1* & *HGS* transcript variants in whole blood & brain

To assess cross-tissue expression variation of transcript variants associated with our topmost hits, we accessed published RNAseq data from 327 samples corresponding to five CNS tissues previously implicated in PTSD (frontal cortex [ba9], hippocampus, amygdala, hypothalamus, brain cortex [54]) as well as whole blood, of the Genotype Tissue Expression Consortium (GTEx) [55]. Expression values for each transcript variant in each sample were quantified by GTEx in fragments per kilobase of exon per million reads, and transcript variants from Ensembl [56] annotations were compared with previously published annotations from the National Center for Biotechnology Information (NCBI) [57] for the relevant genomic loci.

Results

Demographic characteristics

The demographics of each cohort and the combined meta-analytic sample are presented in Table 1. Of the 545 participants, 36% had a current diagnosis of PTSD, 54% were male, 76% were nonsmokers and 63% were African–American. In addition, 23% of the sample was comprised of current smokers. In the combined DNHS and GTP sample for which childhood maltreatment data were available, 29% reported two or more types of maltreatment.

Primary meta-analysis results

A total of 455,405 CpG sites were included in the meta-analysis across all three cohorts. Of these, 25,168 were nominally associated with PTSD, but only two CpG sites remained significant after multiple test correction: cg23637605 in *NRG1* and cg19577098 in *HGS*. Results from the meta-analysis are presented as a Manhattan plot in Figure 1. On average, across all cohorts, participants with current PTSD had lower methylation at both CpG sites than control participants. The forest plots in Figures 2 and 3 present the effect for each locus in each cohort and in the overall meta-analysis. In addition, the top ten CpG sites from the primary meta-analysis are presented in Table 2, along with the β-values in cases and controls, the nominal and FDR-corrected p-values, and Q- and I^2 estimates of between-study heterogeneity. Six of the top ten PTSD-associated CpG sites fall in gene bodies, whereas three are found in intergenic regions and one is found in a 5′UTR. Five of the top ten CpGs show lower methylation in PTSD cases versus trauma-exposed controls, with the CpG in *NRG1* showing the largest difference in β-values between the two groups (~3%).

Table 1. Clinical and demographic description of each cohort.

Variable	DNHS	GTP	WTC	Total
Sample size	100	265	180	545
Mean age (SD)	53.6 (14.0)	41.9 (12.4)	49.7 (8.3)	46.6
Current PTSD	40%	28%	47%	36%
Male	40%	29%	100%	54%
Smoking, %:†				
– Current	32%	30%	9%	23%
– Nonsmokers	67%	70%	91%	76%
Race:				
– White	13%	5%	76%	30%
– Black	87%	94%	4%	63%
– Other	0%	1%	20%	7%
Childhood maltreatment, %:†				
– 2+ types	16%	34%	–	29%
– <2 types	77%	64%	–	68%

†Percentages do not total to 100 due to missing data.
DNHS: Detroit Neighborhood Health Study; GTP: Grady Trauma Project; PTSD: Post-traumatic stress disorder; SD: Standard deviation; WTC: World Trade Center.

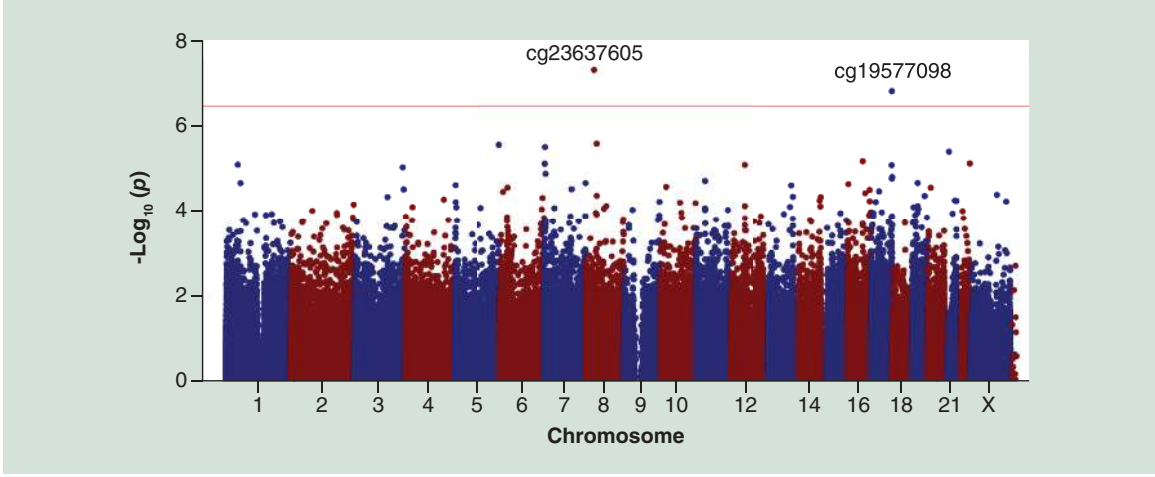

Figure 1. Manhattan plot showing CpG sites associated with post-traumatic stress disorder following false discovery rate correction. X axis depicts the chromosomal location of each CpG site, indicated in circles; Y axis depicts the p-value of each CpG site's association with current post-traumatic stress disorder in negative log scale. cg23637605 = *NRG1*; cg19577098 = *HGS*.

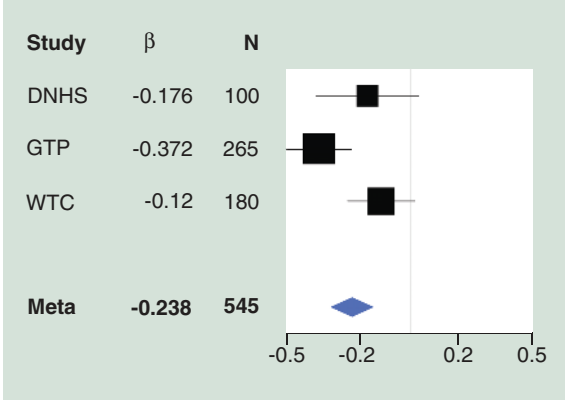

Figure 2. Forest plot showing the β-coefficients for the effect of current post-traumatic stress disorder on methylation at cg23637605 (*NRG1*) from the linear regression within each cohort and the combined effect from the meta-analysis in our primary analysis. DNHS: Detroit Neighborhood Health Study; GTP: Grady Trauma Project; WTC: World Trade Center.

Table 2. Top ten post-traumatic stress disorder-associated CpG sites from the primary meta-analysis.

Genomic information[†]					Primary model results								
CpG	CHR	Position	Gene	Genomic feature	DNAm cases	DNAm controls	Δ DNAm	n	p-value	FDR	Q	Q p-value	I^2
cg23637605	8	31,996,079	*NRG1*	Body	0.832	0.861	-0.029	545	4.66E-08	0.021	7.26	0.027	0.72
cg19577098	17	79,658,554	*HGS*	Body	0.967	0.974	-0.008	545	1.47E-07	0.033	0.38	0.827	0
cg17828057	8	42,037,527	*PLAT*	Body	0.536	0.522	0.015	543	2.48E-06	0.27	2	0.369	0
cg10476773	5	177,412,688	*PROP1*	IGR	0.475	0.459	0.016	543	2.66E-06	0.27	6.59	0.037	0.7
cg19028499	7	3,096,493	*CARD11*	IGR	0.986	0.987	-0.001	545	2.99E-06	0.27	3.38	0.185	0.41
cg15719339	21	17,960,584	*C21orf34*	Body	0.725	0.739	-0.014	544	3.82E-06	0.288	4.52	0.104	0.56
cg10453071	16	57,513,414	*DOK4*	Body	0.509	0.506	0.004	545	6.39E-06	0.298	0.58	0.749	0
cg19051861	22	50,243,786	*ZBED4*	IGR	0.597	0.602	-0.004	545	7.24E-06	0.298	14.12	0.001	0.86
cg02304222	7	2,082,850	*MAD1L1*	Body	0.981	0.981	0.000	545	7.34E-06	0.298	1.21	0.545	0
cg19636224	1	44,176,844	*ST3GAL3*	5′UTR	0.579	0.572	0.007	545	7.73E-06	0.298	8.08	0.018	0.75

[†] Coordinates are provided based on (GRCh37/hg19) assembly.
Δ DNAm: DNA methylation difference; CHR: Chromosome; DNAm: DNA methylation; FDR: False discovery rate; IGR: Intergenic region; I^2: I^2 index; Q: Cochran's Q statistic.

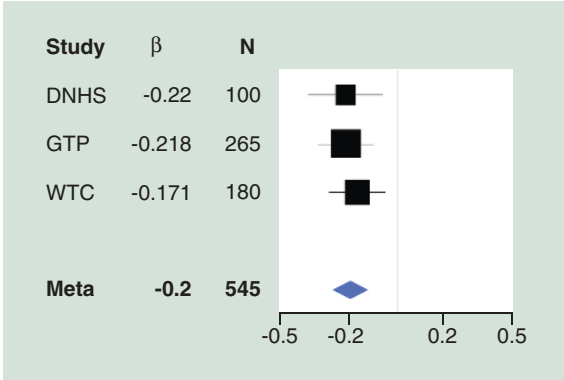

Figure 3. Forest plot showing the β-coefficients for the effect of current post-traumatic stress disorder on methylation at cg19577098 (*HGS*) from the linear regression within each cohort and the combined effect from the meta-analysis in our primary analysis.
DNHS: Detroit Neighborhood Health Study; GTP: Grady Trauma Project; WTC: World Trade Center.

Sensitivity analyses

Table 3 shows that controlling for current smoking did not attenuate the significance of either CpG site identified by our primary analyses. In addition, both CpG sites showed nominally significant differences in association with PTSD in both male- and female-only meta-analyses, with directions of effects consistent with the primary analyses (i.e., lower DNA methylation among those with PTSD; Table 4). Supplementary Table 1 shows that when childhood maltreatment was included as a covariate, the effect of PTSD remained consistent and significant after FDR correction for cg23637605 and nominally significant for cg19577098, but that childhood maltreatment was not significantly associated with either CpG. To assess whether childhood maltreatment moderated the relationship between PTSD and DNA methylation, as suggested by previous work [18], we tested a model that included the PTSD × childhood maltreatment interaction term. Nominally significant associations with PTSD remained for both CpG sites, but neither was significant after FDR adjustment. However, neither the effect of childhood maltreatment nor the interaction term was significant, indicating that the main effect of PTSD is relatively unaffected by exposure to childhood maltreatment for the two CpG sites identified in our primary analyses. Finally, analyses limited to participants of African–American ancestry (n = 336; Supplementary Table 2) showed that DNA methylation at *NRG1* remained associated with PTSD (FDR = 0.04), with the same direction of effect as in our primary model and retained its position as the top site associated with the disorder. Results for *HGS* among African–Americans only were attenuated to nonsignificance (FDR = 0.33) but retained the same direction of effect as in our primary model.

Correlation between methylation & expression of *NRG1* & *HGS*

NRG1 methylation at cg23637605 and expression of *NRG1* in blood correlated significantly in individuals with current PTSD in the DNHS cohort (R^2 = 0.47, p = 0.003), but not in trauma-exposed controls (R^2 = 0.021, p = 0.23; Figure 4). Of note, the *NRG1* probe ILMN_1737252 is annotated to NM_013962, but matches multiple *NRG1* transcript variants. In contrast, analyses of *HGS* in the DNHS cohort showed no significant correlation between DNA methylation at cg19577098 and *HGS* expression either in individuals with current PTSD (R^2 = 0.18, p = 0.1) or trauma-exposed controls (R^2 = 0.0041, p = 0.14). The lack of correlation in *HGS* was also observed in the GTP cohort (cases: R^2 = 0.042, p = 0.27; controls: R^2 = 0.001, p = 0.76).

Expression of *NRG1* & *HGS* in selected tissues

NRG1 is a complex locus that generates six major types of protein, types I through VI, determined through the use of differing 5′ exons, and more than 20 transcript variants that vary in their expression levels among and within tissues, including the brain [58,59]. In blood, the major *NRG1* transcript variant is ENST0000518206 (Supplementary Figure 1). This variant is most similar to a type I transcript variant, which is predicted to produce a 126aa protein that only includes the EGF-like domain that is conserved among all *NRG1* types. The major transcript variant of *NRG1* expressed in the amygdala, cortex, frontal cortex, hippocampus and hypothalamus is ENST0000520502, which uses the type III promoter to produce the *SMDF* transcript variant. The high expression of this transcript variant in these tissues is consistent with previous work showing that a majority of all *NRG1* expression consists of this variant in the cerebral cortex [60]. The major *HGS* transcript variant expressed (fragments per kilobase of exon per million reads ≥10) in amygdala, cortex, frontal cortex, hypothalamus and blood is the full-length variant, ENST000003291388 (NM_004712); a secondary variant, ENST00000573320, which only

Table 3. Results from smoking sensitivity analyses for the top ten post-traumatic stress disorder-associated CpG sites.

Genomic information[†]					Smoking model					
CpG	CHR	Position	Gene	Genomic feature	DNAm cases	DNAm controls	Δ DNAm	n	p-value	FDR
cg23637605	8	31,996,079	*NRG1*	Body	0.832	0.861	-0.029	544	7.53E-08	0.034
cg19577098	17	79,658,554	*HGS*	Body	0.967	0.974	-0.008	544	1.65E-07	0.037
cg17828057	8	42,037,527	*PLAT*	Body	0.536	0.522	0.015	542	6.85E-06	0.354
cg10476773	5	177,412,688	*PROP1*	IGR	0.476	0.459	0.017	542	7.08E-06	0.354
cg19028499	7	3,096,493	*CARD11*	IGR	0.986	0.987	-0.001	544	4.71E-06	0.354
cg15719339	21	17,960,584	*C21orf34*	Body	0.725	0.739	-0.014	543	2.55E-06	0.354
cg10453071	16	57,513,414	*DOK4*	Body	0.510	0.506	0.004	544	2.44E-05	0.445
cg19051861	22	50,243,786	*ZBED4*	IGR	0.597	0.602	-0.004	544	1.10E-05	0.354
cg02304222	7	2,082,850	*MAD1L1*	Body	0.981	0.981	0.000	544	5.06E-06	0.354
cg19636224	1	44,176,844	*ST3GAL3*	5′UTR	0.579	0.572	0.007	544	1.12E-05	0.354

[†] Coordinates are provided based on (GRCh37/hg19) assembly.
Δ DNAm: DNA methylation difference; CHR: Chromosome; DNAm: DNA methylation; FDR: False discovery rate; IGR: Intergenic region.

Table 4. Results from gender-stratified sensitivity analyses for the top ten post-traumatic stress disorder-associated CpG sites.

Genomic information†					Males only					
CpG	CHR	Position	Gene	Genomic feature	DNAm cases	DNAm controls	Δ DNAm	n	p-value	FDR
cg23637605	8	31,996,079	*NRG1*	Body	0.822	0.847	-0.024	298	0.0208	1
cg19577098	17	79,658,554	*HGS*	Body	0.961	0.969	-0.008	298	0.00017	1
cg17828057	8	42,037,527	*PLAT*	Body	0.528	0.522	0.006	298	0.0194	1
cg10476773	5	177,412,688	*PROP1*	IGR	0.442	0.438	0.003	298	0.00141	1
cg19028499	7	3,096,493	*CARD11*	IGR	0.981	0.982	-0.001	298	0.00945	1
cg15719339	21	17,960,584	*C21orf34*	Body	0.737	0.742	-0.005	298	0.00417	1
cg10453071	16	57,513,414	*DOK4*	Body	0.479	0.480	-0.002	298	0.00579	1
cg19051861	22	50,243,786	*ZBED4*	IGR	0.545	0.563	-0.018	298	0.184	1
cg02304222	7	2,082,850	*MAD1L1*	Body	0.976	0.975	0.001	298	0.000749	1
cg19636224	1	44,176,844	*ST3GAL3*	5′UTR	0.542	0.524	0.019	298	1.33E-06	0.295
Genomic information†					**Females only**					
CpG	**CHR**	**Position**	**Gene**	**Genomic feature**	**DNAm cases**	**DNAm controls**	**Δ DNAm**	**n**	**p-value**	**FDR**
cg23637605	8	31,996,079	*NRG1*	Body	0.846	0.877	-0.031	247	3.64E-07	0.071
cg19577098	17	79,658,554	*HGS*	Body	0.975	0.980	-0.005	247	1.88E-03	0.656
cg17828057	8	42,037,527	*PLAT*	Body	0.548	0.521	0.026	245	3.52E-06	0.177
cg10476773	5	177,412,688	*PROP1*	IGR	0.523	0.483	0.040	245	3.81E-04	0.568
cg19028499	7	3,096,493	*CARD11*	IGR	0.994	0.993	0.001	247	2.85E-04	0.568
cg15719339	21	17,960,584	*C21orf34*	Body	0.708	0.735	-0.027	246	2.78E-03	0.669
cg10453071	16	57,513,414	*DOK4*	Body	0.552	0.534	0.019	247	6.79E-04	0.605
cg19051861	22	50,243,786	*ZBED4*	IGR	0.670	0.644	0.026	247	7.80E-06	0.273
cg02304222	7	2,082,850	*MAD1L1*	Body	0.989	0.987	0.001	247	1.47E-03	0.651
cg19636224	1	44,176,844	*ST3GAL3*	5′UTR	0.630	0.625	0.005	247	5.19E-01	0.961

† Coordinates are provided based on (GRCh37/hg19) assembly.
Δ DNAm: DNA methylation difference; CHR: Chromosome; DNAm: DNA methylation; FDR: False discovery rate; IGR: Intergenic region.

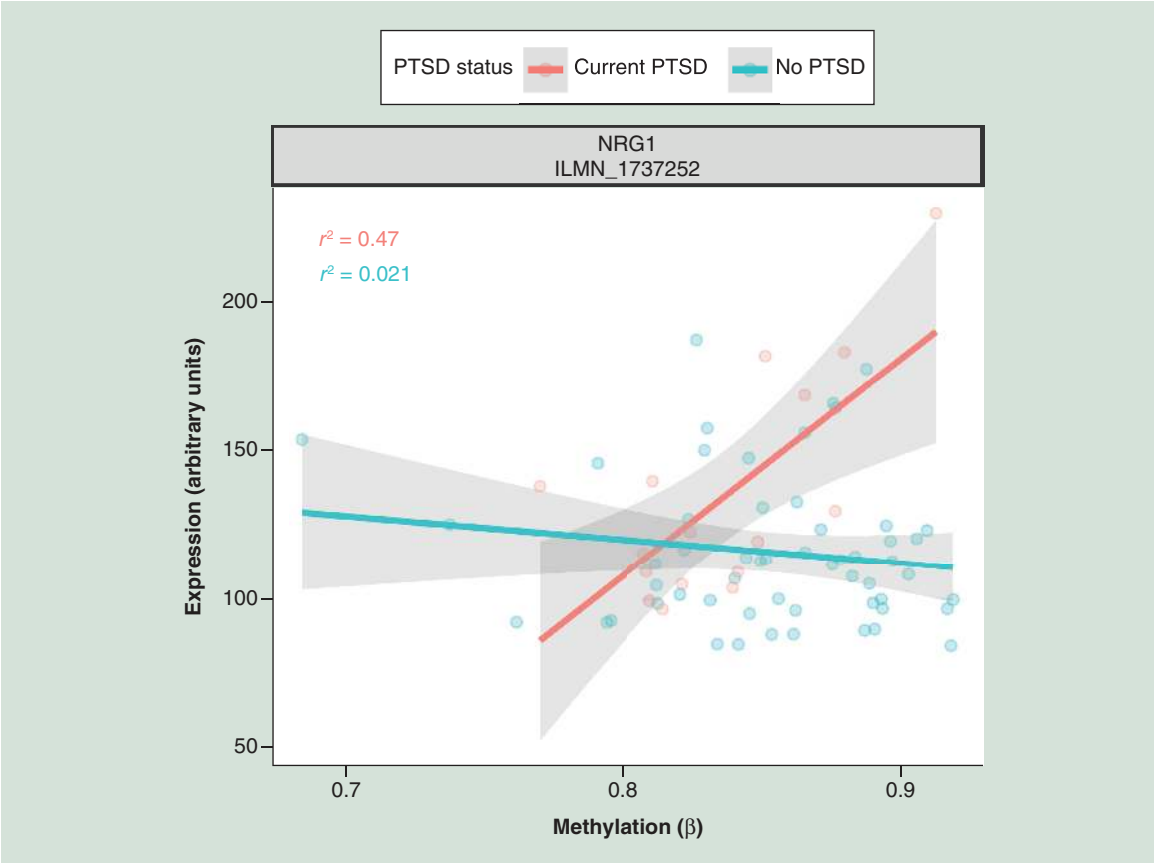

Figure 4. *NRG1* methylation and expression correlation in the Detroit Neighborhood Health Study. Correlation ($p \leq 0.01$) of methylation (β) at cg23637605 and expression (arbitrary units) in n = 16 with current PTSD (red) and n = 55 trauma exposed controls without PTSD (blue) in samples from the Detroit Neighborhood Health Study. Lines represent the linear best fit, and gray areas the 95% CI of the linear best fit.
PTSD: Post-traumatic stress disorder.

contains the 3′ end of the major transcript variant, is also expressed at similar levels in these tissues (Supplementary Figure 2).

Discussion

Trauma exposure is required for a PTSD diagnosis, yet not all trauma-exposed individuals go on to develop the disorder. PTSD therefore affords a unique opportunity to identify characteristics that distinguish individuals with versus without the disorder. Through a meta-analysis of existing EWAS data associated with PTSD in civilian samples, we identified two CpG sites that reached genome-wide significance in our primary analysis: one located in *NRG1*, a gene involved in neural development and synaptic plasticity that is also associated with cardioprotective effects [59,61], and another located in *HGS*, a gene associated with endocytosis and exocytosis [62,63]. These results were robust to adjustment for current smoking and, for *NRG1*, robust to adjustment for exposure to childhood maltreatment, raising the possibility that this locus in particular may serve as a potential biomarker of PTSD even among those with adverse early life experiences; gender-stratified analyses, however, attenuated both sites to nonsignificance, perhaps due to limited power. Taken together, these results provide initial evidence that PTSD is associated with differential methylation within *NRG1* and *HGS* in cohorts comprised of individuals recruited from civilian contexts.

NRG1 is involved in multiple biological processes relevant to neural development and function. Much of its role is enacted through pairing with ErRB receptor tyrosine kinases, which enables signaling to affect neuronal migration, axon guidance, myelination, synapse and neuromuscular junction formation, and oligodendrocyte development [59]. The type III transcript variant, identified here as the likely variant expressed in brain regions

relevant to PTSD, nevertheless plays a prominent role in Schwann cell development and peripheral myelination [64]. Previous behavioral work in rodents has also implicated *Nrg1* dysregulation in altered HPA axis reactivity [65,66] and anxiety-relevant phenotypes [65–68], indicating a role for this gene in not only neural development, but also HPA axis functioning. More specifically, rats with reduced type II *Nrg1* transcript levels showed increased basal corticosterone concentrations, reduced corticosterone concentrations following acute stress, and sustained reactivity to novel environments [65], suggesting that proper expression of this transcript variant in the brain plays an important, possibly protective role in shaping response to stress.

Although the complexity of the *NRG1* locus, along with its wide range of transcript variants that show tissue- [60] and developmental [60,69] expression differences, makes it challenging to infer the precise impact of our observed PTSD-associated differential methylation at this locus, genomic variation across this gene has previously been linked to schizophrenia in multiple meta-analyses [70–73], and type III transcript variants have been reported as being upregulated in leukocytes obtained from individuals with schizophrenia [74]; it has also been linked to bipolar disorder in select candidate gene studies [75,76]. While *NRG1* has not been previously linked to PTSD, we note that the largest genome-wide association study (GWAS) of PTSD published to date identified ErbB-related signaling pathways in three of the five topmost pathways implicated by the GWAS results [77], including ErbB4, the receptor that can interact directly with NRG1 and that plays a preferential role in CNS development and function [64]. Collectively, these findings suggest that genomic variation in *NRG1* may confer a broad risk for mental disorders, including PTSD.

In contrast to our topmost hit, our second hit, *HGS*, has been linked only marginally to mental disorders, specifically Alzheimer's disease, in previous work [78]. In addition, it has not, to our knowledge, been implicated in modulating HPA axis signaling or function. Nevertheless, its protein product does appear to play a role in both CNS- and immune-related functions, both of which have been implicated in PTSD [79]. HGS is a key protein involved in the endosomal sorting complex required for transport, a multiprotein complex that serves to deliver G protein-coupled receptors, including neuromodulators such as δ-opioid and β-adrenergic 2 receptors for downregulation and/or degradation, often following ubiquitination [80,81]. The gene has been implicated in peripheral development and activation of B cells [82] and in the secretion of exosomes by dendritic cells [83]. Deficiency in *HGS* expression has been linked to increases in both IL6 and TNF-α signaling [83,84], suggesting the gene plays a role in immune and inflammatory processes. Intriguingly, the gene has been annotated as belonging to the Internalization of ErbB1 pathway [85], further implicating ErbB-related signaling pathways in PTSD pathophysiology. Although the relevance of our blood-based findings in relation to the brain is unclear at this time, it is worth noting that in mice with neuron-specific knockout of *HGS*, ubiquitinated proteins were observed to accumulate in brain tissue at 5 weeks, and hippocampal neurodegeneration was detectable at 8 weeks, suggesting a key role for the gene in promoting neuronal survival [86]. Thus, although existing studies have not implicated this locus in PTSD etiology *per se*, findings from the studies described here provide evidence that *HGS* is involved in multiple biological processes whose dysregulation has been previously linked to PTSD [79].

Of note, our secondary analyses showed that methylation and expression were correlated at *NRG1* but not *HGS*. In the DNHS, blood-based expression of *NRG1* appears to be actively regulated by DNA methylation measured in the same tissue, but only among individuals with current PTSD; trauma-exposed individuals showed no such association. In contrast, DNA methylation of our *HGS* hit was not significantly associated with expression in either the DNHS or GTP samples. Although these preliminary *NRG1* findings await replication, these results suggest that PTSD-associated DNA methylation has varying effects on gene expression, with effects that are sometimes, but not always, evident at the transcript level.

Our findings should be interpreted in light of a number of study limitations. First, our investigation was necessarily limited to CpG sites included on the 450K BeadChip, which represent only a fraction of the CpG sites in the human genome; thus, we are likely missing other, important CpG sites associated with PTSD that are not present on this array. Future work using the more comprehensive MethylationEPIC BeadChip DNA methylation microarray, or whole genome bisulfite sequencing of entire genomes, should help to address this shortcoming. Second, our findings are based on blood-derived DNA, and we are currently unable to make inferences about DNA methylation levels among individuals with PTSD at the identified sites within the target organ of interest, that is, the brain. Future investigations of brain tissue from PTSD Brain Banks, presently under development, may help to illuminate the correspondence between our observed PTSD-associated biomarkers in direct findings in the brain. Third, the DNHS and GTP studies assessed PTSD for worst exposure occurring across the life course, while for the WTC cohort, PTSD was determined only in relation to WTC exposures. Thus our sensitivity analysis adjusting

for childhood exposure and investigating its potential moderating effects in our top hits was limited to the former two cohorts. Future studies are needed to confirm our finding that PTSD is associated with DNA methylation in *NRG1* when adjusting for childhood maltreatment in samples that include the WTC as well as other civilian cohorts. Fourth, our secondary analyses of DNA methylation and expression were limited to cohorts with available data and to probes that passed QC filters; as such our observations for this portion of the study are based on a more limited sample than the meta-analysis, and should be interpreted with caution. We anticipate an increase in the availability of paired DNA methylation-expression data through ongoing work within the PGC PTSD workgroup that will help determine the replicability of the current results. Fifth, we did not control for possible effects of comorbid conditions on DNA methylation, due in part to a lack of good measures across a range of psychiatric diagnoses in addition to PTSD. Finally, a limitation of the results from our isoform analysis is that, for *NRG1*, the Ensembl and NCBI annotations in these regions do not match completely. Ensembl is missing many of the types II and V isoforms that are present in NCBI, and NCBI is missing some of the novel isoforms that are present in Ensembl. Future work to resolve the annotation of this genomic region, which is strongly implicated in disorders such as PTSD and schizophrenia, will help the comparison of future high-throughput analyses with each other and with existing literature.

Despite these limitations, our study includes several strengths, including the use of a well-developed, standardized preprocessing pipeline applied across multiple cohorts [36] and the use of meta-analytic techniques to increase the power of our PTSD-related analyses. Our efforts have identified preliminary evidence for two DNA methylation-based epigenetic associations with current PTSD across three civilian cohorts; one of these, *NRG1*, falls in a gene previously implicated in schizophrenia, and the other, *HGS*, is involved in critical biological processes that impact immune function and neuronal survival. These findings should be replicated in larger, independent samples and in additional cohorts as they become available through the PGC PTSD; if they hold, future, longitudinal studies should be conducted to determine whether these PTSD-associated DNA methylation differences exist prior to trauma exposure and thus serve as a vulnerability marker or biomarker of risk prognosis [87], or whether they arise following onset of the disorder.

Summary points

- Post-traumatic stress disorder (PTSD) is a debilitating mental disorder that occurs following exposure to a life-threatening event.
- The marked difference in prevalence between trauma exposure and PTSD onset suggests that distinct characteristics, including epigenetics, may distinguish between individuals with versus without the disorder.
- We used meta-analysis to test whether current PTSD is associated with differential methylation in cohorts comprised of individuals recruited from civilian settings. We focused specifically on civilian contexts in order to maximize our potential to discern PTSD-associated differential methylation in noncombat situations.
- Blood-based DNA methylation measures from epigenome-wide association studies of PTSD were obtained from the 545 participants drawn from three civilian cohorts participating in the epigenome-wide association studies arm of the PTSD working group of the Psychiatric Genomics Consortium.
- DNA methylation at two CpG sites significantly associated with current PTSD, controlling for gender, age, blood cell composition and ancestry: cg23637605, located in *NRG1* (false discovery rate $p = 0.021$); and cg19577098 located in *HGS* (false discovery rate $p = 0.033$).
- Results were robust to adjustment for smoking and, in *NRG1*, exposure to childhood maltreatment.
- Complimentary analyses in a subset of samples showed significant associations between DNA methylation and mRNA expression in *NRG1* but not *HGS*.
- *NRG1* is important in neural development and synaptic plasticity and has been previously implicated in schizophrenia; *HGS* plays a role in CNS and immune-related functions.
- This is the first report of PTSD-associated variation at both of the identified loci.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: https://www.futuremedicine.com/doi/suppl/10.2217/epi-2018-0049

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Financial & competing interests disclosure

KJ Ressler has a patent US7655655 B1 – method for facilitating extinction training using D-cycloserine issued to Extinction Pharmaceuticals, a patent WO 2005016319 A2 – acute pharmacologic augmentation of psychotherapy with enhancers of learning or conditioning issued to Extinction Pharmaceuticals, a patent WO 2012106407 A2 – diagnostic and therapeutic methods and products related to anxiety disorders pending, a patent US 20140255517 A1 – managing post-traumatic stress disorder pending, and a patent US 20170296528 A1 – methods of managing conditioned fear with neurokinin receptor antagonists pending. DE Wildman is Editor in Chief of *Molecular Phylogenetics and Evolution*, an Elsevier Journal, for which he receives an honorarium. The remaining authors (M Uddin, A Ratanatharathorn, D Armstrong, P Kuan, AE Aiello, EJ Bromet, S Galea, KC Koenen, B Luft, CM Nievergelt and A Smith) report no biomedical financial interests or potential conflicts of interest. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

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