Methylome-wide association studies of traumatic physical injury identifies differential DNA methylation of synaptic plasticity and GABAergic-signalling.

Brown J[1#], Marshall S[2#], Walker RM[2,3], Campbell A[2], Hayward C[4], McIntosh AM[5], Evans KL[2], **Thomson PA**[2]

Author Affiliations:

1. Edinburgh Medical School, University of Edinburgh

2. Centre for Genomic and Experimental Medicine, Institute of Genetics and Cancer, University of Edinburgh

3. School of Psychology, University of Exeter, Exeter, United Kingdom.

4. MRC Human Genetics Unit, Institute of Genetics and Cancer, University of Edinburgh

5. Centre for Clinical Brain Sciences (Division of Psychiatry), University of Edinburgh

Corresponding Author:

Name: Pippa Thomson

Telephone: 0131 651 8724

Email: pippa.thomson@ed.ac.uk

Address: Institute of Genetics and Cancer, University of Edinburgh, Western General Hospital, Crewe Road, Edinburgh, EH4 2XU, United Kingdom

# These authors contributed equally to this study.

Abstract

Physical trauma is often associated with psychological trauma and is a risk factor for the development of major depressive disorder (MDD). We derived a traumatic physical injury phenotype in the Generation Scotland cohort and showed that it was significantly associated with a diagnosis of recurrent major depression and measures of related symptoms, particularly disorganised thought. Blood-based methylome-wide analyses of traumatic injury were performed in groups of individuals with or without diagnoses of MDD. Nominally significant differences in DNA methylation were identified at 40,003 CpG sites in the effect size of the association of DNA methylation in individuals with and without MDD. Individuals with recurrent MDD showed a significant higher levels of DNA methylation at CpG sites associated with the first exon and lower levels associated with exon boundaries. This may suggest alterations in gene expression and splicing in individuals with recurrent MDD compared to controls. Analyses at the level of CpG site, genes and gene ontologies implicated dysregulation in neuronal development, e.g. *MATN2* and *ZEB2,* and processes related to synaptic

plasticity, including dendrite development, excitatory synapse and GABAergic signalling. Analyses of brain-expression patterns highlight the limbic lobe and supraoptic nuclei, brain regions associated with fear memory encoding. The results suggest that traumatic injury is associated with patterns of DNA methylation but that the direction of these associations differ in individuals with MDD compared to controls, highlighting the need for novel analysis approaches.

Introduction

Major depressive disorder (MDD) is a multifactorial disorder with highly polygenic heritability and strong environmental risk factors including stressful life events and trauma. There is evidence that genetic predisposition and adverse life events interact to determine individual risk. Differential DNA methylation is associated with both self-reported traumatic events and MDD [1, 2] This suggests that DNA methylation may be used to identify the biological pathways leading to increased risk of MDD.

Studying environmental factors that could be a cause or consequence of MDD has been problematic, some have grouped life events into dependent/independent [3-10], but relatively few have looked objectively at external traumatic risk factors, such as traumatic injury. These can be measured objectively, in terms of the injury sustained, but can also reflect an individual's perception of the seriousness of the incident. The perception of trauma may be important for poor mental health outcomes and treatment response.[11, 12]

Traumatic injury is a physical injury that requires urgent medical attention and can be the result of road traffic accidents, falls, violence, or sports injuries. According to the WHO, traumatic injury affects tens of millions of individuals globally each year resulting in significant long-term disability (WHO, Key Facts Injuries and violence, 19 March 2021, https://www.who.int/news-room/fact-sheets/detail/injuries-and-violence). Approximately 30-40% of survivors of traumatic injury develop increasing psychological problems including 9%-15% of whom develop MDD.[12-15] A prior history of mental health issues, persistent rumination about the trauma, perceived threat to life, lack of social support and persistent physical problems are predictors of poor mental health outcomes.[15-18] Increased understanding of the effects of physical trauma on mental health has led to the development of a number of screening tools for the prediction of individuals at increased risk of generalised anxiety disorder, depression or post-traumatic stress disorder (PTSD)[19-22], and to the development of NHS guidance for psychological support after trauma even in the absence of physical injury. Response to treatment of psychological distress is, however, heterogeneous.[14, 23]

We hypothesised that: i) self-reported traumatic injury may be associated with differential methylation and may identify biological processes that may mediate the effect of trauma, ii) investigating differential methylation in both individuals with diagnoses of lifetime major depression and healthy controls separately may identify the individual differences in response to trauma leading to mental health problems.

A traumatic injury phenotype incorporating both physical injury (bone fracture) and self-reported psychological distress was derived in the Generation Scotland cohort.[24, 25] Preliminary phenotypic analyses confirmed a significant association between a positive traumatic injury response and lifetime diagnoses of MDD. Methylome-wide association studies (MWAS) were performed for differential DNA methylation associated with traumatic injury in three groups: healthy controls (noMDD), combined single or recurrent episode MDD (MDD) and recurrent only MDD (rMDD). The results of the MWAS were analysed to investigate pathway-specific enrichment and to identify differences in differential DNA methylation between the groups (methylome-wide x environment interaction studies, MWEIS: xMDD, xrMDD) and associated with traumatic injury across all individuals (META). Pathway-specific polygenic risk scores were used to assess the association of traumatic injury-related biological processes with MDD in an independent subgroup of Generation Scotland participants. Finally, regional-brain expression patterns over-represented in the genes associated with differential DNA methylation were investigated.

Material and methods

Study cohort

Generation Scotland is a population-based cohort of approximately 24,000 participants.[24-26] Participants were recruited via general medical practices in Scotland between 2006 and 2011 and

completed physical test and health questionnaires (data available on request: http://www.generationscotland.co.uk). All participants have given informed consent. Ethics approval for Generation Scotland was given by the NHS Tayside committee on research ethics (reference 05/S1401/89).

Phenotypes

Information on traumatic incident that resulted in bone fracture was collected as part of the Generation Scotland questionnaire on musculoskeletal history in which participants were asked whether they had ever broken a bone and, if so, whether the fracture had occurred “in a road traffic accident or other serious incident”. This question was added part way through recruitment, as a result only approximately half the cohort provided data on traumatic injury. In total, approximately 11,800 participants answered this question, of whom 11,387 reported at least one fracture. An individual was classified as having had traumatic injury if they responded yes to the second question (N=1,010).

A diagnosis of major depressive disorder (MDD) was made using the structured clinical interview for DSM-IV disorders (SCID).[27] Participants who answered yes to either of the two screening questions were invited to continue the interview, which provided information on the presence or absence of a lifetime history of MDD and number of depressive episodes. Participants who answered no to both screening questions or who completed the SCID but did not meet the criteria for depression were assigned control status. Participants with diagnoses of schizophrenia or bipolar disorder were excluded from the analyses.

The age and sex of participants, and additional questionnaires to assess cognitive [28-30] ability and personality [31] and traits reflecting symptoms associated with depression (GHQ)[32], bipolar disorder (MDQ)[33] and schizophrenia (SPQ-BR) were collected at baseline.[34, 35]

Phenotypic analyses

Logistic regression analyses of traumatic injury were performed in R ([36]) using the *glm* function in MASS.[37] The phenotypic sample consisted of 2,946 individuals including 398 MDD individuals (72% female) and 2,548 controls (59% female). Phenotypic analyses were adjusted for sex and age at baseline. Variables from SPQ, MDQ and GHQ were log2 transformed to improve the normal distribution of the residual variance. Coefficients are reported on the non-log2 scale.

MWAS & MWEIS

Whole blood DNA methylation was profiled using the Infinium MethylationEPIC BeadChip (Illumina Inc.) in two sets of GS participants at two separate times by the Wellcome Trust Clinical Research Facility, Edinburgh. The natural discovery sample comprised 5190 individuals and the natural replication sample included 4583 individuals, as described previously.[1, 38, 39] The two samples were normalised, and the data was converted to M-values. Individuals in the replication sample were unrelated to those in the discovery set (SNP-based relatedness <0.05). The data was quality controlled prior to the analyses to remove~~:~~ poor-performing probes, sex chromosome probes, individuals with unreliable self-report data, suspected XXY genotype, as described in Walker et al.[40]

Individual MWAS were performed for traumatic injury in three groups: healthy controls, MDD and recurrent (rMDD, see Supplementary Table 1). This design resulted in complete overlap between samples used for the analyses of MDD and rMDD, which was a subset of MDD; but with no overlap between the MDD groups and the healthy controls. *Limma* was used to calculate empirical Bayes moderated t-statistics from which the P-values were obtained.[41] Covariates were fitted to the discovery and replication sample following Walker et al.[40] The discovery sample M-values were pre-corrected for relatedness, estimated cell count proportions (granulocytes, natural killer cells, B lymphocytes, CD4+ T lymphocytes, and CD8+ T lymphocytes) and processing batch. This sample was also corrected for age, sex, smoking status, pack years, 20 methylation PCs and 20 genetic PCs.

The replication sample M-values were corrected for age, sex, smoking status, pack years, estimated cell count proportions, processing batch, 20 methylation principal components and 20 genetic principal components.

The MWAS results for healthy controls (noMDD) and MDD were further meta-analysed using an inverse standard error-weighted fixed model implemented in METAL to give the data set META. The meta-analysis included 997,608 loci from 4,308 individuals.

MWEIS were performed using a two-sample Z-test to test for significant differences in the effect size (fold-change in the ratio of unmethylated to methylated CpG sites) of the association with traumatic injury in the healthy control versus MDD groups to give the datasets xMDD and xrMDD.

A genome-wide significant P-value threshold, P-value < 9.42×10^{-8}, was applied to all MWAS and MWEIS results within the study, following Mansell et al.[42].

Functional enrichment for *MATN2* and *ZEB2* co-expression and protein-interaction network analyses

Co-expression partners were identified using GeneCards.[43].The top 25 human protein-interaction network partners were identified using STRING.[44] Enrichment analyses were performed in STRING using the whole genome as the background. STRING assesses enrichment within categories defined from external sources and p-values are corrected for multiple testing within each category using the Benjamini–Hochberg procedure. The top five most FDR-significant functional enrichments are reported.

Gene set enrichment analyses

CpG sites were mapped to the nearest genes based on nearest transcriptional start site within 10 Kb. Over-representation analyses for gene ontology terms were performed using *WebGestaltR*.[45] Gene ontologies were considered significant at a false discovery rate (FDR) of p-value < 0.05 and a minimum of five genes in the target list.

Gene set expression enrichment in adult human brain regions was assessed by hypergeometric tests using *ABAEnrichment*[46] at three MWAS p-value thresholds (T1 $p<2.668\times10^{-6}$, -logP =5.5738; T2 $p<1\times10^{-5}$, -logP =5; T3 p<0.005, -logP =2.3) and three expression quantiles: 0.5, 0.7, and 0.9. Brain regions were considered significant at FWER<0.05.

Polygenic risk scores (PRS) analyses

GWAS of adult trauma was performed in UK Biobank[47, 48] using Plink V1.9b4[49] fitting the adult trauma score as a continuous variable (See Supplementary information for full details). The UKB genotype data used 74,224 unrelated UK white British individuals who had completed the MHQ. (no trauma = 36,525, at least one reported trauma = 37,699). Female participants were more likely to report traumatic events in adulthood than males, giving a final sample that was 56% female. Age, sex, and 20 principal components were fitted as covariates. MAGMA, within FUMA, was used to perform gene-based tests using the GWAS summary statistics, mapping input SNPs to protein coding genes (FUMA v1.5.2, Watanabe et al. 2017). SNPs were mapped to genes when they were within 10kb. FUMA conducts analysis of gene expression by tissue, tissue specificity for mapped genes using; ‘GTEx v8: 54 tissue types’ and ‘GTEx v8: 30 tissue. The significance threshold used was a Bonferroni corrected p-value of P < 0.05. See Supplementary Table 13 and Supplementary Figures 14-16 for full results.

Generation Scotland genome-wide genotype data were generated using the Illumina HumanOmniExpressExome-8 v1.0 DNA Analysis BeadChip (San Diego, CA, USA) and Infinium chemistry by the Clinical Research Facility, University of Edinburgh. Full details can be found (refs).[26, 50] UK Biobank genome-wide genotype data details can be found (refs),[47, 48] following Howard et al., individuals, or relatives of individuals, also in Generation Scotland were removed prior to the GWAS analysis.[50]

Polygenic risk scores were calculated using PRSice-2 (v2.3.5)[51] PRSet functions to calculate genome-wide and pathway specific PRS weighted by genome-wide summary statistics from a GWAS of adult trauma in UK Biobank or the 2018 PGC MDD meta-analysis (excluding Generation Scotland, Wray et al.). Independent SNPs were selected using clumping with a window of 250 kb, threshold p-value = 1 and an r^2 = 0.1. PRS were derived for six p-value thresholds (0.00000005, 0.00001, 0.001, 0.05, 0.5, 1). Gene regions were defined using hg19 with 2 kb flanking regions 5' and 3' of the transcription start and stop sites. Significance was accepted at $P < 0.05$ using the competitive p-value derived after 10,000 set permutations. The competitive permutations test for enrichment of signals in the tested pathways compared to match numbers of post-clump SNPs selected from the whole genome (Base set). Analyses were corrected for age, sex and 10 genetic PCs.

PRS analyses were performed on two independent sets of unrelated individuals from Generation Scotland. The first set contained unrelated individuals who answered the questionnaire version that contained the traumatic injury question (SetA, N = 3,522, including: 3,185 controls and 327 individuals reporting traumatic injury; 3,010 controls and 512 MDD cases of which 253 were rMDD cases). These individuals contributed to the MWAS/MWEIS analyses. The second set of individuals had no information on traumatic injury defined from the musculoskeletal history (SetB, N = 1,515, including 1,274 controls, 241 MDD cases of which 117 were rMDD cases). This second set were not included in the MWAS/MWEIS analyses and are unrelated to those individuals in the first set.

Results

Association between traumatic injury and psychiatric traits

Traumatic injury was significantly associated with a lifetime diagnosis of depression (p = 0.0048, OR = 1.67 95% CI =1.17-2.39), specifically in those individuals with evidence of recurrent episodes (p = 0.0016, OR = 2.10 95% CI =1.33-3.33), but not in those reporting single episodes (p = 0.30, OR = 1.31 95% CI =0.78-2.20, Table 1). Traumatic injury was associated with symptom measures and personality traits relevant to some aspects of psychiatric disease; with individuals reporting traumatic injury having significantly higher scores on the depression subscale of the General Health Questionnaire; the Mood disorder questionnaire and the Schizotypal Personality Questionnaire-Brief Revised scale, particularly for the disorganised factor (Table 1). This schizotypal factor assesses eccentric behaviour and odd speech, and, of the three factors, and is the most strongly associated with a positive psychiatric history.[34] No significant association was seen with personality factors (neuroticism and extroversion) or cognitive ability traits, except for digit symbol coding score.[52, 53]

Table 1. Phenotypic analyses of traumatic injury

Variable	Traumatic injury	No traumatic injury	OR (95% CI)	P
N	258	2688		
Age mean (sd)	50.09 (11.63)	52.23 (12.6)	1.04 (1.04-1.05)	**9.0×10^{-10}**
Sex (% Female)	72%	59%	2.95 (2.26-3.84)	**1.3×10^{-15}**
N. MDD (MDD/noMDD)	258 (43/215)	2548 (355/2333)	1.67 (1.17- 2.39)	**0.0048**
N. MDD Single episode (MDD/noMDD)	233 (18/215)	2522 (189/2333)	1.31 (0.78-2.20)	0.3041
N. MDD Recurrent episodes (MDD/noMDD)	240 (25/215)	2499 (166/2333)	2.10 (1.33-3.33)	**0.0016**

	Traumatic injury	No traumatic injury	Beta (95% CI)	
Eysenck Neuroticism Mean (std.dev)	3.54 (3.22)	3.59 (3.18)	0.386 (-0.019-0.791)	0.0621
Eysenck Extroversion Mean (std.dev)	7.34 (3.51)	7.46 (3.54)	0.123 (-0.334-0.581)	0.5972
Vocabulary Mean (std.dev)	14.17 (4.71)	14.42 (4.85)	-0.475 (-1.146-0.197)	0.1660
digit symbol coding Mean (std.dev)	15.57 (4.05)	16.20 (4.09)	-2.540 (-4.574 - -0.505)	**0.0145**
verbal total Mean (std.dev)	64.97 (16.15)	71.85 (17.64)	0.452 (-1.147-2.051)	0.5790
Logical memory delayed Mean (std.dev)	13.66 (4.65)	13.44 (4.60)	0.310 (-0.887-0.267)	0.2670
GHQ total# Median (IQR)	19 (16)	13 (7)	0.054 (-0.014-0.127)	0.1220
GHQ somatic symptoms# Median (IQR)	5 (5)	3 (4)	0.002 (-0.078-0.088)	0.9670
GHQ anxiety & insomnia# Median (IQR)	5 (5)	3 (4)	0.110 (0.004-0.228)	0.0425
GHQ social dysfunction# Median (IQR)	7 (2)	7 (1)	0.021 (-0.023-0.068)	0.3574
GHQ depression# Median (IQR)	0 (4)	0 (0)	0.103 (0.014-0.200)	0.0218
MDQ Total Median (IQR)	2 (5)	1 (3)	0.200 (0.077-0.336)	**0.0010**
SPQ total Median (IQR)	16 (0-25)	3 (4.75)	0.183 (0.062-0.316)	**0.0022**
SPQ cognitive-perceptual Median (IQR)	2 (3)	1 (2)	0.142 (0.050-0.242)	**0.0020**
SPQ interpersonal Median (IQR)	2 (0-12)	1 (3)	0.077 (-0.018-0.182)	0.1138
SPQ disorganised Median (IQR)	8 (0-12)	0 (0)	0.111 (0.049-0.177)	**0.0004**

#GHQ likert scoring. All analyses fitted age and sex as covariates. Std.dev = standard deviation. IQR = interquartile range.

Genome-wide assessment of differential DNA methylation associated with traumatic injury

Genome-wide, 40,003 CpG sites, out of 756, 971, showed nominally significant differences in the fold change of methylated to unmethylated CpG sites associated with traumatic injury in noMDD versus rMDD (xrMDD $p < 0.05$, 40,003/756, 971 = 0.052). Significant depletion of these sites was seen for CpG sites associated with the 1st exon (3.2% v 24.4%, $P < 1 \times 10^{-16}$). Across all CpG sites there was no significant difference in the methylation levels in noMDD, rMDD or xrMDD (Table 2, Supplementary table 2). At sites associated with the first exon, however, the average DNA methylation was higher in individuals with rMDD who reported traumatic injury compared to those who did not report traumatic injury. This pattern was reversed in controls who reported traumatic injury compared to those not

reporting traumatic injury (Table 2). The reversal of the pattern was also seen at exon boundaries, with higher levels of DNA methylation in controls reporting versus not reporting traumatic injury and lower levels of DNA methylation in those with rMDD reporting versus not reporting traumatic injury.

Table 2: Mean difference in M-values at CpG sites with significant differences in the association of traumatic injury with differential methylation in controls (noMDD) compared to rMDD.

Feature	**N. CpG sites Genome**	**N P<0.05 xrMDD**	**% Genome / xrMDD**	**noMDD (Δ M-val)**	**rMDD (Δ M-val)**	xrMDD (Δ M-val)	P.val xrMDD
	214550	11043	21.51 / 27.61	-0.00068	0.004082	**0.00477**	**$2x10^{-4}$**
1stExon	243225	1292	24.38 / 3.23[#]	**-0.00051**	**0.007831**	**0.00835**	**$7x10^{-62}$**
3'UTR	19499	996	1.95 / 2.49	**-0.00151**	0.000904	**0.00241**	**$1x10^{-10}$**
5'UTR	67698	3466	6.79 / 8.66	-0.00013	0.006124	**0.00625**	**$2x10^{-18}$**
Body	288428	14710	28.91 / 36.77	-0.00054	0.004521	**0.00506**	**$3x10^{-3}$**
ExonBnd	5310	255	0.53 / 0.64	**0.00176**	**-0.01070**	**-0.01246**	**$2x10^{-174}$**
TSS1500	98047	5009	9.83 / 12.52	-0.00026	0.002301	**0.00256**	**$4x10^{-3}$**
TSS200	60851	3232	6.10 / 8.08	**-0.00070**	0.005653	**0.00635**	**$1x10^{-16}$**
ALL	997608	40003		-0.00055	0.00392	0.00447	$7x10^{-2}$

[#] Significantly depleted in xrMDD sites $p < 1 \times 10^{-16}$; Bold type = significantly different from 0 at $p < 0.05$.

Methylome-wide association and methylome-wide environment interaction studies of traumatic injury

The association of traumatic injury with DNA methylation was assessed separately in controls (noMDD, Supplementary Table 2) and in individuals with a lifetime diagnosis of MDD (MDD, Supplementary Table 3) or only those with rMDD (Supplementary Table 4). Evidence of differential methylation in the healthy controls versus the MDD groups (MWEIS) was also investigated (xMDD and xrMDD, Supplementary Tables 5 & 6). Finally, meta-analysis of the noMDD and MDD set was performed to identify consistent evidence for differential DNA methylation associated with traumatic injury across all individuals (META, Supplementary Table 7).

Two CpG sites showed methylome-wide significant differential DNA methylation in either the MWAS or MWEIS of traumatic injury (cg14764459, cg02101279; MWAS noMDD and META or xrMDD, respectively; Supplementary Figure 1). Sixty-six CpG sites had p-values less than a suggestive significance level ($p < 1 \times 10^{-5}$, Supplementary Table 7). No CpG sites had suggestive levels of significance in the noMDD as well as either of the MDD groups. However, three CpG sites show suggestive significance across multiple MWAS/MWEIS groups (cg02101279, cg04640885, cg13395761). CpG site cg02101279, maps to a lincRNA *RP11-46E17.6*, and may show lower levels of DNA methylation in the rMDD individuals reporting traumatic injury. MWEIS analyses indicate that both the MDD and rMDD group differ significantly from the noMDD group in the differential methylation levels associated with traumatic injury at this site, with higher levels of DNA methylation in the MDD groups and decreased DNA methylation in the control group reporting traumatic injury. The opposite pattern was observed for cg04640885 that maps to *ZEB2*, with higher levels of DNA methylation in the control group versus lower levels of DNA methylation in MDD, when they reported

traumatic injury and in both MWEIS analyses (Figure 1a, 1c). Finally, cg13395761 maps within the *MATN2* and *RPL30* genes, and shows no evidence for differential DNA methylation controls, but may show an association with higher levels of DNA methylation in the MDD individuals reporting traumatic injury (Figure 1b, 1c). Although, a nominally significant higher level of DNA was seen in controls reporting traumatic injury at an exonic CpG site within *MATN2* (cg01504940, p = 0.031, Figure 1b, 1c). Analysis of MATN2 and ZEB2 co-expression and protein-interaction networks suggests that they function in NOTCH signalling and negative regulation of transcription, respectively (Supplementary Tables 9-12). However, these results are not genome-wide significant and will require replication. There was little overlap between the genes mapping to CpG sites with suggestive significance in controls and rMDD (Supplementary Figure 2*).*

Meta-analysis of the MWAS results from the healthy control and MDD groups identified six CpG sites with suggestive significance (META $P < 1 \times 10^{-5}$). Differential DNA methylation of four of these were suggestive significant in healthy controls alone, and only cg07615506 (xMDD p = 0.019, xrMDD p = 0.44), showed a nominal significant difference between noMDD and MDD groups (cg14764459, *LOC100287944*; cg00215521; cg06679515, *THSD7A; xMDD & rMDD p > 0.05*). The remaining two CpG sites (cg24805069, *STRIP2; FAM40B*; cg22099236, *ORC3L;RARS2;ORC3*) were not suggestive significant in the individuals groups nor nominally significant in the between group comparisons. CpG site, cg14764459 was the only genome-wide significant site in the control individuals and META analyses ($p = 5.14 \times 10^{-8}$ & 6.76×10^{-8} respectively, Figure 1c).

MWAS/MWEIS gene set enrichment analyses

Gene set enrichment analyses, performed on ranked list of mapped genes in WebGestalt[45], identified 808 gene ontology (GO) terms at an FDR significant p-value < 0.05 in at least one MWAS/MWEIS of traumatic injury, of which 205 terms were significant in all six (noMDD, MDD, rMDD, xMDD, xrMDD, META; Supplementary Table 13). Multicellular organismal response to stress (GO:0033555) was significant at an FDR P < 0.05, for all the analyses except xMDD. xrMDD showed the greatest enrichment of this term (33 leading edge genes, normalized enrichment values = 1.89, FDR P = 0.011).

Comparison of the gene ontology terms found to be enriched at FDR p < 0.05 across the MWAS and MWEIS shows that the majority that are shared in the analyses of both noMDD and rMDD (325/563 GO terms for rMDD are shared with noMDD). However, the majority of these terms are also enriched in xrMDD, suggesting enrichment for differences in effect sizes for the association in noMDD versus rMDD (371/563 are FDR P < 0.05, Figure 2, Supplementary Figure 3-8).

Normalized enrichment values > 2 were found for 18 terms (Supplementary Table 14), of which 16 were FDR significant in all six MWAS/MWEIS (hypergeometric p-value = 1.7×10^{-8}). These terms included biological processes and molecular functions associated with neuron projection development, neuron projection organization and cell surface/transmembrane receptor signalling pathways (negative chemotaxis, cyclic nucleotide metabolic process, semaphorin-plexin signaling pathway, gamma-aminobutyric acid signaling pathway, transmembrane receptor protein kinase activity and glutamate receptor activity). The only GO cellular component term included in this set was that of Schaffer collateral - CA1 synapse. This suggests that these gene ontologies show consistent enrichment for differential DNA methylation, but that those associations are significantly different in individuals with MDD. The two pathways not significant in all six MWAS/MWEIS were excitatory synapse (FDR significant in all except MDD) and tau-protein kinase activity (FDR significant in META, rMDD, xMDD and xrMDD).

There were three terms with normalized enrichment values > 2 in the META analysis. These were gamma-aminobutyric acid signaling pathway, neuron projection guidance and mesenchymal cell proliferation (FDR P = 0.0040, 0.0010, 0.0025, respectively). A further six terms were FDR significant in META, noMDD, MDD and rMDD, but not in either of the MWEIS. These were: mismatch repair, Notch signaling pathway, beta-catenin-TCF complex assembly, beta-catenin binding, hepaticobiliary system development and telomere organization. Only two terms, histone deacetylase binding and

cyclase activity, were significantly enriched only in noMDD, MDD and rMDD, but not in the META or either MWEIS.

Twenty-two GO terms were FDR significant in the MDD-related MWAS and both MWEIS (but not in noMDD or META) consistent with an MDD-specific pattern. These included: regulation of neurological system process, amyloid precursor protein metabolic process, response to ischemia and one cellular component: cell division site. Twenty-six GO terms were FDR significant in four MWAS/MWEIS including META, rMDD and xrMDD, consistent with greater enrichment of putative differentially methylated genes in rMDD. These included: apical part of cell, midbrain development, neural precursor cell proliferation, semaphorin receptor binding and tau-protein kinase activity.

Of note are those terms with FDR p-values < 0.05 in all six MWAS/MWEIS analyses that contain *MATN2* or *ZEB2*. These included for *MATN2*: axon development, dendrite development, gliogenesis, neuron migration and neuron projection guidance; *ZEB2*: axon development, cell-cell signaling by WNT, central nervous system neuron differentiation, forebrain development, regulation of cell morphogenesis, regulation of neuron projection development, and regulation of protein serine/threonine kinase activity. *MATN2* containing GO terms significant in more than one group included: extracellular matrix, extracellular matrix structural constituent, regeneration, and response to axon injury. *ZEB2*-related GO terms further included, neural precursor cell proliferation, which was FDR significant in four MWAS/MWEIS: META, rMDD, TIxMDD, and TIxrMDD; and two further terms: phosphatase regulator activity and somite development that were significant in more than one analysis.

Enrichment of MWAS/MWEIS pathways for common variants associated with adult trauma or MDD

Pathway-specific polygenic risk score analyses were performed to assess the enrichment of association signals for traumatic injury, MDD and related traits. Eighteen pathways that had normalized enrichment values > 2 and FDR p < 0.05 in any one MWAS/MWEIS analysis were selected (including 11 biological processes, two cellular components and five molecular functions, see Supplementary Table 14).

Polygenic scores were weighted by a GWAS for adult trauma in analysed in UK Biobank (PRS_{AT}, Supplementary Table 15; Supplementary material, Supplementary Table 16; Supplementary Figures 10-12). For PRS_{AT}, neither the whole genome base set (p = 0.68) nor any pathway significantly predicted traumatic injury (competitive p-value > 0.05). PRS_{AT} was significantly associated with MDD (single and recurrent episode, competitive p = 0.0087) and recurrent MDD (competitive p = 0.0014) in SetA but not SetB (competitive p = 0.073 and p = 0.23, respectively). Across both SetA and SetB, enrichment for associated variants was detected for only one PRS_{AT} pathway, that of dendrite development, (competitive p-values SetA-rMDD = 0.040, SetB-MDD = 0.0040, SetB-rMDD = 0.024). This suggests that the dendritic development pathway is enriched for common variants associated with adult trauma that may predict lifetime MDD. Notably, this pathway is one of the three selected pathways that include *MATN2*. Four further pathway-specific PRS_{AT} were associated with MDD or rMDD in SetB: cellular response lipoprotein (competitive p MDD = 0.04), cyclic nucleotide metabolic process (competitive p MDD = 0.029 and rMDD 0.029), transmembrane receptor protein kinase activity (competitive p MDD = 0.012), and excitatory synapse, (competitive p rMDD = 0.023).

Polygenic scores were next generated for the whole genome weighted by the PGC GWAS for MDD (PRS_{MDD}) 2018, [54], Supplementary Table 15). The whole genome base set significantly predicted traumatic injury (p = 0.0047, SetA only) and, as expected, both MDD phenotypes ($P < 8.1 \times 10^{-26}$). Enrichment for associated variants was detected for only one PRS_{MDD} pathway across both traumatic injury and both MDD phenotypes in SetA and SetB, that of the gamma-aminobutyric acid (GABA) signaling pathway, (competitive p-values SetA-TI = 0.045, SetA-MDD = 0.010, SetA-rMDD = 0.0027, SetB-MDD = 0.016). This suggests that the GABA-signaling pathway is enriched for common variants associated with MDD. Enrichment in SetB was also seen for semaphorin receptor binding (competitive p-value MDD = 0.021); and neuron projection organisation (competitive p-value rMDD = 0.0003).

Axon development, a pathway that includes *MATN2* and *ZEB2*, was the only pathway that showed significant enrichment for the same depression phenotype, rMDD, in both the adult trauma and MDD-weighted PRS (SetA competitive p-value = 0.034, SetB competitive p-value = 0.022). Although, the best p-value threshold for PRS_{AT} was much lower, i.e. more significant, and included fewer variants, than that for PRS_{MDD}.

Gene set expression enrichment in adult human brain regions

Mapped genes from the MWAS/MWEIS were assessed for gene expression enrichment in adult human brain regions using data from the Allen Brain Atlas. Brain regions significant at a family-wise error rate (FWER) of <0.05 in one MWAS/MWEIS are shown with the comparative results for that region in the other MWAS/MWEIS (Table 3, Supplementary Table 17). Three regions were FWER significant in the genes from MWAS of traumatic injury in individuals with rMDD: limbic lobe (MWAS/MWEIS p-values threshold (pT) = 0.005, N. genes =3,307, overlap = 1,658) and both left and right supraoptic nucleus (pT = 1 x 10^{-5}, N. genes = 19, overlap = 10 genes including *MATN2*). The limbic lobe was also FWER significant in the MWEIS of MDD (pT = 0.005, N genes = 2,880, overlap=1,447).

Table 3 – Results of regional gene-expression enrichment analyses

MWAS/ MWEIS	pT	Allen ID:	Structure	FWERs for expression quantiles: 0.5, 0.7, 0.9	Min FWER
TIxMDD	**2.3**	**4219**	**LL_Limbic_Lobe**	**0.027;0.543;0.602**	**0.027**
rMDD	**2.3**	**4219**	**LL_Limbic_Lobe**	**0.028;0.964;1**	**0.028**
MDD	2.3	4219	LL_Limbic_Lobe	0.125;0.91;0.935	0.125
noMDD	2.3	4219	LL_Limbic_Lobe	0.605;0.992;0.453	0.453
TIxrMDD	2.3	4219	LL_Limbic_Lobe	0.595;1;0.983	0.595
META	2.3	4219	LL_Limbic_Lobe	0.855;0.944;0.727	0.727
rMDD	**5**	**4624**	**SO_Supraoptic_Nucleus_Right**	**0.033;0.647;1**	**0.033**
rMDD	**5**	**4593**	**SO_Supraoptic_Nucleus_Left**	**0.034;0.3;1**	**0.034**
TIxrMDD	5	4624	SO_Supraoptic_Nucleus_Right	0.581;1;0.995	0.581
TIxrMDD	5	4593	SO_Supraoptic_Nucleus_Left	0.616;0.997;0.985	0.616
MDD	5	4593	SO_Supraoptic_Nucleus_Left	0.66;0.992;0.96	0.66
noMDD	5	4593	SO_Supraoptic_Nucleus_Left	1;0.99;0.815	0.815
noMDD	5	4624	SO_Supraoptic_Nucleus_Right	0.972;0.99;0.877	0.877
MDD	5	4624	SO_Supraoptic_Nucleus_Right	0.887;0.992;0.975	0.887
TIxMDD	5	4593	SO_Supraoptic_Nucleus_Left	0.889;1;1	0.889
TIxMDD	5	4624	SO_Supraoptic_Nucleus_Right	0.984;1;1	0.984
META	5	4593	SO_Supraoptic_Nucleus_Left	1	1
META	5	4624	SO_Supraoptic_Nucleus_Right	1	1

pT = -log10(p-values) threshold for gene selection.

Discussion

Trauma is associated with the onset of mental health issues such as PTSD and major depressive disorder (MDD). In this study, we investigated the association of self-reported traumatic physical injury, bone fracture occurring in traumatic circumstances, with depression and related traits. We

performed a methylome-wide analysis of traumatic physical injury and DNA methylation in individuals with no mental health diagnoses and in those with major depressive disorder. Further, we compared the strength of those associations between the two groups and examined the potential functional effects through analyses of biological pathways, polygenic scores and brain regional expression.

Traumatic physical injury was associated with a lifetime diagnosis of MDD, particularly of a recurrent course, and with symptoms associated with psychiatric illness. There was no association with cognitive traits, apart from with digit symbol coding score, a measure of processing speed which is associated with schizophrenia spectrum disorders.[52] The strongest association was seen with the SPQ cognitive disorganization factor which is strongly positively associated with an individual's personal and familial psychiatric history.[34] Childhood trauma score has previously been associated with both SPQ total score and subscales.[55] A thought disorder factor has also been associated with poor mental health, particularly physical abuse, and intrusive memories following trauma.[56-60] SPQ and thought disorder are related symptoms psychosis, but are not common in MDD, suggesting that the phenotype is suitable for the detection of the effects of trauma.

On average, when comparing the MDD and control groups, we identified opposing patterns of DNA methylation across the approximately 40,000 sites nominally associated with traumatic injury. This pattern was most prominent at the first exon and exon boundaries, despite a significant of differentially methylated sites at the 1st exon in the MDD group. Although, the association between levels of DNA methylation and gene expression are difficult to predict on such a wide scale this may reflect a reduction in gene expression and/or increased alternative splicing at key genes.

Examination of the DNA methylation profiles associated with traumatic injury identified two CpG sites with genome-wide significant differential DNA methylation: cg14764459 (noMDD $p = 5.14 \times 10^{-8}$, META $p = 6.76 \times 10^{-8}$) and cg02101279 (rMDD $p= 8.60 \times 10^{-8}$). Although cg14764459 maps to LOC100287944, it lies in an ENCODE distal enhancer-like signature (cCRE ENCODE Accession: EH38E1641208) linked to *RIC8B* and containing a putative glucocorticoid response element.[61] RIC8B is a non-canonical GEF for heterotrimeric G protein alpha subunits. It is expressed in neural crest cells, astrocytes, Purkinje cells, the cerebellum, hippocampus and adrenal gland. It is associated with neurodevelopment, cortical surface area, cortical thickness and may regulate synapse formation.[62, 63] The second genome-wide significant CpG site, cg02101279 (rMDD $p= 8.60 \times 10^{-8}$), is also associated with a distal enhancer-like sequence with NR3C1 binding potential (EH38E2155469). However, the CpG site is six base-pairs from the enhancer and there is no gene linked to this sequence in the ENCODE SCREEN database.[61]

Downstream analyses of the MWAS/MWEIS showed significant overlap in the gene ontology pathways, but less so individual CpG sites, enriched for signals of differential methylation associated with physical trauma in individuals with and without MDD. Although this initial study suggests that epigenetic response to traumatic injury may be associated with the same functional processes in individuals regardless of their mental health diagnoses, the magnitude, and indeed the direction, of the association was may differ at the levels of individual CpG sites, genes and functional pathways. Gene set enrichment analyses identified enriched DNA methylation signals in key neuronal development and neuronal signalling pathways including axon development, dendrite development and GABA-ergic signalling implicating functional effects on synaptic plasticity.[64, 65] These pathways included *MATN2* and//or *ZEB2,* genes linked to CpG sites with suggestive levels of differential methylation in MDD groups, and were enriched for common variants associated with MDD and adult trauma. GABA-ergic signalling has previously been associated with depression[66] and trauma, particularly involving the limbic system.[67-70] and may be important in the response to cognitive therapy targeting the suppression of traumatic memories.[71] A recent study of depression in twins also identified glutamate and GABA-ergic, as well as ECM-receptor interaction, as enriched in a weighted-gene co-expression network cluster associated with depressive symptoms and disease state (Wang et al., 2021).[72] This study also highlighted the regulation of NOTCH-signalling, a pathway strongly associated with *MATN2*.

Finally, regional brain enrichment analyses suggested that the genes mapping to CpG sites with differential DNA methylation p-values $<1 \times 10^{-5}$ in individuals with rMDD were enriched for genes expressed in the limbic lobe (Allen Brain Atlas ID: 4219, containing cingulate gyrus, hippocampal formation, parahippocampus, piriform cortex) and supraoptic nuclei (Allen Brain Atlas ID: 4593, 4624; part of the anterior hypothalamic region). This is consistent with the enrichment of gene ontology term

Schaffer collateral - CA1 synapse in all the MWAS/MWEIS analyses. Lesions of hippocampal CA1 neurons significantly effects retrieval of episodic memories[73, 74] and these neurons are important for fear extinction.[75] The limbic and hippocampal regions have previously been linked to anxiety, fear memory formation and PTSD.[67] The supraoptic nuclei contain vasopressin and oxytocin-producing cells and has been implicated in responses to acute restraint.[76] Vasopressinergic neurons in the SON sending projections to limbic forebrain structures such as the dorsal hippocampus, ventral hippocampus, BNST, and amygdala,[77] and can control hippocampal excitability and response to acute restraint stress.[76]

The potential regulation of both *MATN2* and *ZEB2* expression by valproic acid (VPA) may indicate a therapeutic role for histone deacetylase inihibitors (HDACi) for psychiatric symptoms resulting from traumatic injury.[78] (See: Supplementary Notes). VPA is a relatively nonselective HDAC Classes I and II inhibitor, known to enhance histone acetylation to promote synaptic plasticity, thereby facilitating fear extinction learning.[79] VPA may increase GABAergic transmission, and normalise glutamate neurotransmission.[80, 81] VPA also upregulates NOTCH1 signalling, at least in proliferating cells. In a large animal brain injury model VPA treatment reduced injury and improved recovery,[82] but trials in humans indicate heterogeneity in response to HDAC inhibition.[83] Evidence, although limited, suggests that use of VPA in conjunction with cognitive behaviour therapy may reduce symptoms of panic disorders and improve fear extinction.[84-86] Roseberry *et al*, suggest that Valproate could provide a possible therapeutic route for anxiety disorders.[87] This is consistent with VPA, prescribed for the treatment of bipolar disorder, as a possible therapeutic approach to trauma, particularly for irritability associated with trauma.[88]

The restriction of these analyses to white British individuals only, reduces the representativeness of the results. The use of Generation Scotland participants may also have resulted in the individuals having less severe depression, higher income, and higher cognitive ability on average than in the general population, as is common for population-based samples. A major limitation of this study is the sample size, particularly of the MDD groups, replication is required in larger samples. The trauma weighted PRS analyses was limited by the availability of a suitable adult trauma genome-wide association study. We used a previously defined adult trauma phenotype, but this phenotype may differ from the traumatic physical injury phenotype. This study examined DNA methylation in whole blood and, although whole blood has shown some correlation with DNA methylation changes in the brain particularly those sites under genetic control, this may reduce the power to detect regional or cell types important to response to traumatic injury. Analysis of whole blood increases the available sample size and may provide a biomarker to guide treatment and/or for treatment response.[89]

We were unable to examine the temporal association of the phenotypes. The traumatic physical injury may have occurred before or after the onset of major depression and current diagnostic symptoms were not included as covariates in the analyses. The differences in DNA methylation levels may therefore reflect genetic susceptibility loci, whist others may reflect the disease state or medication. The identification of differential methylation in those individuals without a diagnosis of mental illness may, however, suggest a direct association with traumatic injury. However, the collection of samples pre- and post-event would be required to distinguish between potential causal mechanisms.

In conclusion, whilst these results suggest that traumatic physical injury is associated with widespread differential DNA methylation and that these associations are enriched in glutamate and GABA-ergic signalling pathways particularly in regions of the brain associated with encoding fear memory. These results require replication in larger cohorts and the use of longitudinal study designs. Such studies may help to identify markers for the long-term effects of traumatic injury, particularly on mental health, and identify therapies and treatments.

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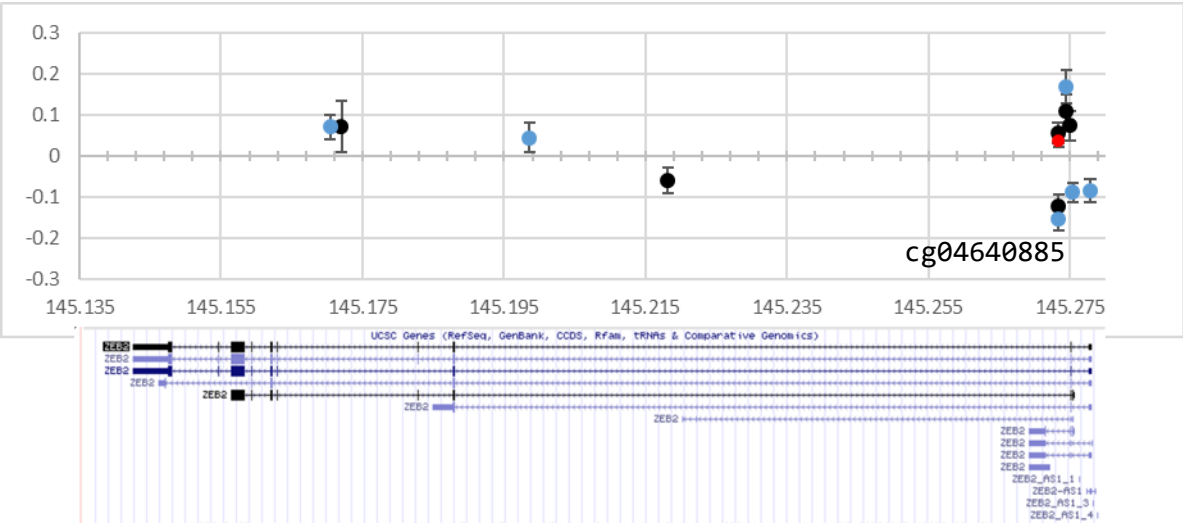
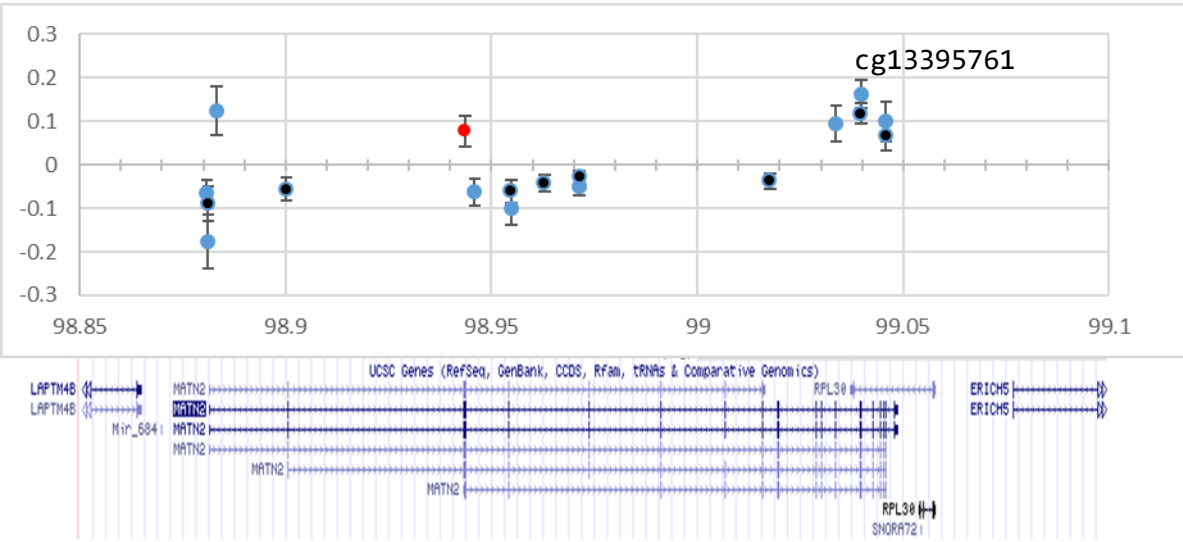
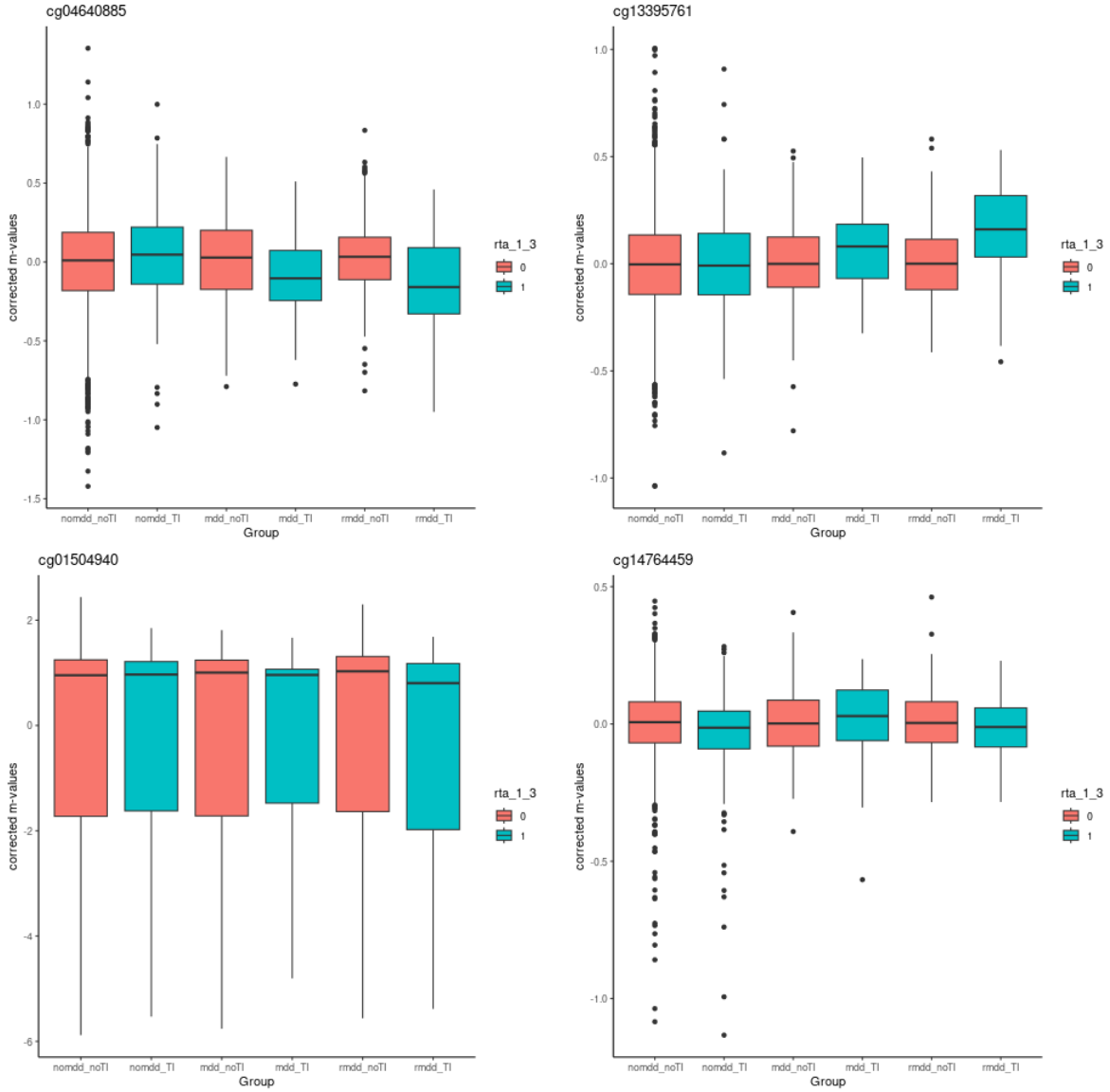
Figure 1: Difference in M-value at CpG sites with nominally significant differential methylation (P < 0.05) associated with traumatic injury in a) *ZEB2* and b) *MATN2*; for noMDD (red), any MDD (black) and rMDD (light blue). X-axis – position in Mbp. Y-axis – difference in M-value between individuals reporting traumatic injury and those not reporting traumatic injury, Error bars – standard error. Lower panel shows the genes in the region. c) Boxplot of M-values (corrected for covariates) for cg04640885 (*ZEB2*), cg13395761 (*MATN2*), cg01504940 (*MATN2*) and cg14764459 (*LOC100287944*), by group: nomdd_noTI, nomdd_TI, smdd_noTI, smdd_TI, rmdd_noTI, rmdd_TI.

a) *ZEB2*



b) *MATN2*



c) cg04640885, cg13395761, cg01504940, cg14764459