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Acute *Brain-Derived Neurotrophic Factor* DNA Methylation Trajectories in CSF and Associations with Outcomes following Severe Traumatic Brain Injury in Adults

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Abstract

Background: Epigenetic biomarkers have the potential to explain outcome heterogeneity following traumatic brain injury (TBI) but are largely unexplored.

Objective: This exploratory pilot study characterized *brain-derived neurotrophic factor* (*BDNF*) DNA methylation trajectories following severe TBI.

Methods: *BDNF* DNA methylation trajectories in cerebrospinal fluid (CSF) over the first five days following severe TBI in 112 adults were examined in association with 3- and 12-month outcomes.

Results: Group-based trajectory analysis revealed low and high DNA methylation groups at two *BDNF* CpG targets that showed suggestive associations ($p < .05$) with outcomes. Membership in the high DNA methylation groups was associated with better outcomes after controlling for age, sex, and injury severity. Associations of age x trajectory group interactions with outcomes at a

third CpG site revealed a pattern of the same or better outcomes with higher ages in the high DNA methylation group and worse outcomes with higher ages in the low DNA methylation group.

Conclusions: Although no observed associations met the empirical significance threshold after correcting for multiple comparisons, suggestive associations of the main effect models were consistent in their direction of effect and were observed across two CpG sites and two outcome time points. Results suggest that higher acute CSF *BDNF* DNA methylation may promote recovery following severe TBI in adults, and this effect may be more robust with higher age. While the results require replication in larger and racially diverse independent samples, *BDNF* DNA methylation may serve as an early postinjury biomarker helping to explain outcome heterogeneity following TBI.

Keywords

brain-derived neurotrophic factor; clinical outcomes; DNA methylation; epigenetics; TBI

INTRODUCTION

Globally, approximately 5.5 million people sustain a severe traumatic brain injury (TBI) each year, with ~15% mortality and a high number of survivors living with chronic functional disability.^{1,2} Current prediction models explain only a third of the variance in patient outcomes.³ The remaining unexplained heterogeneity in outcomes is a critical barrier to accurate prognostication and hinders the development of evidence-based interventions.^{4,5} The investigation of personal biological factors or “biomarkers” is a promising avenue that may explain additional variance and identify mechanistic targets for treatment development. The field of epigenetics has become central to studying the modulation of genetic phenotypes by environmental factors. The most well-studied epigenetic mechanism is DNA methylation (DNAm), which involves the addition of a methyl group to a cytosine-phosphate-guanine (CpG) site, with higher methylation often rendering the gene less transcriptionally active.⁶ Characterizing DNAm representing the central nervous system (CNS) environment following TBI has great potential to elucidate mechanistic differences that may explain outcome heterogeneity.

Experimental models indicate robust DNAm changes in various brain regions and cell types post-TBI. These dynamic methylomic changes have been observed in both pediatric and adult TBI rat models during the acute/sub-acute^{7–11} and chronic^{12,13} periods following brain injury. Both differentially hypomethylated and hypermethylated loci, and associated changes in gene expression, have been observed relative to sham controls. Systems biology approaches have identified enrichment of differentially methylated genes following experimental TBI in cell death and survival, nervous system development and function, inflammation, metabolism, and cell communication pathways.^{10,12}

The present exploratory pilot study aimed to characterize methylation trajectories in DNA extracted from serial cerebrospinal fluid (CSF) samples collected over the first five days following severe TBI in adults and examine associations of inferred trajectory groups with short- and long-term patient outcomes. We chose a candidate gene approach, targeting the brain-derived neurotrophic factor (*BDNF*) gene. *BDNF* is a well-studied neurotrophin

growth factor that is released in an activity-dependent fashion from pre- and post-synaptic neurons. Synthesized as pro-BDNF and subsequently cleaved to mature BDNF, pro-BDNF and mature BDNF function antagonistically to regulate neuroplasticity.¹⁴ *BDNF* gene expression is regulated in part by DNAm, with higher methylation associated with lower gene expression¹⁵ and neuronal synthesis of BDNF¹⁶.

The examination of *BDNF* DNAm in CSF in the acute period following severe TBI may provide new information regarding the role of BDNF in outcome heterogeneity and the epigenetic influence of the CNS environment on *BDNF* gene expression. We characterized *BDNF* DNAm trajectories in CSF over the first five days following severe TBI in adults and examined associations of inferred trajectory group membership with patient outcomes 3- and 12-months post-injury. Given prior TBI studies suggesting differential effects of BDNF with age^{17,18}, we also explored the moderating effect of age on *BDNF* DNAm trajectories and their association with outcomes.

METHODS

Study Design and Participants

We derived data from a prospective, longitudinal, observational study of adults with severe TBI. The study was approved by the University of Pittsburgh Institutional Review Board and consent obtained from legal authorized proxy individuals and re-consent obtained when appropriate. Inclusion criteria included severe head injury, defined as hospital-admission Glasgow Coma Scale (GCS) score ≤ 8 prior to the administration of paralytics or sedatives; age 16 to 80 years; and CSF sampling via external ventricular drain (EVD) during standard care. Individuals with cardiovascular disease or conditions predisposing them to cardiovascular disease were excluded to control for differential DNAm relating to these comorbidities¹⁹ rather than TBI, and to control for the effect of such comorbidities on TBI recovery. Additional exclusion criteria were penetrating head injury or brain death.

DNA Methylation Data Collection

Genome-wide DNAm data were collected from bisulfite converted DNA extracted from CSF collected via EVD into a sterile ventriculostomy bag over the first six days post-TBI. CSF samples were centrifuged, and the cellular pellet and aliquoted supernatant were stored in a -80 degree freezer. DNA was extracted from the cellular pellet using the Qiaamp DNA extraction kit from Qiagen Corp (Qiagen, Valencia, CA, USA).

The Infinium Human Methylation450 Beadchip and Illumina iSCAN (Illumina, Incorporated, San Diego, CA, USA) were used to generate and scan genome-wide DNAm data at the Center for Inherited Disease Research. As part of laboratory quality control (QC), technical replicates and DNAm controls were included, participants were randomly distributed across the batches, and all samples from a participant were run on the same chip and within the same batch.

Genome Studio Software (Illumina, Incorporated, San Diego, CA, USA) was used to analyze the raw genome-wide DNAm data. Data cleaning and QC were performed as previously described.²⁰ Briefly, we used the *Minfi*,²¹ *Enmix*,²² and *funtooNorm*²³ packages

in R to remove poorly performing samples and probes and perform functional normalization. Our final data set included DNAm data for 419,895 probes in 120 participants at up to three cross-sectional time points. Following data cleaning, *BDNF* DNAm data were extracted from the gene transcript region $\pm 5,000$ kb upstream and downstream (GRCh37/hg19, chr11:27671442–27727600).

Patient Outcomes

Outcomes of interest examined at 3 and 12 months post-injury included mortality, global outcome as measured by the Glasgow Outcome Scale (GOS),²⁴ neurobehavioral dysfunction as measured by the Neurobehavioral Rating Scale-Revised (NRS-R),²⁵ and functional outcome as measured by the Disability Rating Scale (DRS).²⁵

Statistical Analyses

Preliminary analyses—Statistical analyses were conducted using R (version 4.0.1, Vienna, Austria) and SAS (version 9.4, SAS Institute Incorporated, Cary, NC, USA). The NRS-R was treated as continuous. The DRS was collapsed into categories and treated as ordinal (0, no disability; 1–11, mild disability; 12–29, severe disability; 30, death), consistent with prior studies²⁶ and to maintain clinical meaningfulness. Both GOS and death were treated as binary after dichotomizing GOS (good, 4 to 5; poor, 1 to 3).^{27,28} GCS was dichotomized (severe, 6–8; very severe, 3–5) due to small cell sizes.^{28,29}

For DNAm data, M values were used and participants with only one DNAm data observation were removed. Standard descriptive statistics and data screening were performed. DNAm observations identified as an outlier >3 times the interquartile range (IQR) within each day were score adjusted. Regression assumptions were evaluated, and data were transformed to normality as necessary.

Group-based trajectory analyses—DNAm trajectory groups were inferred for each CpG site using group-based trajectory analysis (GBTA) and censored normal models implemented with the Proc TRAJ macro in SAS.^{30,31} We largely automated our analyses using R, SAS, and an objective protocol as previously described.³² Briefly, our criterion for best model selection included evaluation of Bayesian Information Criterion, a model simplicity score, and posterior model selection QC including requirement of an average posterior probability of >0.7 , odds of correct classification >5 , and estimated group membership of $>5\%$.³²

Cell-type heterogeneity—Differences in cell-types within biospecimens can vary between individuals, across time and tissues, and can confound relationships in DNAm studies.³³ The overall DNA methylation level represents a composite value of cell-type specific methylation levels weighted by proportion. In cases where genome-wide DNAm data are generated, existing algorithms can be used to infer cell-type proportions for individual samples so this heterogeneity can be controlled for. Because there are no reference DNAm data to infer cell-type heterogeneity (CTH) found in CSF, we used Houseman's reference-free method and the *RefFreeEWAS* package in R³⁴ to generate CTH data as percentages of major cell-types. While this method has been shown to have high

accuracy resulting in putative “cell-types” defined by cell-type specific methylomes³⁴, and cell-types found in peripheral blood are observed within CSF following TBI, the true cell-type identities cannot be confirmed. However, it is currently unlikely that genome-wide and CTH data would be generated for a clinical biomarker due to cost, time, and laboratory burden. To explore the potential clinical utility of *BDNF*DNAm unadjusted for CTH, as well as potential confounding by cell-type, our GBTA protocol was implemented twice for all CpG sites to compute trajectories both unadjusted and adjusted for CTH.

Association analyses—Following GBTA, for CpG sites with trajectory models passing posterior model QC described above, the relationships between inferred trajectory group assignment and patient outcomes were tested in R while controlling for age, sex, and dichotomized GCS using linear (NRS-R), ordinal logistic (DRS), and binary logistic (GOS and death) regression. *P*-values <.05 were considered suggestive. Given the correlation between patient outcomes selected for this study, we did not adjust for multiple testing. However, we did compute an empirical significance threshold, p_{emp} , using 10,000 permutations as previously described.³² For CpG sites associated with outcomes, participant characteristics by trajectory groups were compared using *t*-tests for continuous variables, and Chi-square or Fisher’s exact test for categorical variables. Given prior TBI studies suggesting differential effects of BDNF with age^{17,18}, we also explored the moderating effect of age on *BDNF*DNAm trajectories and their association with outcomes. Analyses mirrored the above but also included an age-trajectory group interaction term for each model.

RESULTS

Preliminary analyses

Serial genome-wide DNAm data between days zero and six post-TBI were available for 120 participants. Days zero and ten were removed due to low sample sizes. Three participants were removed because they had only one DNAm observation, precluding GBTA, and five participants were removed due to missing phenotype/covariate data. Our final sample size consisted of 112 participants with DNAm observations available at 2 (n=19) or 3 (n=93) time points over five days post-TBI. Our sample had a mean age ($\pm$ SD) of 37.8 ($\pm$ 16.9) years, was 79.5% male, and all participants self-reported their race as White. Detailed sample characteristics are presented in Table 1.

From the genome-wide DNAm data, data for 45 CpG sites were included in our analyses. During DNAm outlier screening, 43 observations across the five days were identified as extreme outliers >3 times the IQR and score adjusted. NRS-R data were transformed to normality using a square root transformation. In bivariate analyses, age was associated with square root-transformed NRS-R at 3 and 12 months ($p=0.005$ and 0.006 , respectively), DRS at 3 and 12 months ($p=0.003$ and $p<0.00005$, respectively), GOS at 12 months ($p=0.002$), and death at 3 and 12 months, (both $p<0.00005$) and worse GCS was associated with DRS at 3 months ($p=0.02$). No associations between sex and outcomes were observed.

Group-based trajectory analyses

Of the 45 CpG sites available, DNAm trajectory groups were inferred at 32 CpG sites unadjusted for CTH and 39 CpG sites adjusted for CTH as shown in Supplementary Table 1. Trajectory plots for all CpG sites with models passing QC have been included in the Supplementary Material. Of the models that passed QC, 14 unadjusted models and 36 CTH-adjusted models consisted of only one trajectory group and were excluded from further analyses. Therefore, of the models passing QC, 18 unadjusted and 3 CTH-adjusted models with more than one trajectory group were carried forward for patient outcome association tests.

Association analyses

Primary analyses—Associations between inferred trajectory group assignment and patient outcomes were tested using regression while controlling for age, sex, and dichotomized GCS. Given the scale of this study, we have only presented results for CpG sites with suggestive associations with patient outcomes (cg26057780 and cg25328597). Results for all CpG sites can be found in Supplementary Table 1. At cg26057780 and cg25328597, two distinct trajectory groups were inferred at each CpG site including a ‘low DNAm’ group and a ‘high DNAm’ group. Inferred participant group assignment for each site included: cg26057780 (n=105 (93.8%) low DNAm, n=7 (6.2%) high DNAm); and cg25328597 (n=98 (87.5%) low DNAm, n=14 (12.5%) high DNAm). Observed and estimated DNAm trajectories are shown in Figure 1.

At CpG sites cg26057780 and cg25328597, both the low and high DNAm groups showed no change over time (Figures 1A and 1B, respectively). Participant characteristics by inferred trajectory groups are presented in Table 2. For both cg26057780 and cg25328597, significant differences in age were observed between trajectory groups ($p = 0.02$ and $p = 0.04$, respectively) with higher mean age observed in the high methylation trajectory groups relative to low methylation trajectory groups, as shown in Supplementary Figure 2.

Results of trajectory group—patient outcome association tests are presented in Table 3. At cg26057780, we observed suggestive associations with NRS-R at 3 months ($p=0.04$), DRS at 12 months ($p=0.01$), and death at 12 months ($p=0.04$). At CpG site cg25328597, we observed suggestive associations with DRS at 3 months ($p=0.02$), DRS at 12 months ($p=0.001$), GOS at 12 months ($p=0.03$), death at 3 months ($p=0.01$), and death at 12 months ($p=0.003$). Overall, for both cg26057780 and cg25328597, participants assigned to the high DNAm groups had consistently better outcomes at 3 and 12 months relative to participants assigned to the low DNAm groups. No observed associations met the empirical significance threshold computed using permutation testing ($p_{emp}=0.0008$). No associations were identified in the trajectory models adjusted for CTH.

Interaction analyses—To explore potential interactions between age and DNAm, we used regression while controlling for age, sex, dichotomized GCS, trajectory group, and age x trajectory group interaction as described above. We observed suggestive associations for age x trajectory group interactions with patient outcomes at one CpG site (cg25962210). At cg25962210, two distinct trajectory groups were inferred (n=75 (67.0%) low DNAm,

n=37 (33.0%) high DNAm) with no change over time as shown in Figure 2. Participant characteristics by inferred trajectory groups are presented in Table 4. No differences in participant characteristics were observed by trajectory groups. Detailed results for all CpG sites can be found in Supplementary Table 2.

Results of age x trajectory group interactions for cg25962210 are presented in Table 5. At cg25962210, we observed suggestively significant age x trajectory group interactions for NRS-R at 12 months ($p=0.004$), DRS at 3 months ($p=0.01$), DRS at 12 months ($p=0.002$), and GOS at 12 months ($p=0.02$).

To visualize these interactions, an age x trajectory group interaction plot for NRS-R at 12 months is shown in Figure 3 and interaction plots for all outcomes are shown in the Supplementary Material. In general, participants in the high DNAm group had the same or better outcomes at higher ages while participants in the low DNAm group had worse outcomes at higher ages.

DISCUSSION

The present exploratory pilot study characterized *BDNF*DNAm trajectories in CSF over the first five days following severe TBI in 112 adults and examined associations of inferred trajectory group membership with patient outcomes 3- and 12-months post-injury. Trajectory analysis revealed low and high DNAm groups at two *BDNF*CpG targets (cg26057780 and cg25328597) that showed suggestive associations ($p<.05$) with patient outcomes in models unadjusted for CTH. DNAm trajectories at these two sites were stable across the first five days post-injury. At both CpG sites, a small minority of participants had inferred group assignment in the high DNAm trajectory and participants in this trajectory group were significantly older. Despite their older age, results showed suggestive associations between the high DNAm trajectory groups and better outcomes at 3 and 12 months relative to participants assigned to the low DNAm trajectory groups after controlling for age, sex, and injury severity (GCS). When we explored the moderating effect of age, a third CpG site showed suggestive associations with patient outcomes in models unadjusted for CTH. Specifically, at cg25962210, we observed a similar trajectory pattern to that described above of low and high DNAm trajectories with no change over time. Associations of age x trajectory group interactions with outcomes revealed a relatively consistent pattern of the same or better outcomes with higher age in the high DNAm group and worse outcomes with higher age in the low DNAm group. Although no observed associations met the empirical significance threshold after correcting for multiple comparisons, suggestive associations of the main effect models were consistent in their direction of effect and were observed across two CpG sites and two outcome time points. No associations were identified with patient outcomes in trajectory models adjusted for CTH.

The results suggest that during the acute phase following severe TBI in adults, higher *BDNF*DNAm in CSF, which is assumed to be associated with lower *BDNF* expression¹⁵ and neuronal synthesis,¹⁶ may promote recovery; and conversely, lower *BDNF*DNAm, with presumably higher *BDNF* expression and synthesis, may hinder recovery. Further, our results suggest that this effect may be more robust with higher age at a third suggestive CpG

site. The findings are generally consistent with prior BDNF protein level and genetic studies in cohorts of adults with moderate to severe TBI. Relative to non-brain-injured controls, some studies have shown that BDNF levels during the first week following TBI in adults are higher in CSF,^{18,35} and significantly lower in serum.^{18,36} Along the same lines, CSF and serum BDNF levels are marginally negatively correlated.¹⁸ Higher acute BDNF levels in CSF, and lower acute and chronic levels in serum/plasma, have been associated with poorer outcomes, including mortality,¹⁸ global functioning,^{36,37} and long-term functional cognition and depression.^{35,38} Similarly, initial genetic studies have shown a protective effect of the BDNF Val66Met Met allele (associated with reduced activity-dependent secretion of BDNF) on outcomes following moderate to severe TBI.^{39,40}

Together, these emerging BDNF biomarker studies demonstrating poorer outcomes with greater BDNF action in the CNS suggest that the apoptotic action of pro-BDNF predominates over the survival signaling of mature BDNF in adults with TBI. The risk conferred by greater BDNF action, however, may be moderated by age, possibly related to reductions in BDNF levels⁴¹ and regional balances of BDNF receptor ratios across the lifespan,^{42,43} as has been previously suggested.^{17,18} It is interesting that the detrimental effect of higher *BDNF* action found in adults with TBI contrasts with most evidence in non-brain-injured populations in which lower *BDNF* action appears to confer risk for psychiatric and neurological conditions.^{44–56} This suggests brain-injury-specific involvement of BDNF, possibly related to shifts in BDNF receptor balances in response to injury.^{17,18,57}

Regarding location and functionality of our suggestive CpG sites, all three are annotated to a *BDNF* promoter region (GRCh37/hg19, depending on the transcript of interest: cg26057780, chr11:27721277 north shore in gene body, 5' untranslated region [UTR], or transcription start site [TSS]; cg25328597, chr11:27722638 CpG island in gene body, 5'UTR, or TSS; cg25962210, chr11:27721222 north shore in gene body, 5'UTR, or TSS). Examining the functionality of these CpG sites is outside the scope of the present study and reviewing prior literature for functionality of specific CpG sites is challenging as specific CpG sites are inconsistently included in searchable content (e.g. title, abstract, keywords) in literature databases. Nevertheless, we found several studies reporting significant associations of our three suggestive CpG sites with brain-related outcomes or correlates. DNAm in blood at both cg25328597 and cg25962210 was positively associated with age in a cohort of low-income, primarily African American adults.⁵⁸ In another study, DNAm in blood at cg25328597 was differentially methylated in pre-symptomatic dementia cases versus controls.⁵⁹ In a third study, DNAm in placenta at cg25962210 was positively associated with maternal exposure to war trauma and maternal chronic stress.⁶⁰ Finally, using the Match algorithm and TRANSFAC matrices in geneXplain (genexplain.com), we identified putative transcription binding sites at each of our three suggestive CpG sites (see Supplement 3), suggesting that these sites may be functionally relevant for gene transcription. More specifically, our third CpG site at which we observed a suggestive interaction of age x trajectory group, is situated at a binding site for transcription factor MeCP2, which is a known transcriptional regulator of *BDNF*, affecting BDNF-induced myelin repair mechanisms.⁶¹

Our study selected CSF as the tissue of interest. Because DNAm patterns are tissue-specific and reflective of the local environment of each cell type, and examination of brain tissue is not feasible in patients who survive their injuries, determining which types of tissues serve as effective proxies for the CNS environment is an important methodological consideration. Several studies have shown that DNAm in peripheral tissues, including blood and saliva, is correlated with DNAm in brain tissue or CSF,^{62–64} for several target genes, including *BDNF*.^{65,66} To our knowledge, however, no studies have investigated concordance of DNAm in CSF and brain. Although it is difficult to make inferences about the brain itself without such understanding, to be useful as a clinical biomarker, it is also essential that markers be found in readily accessible tissues or cells. CSF is often accessible in individuals with severe TBI via collection from an EVD placed for intracranial pressure monitoring and CSF diversion as standard of care. However, because the blood brain barrier is disrupted during injury, CSF from individuals with TBI is likely to contain blood, further exacerbating CTH. The influence of CTH in CSF DNAm remains understudied and is likely of critical importance in CSF, in particular, because of injury- and recovery-specific temporal changes. An interesting observation in this study was that, once we controlled for CTH during GBTA, trajectory groups were inferred with high accuracy at a larger number of sites compared with the unadjusted analyses (i.e., 39 versus 32; see Supplementary Table 1). Further, a majority of these CTH-adjusted models included only one group and, of the three carried forward for patient outcome association testing, no associations were identified. These observations suggest that CTH is likely an important factor to variability of CSF *BDNF* DNAm and should be considered in future studies. Of note, however, the examination of CTH is only possible in genome-wide investigations and not in targeted DNAm studies.

Limitations

Several important limitations of the present study should be considered. First, because of the relatively small sample size of the present pilot study, we were unable to examine potential effects of injury heterogeneity, such as brain regions affected and type of injury (e.g. DAI, SDH, etc.) on the results. Next, by selecting DNAm targets within the relatively narrow window of $\pm 5,000$ kb upstream and downstream of the *BDNF* gene region, we could have missed potentially important regulatory regions of the gene. In addition, *BDNF* genotype, which has been inconsistently shown to modulate *BDNF* DNAm,^{47,67–69} was not examined. We focused our exploratory pilot study on epigenetic variability in *BDNF* to the exclusion of other potentially relevant candidate genes for TBI recovery. Interpretability of the results is limited without considering interactions with a larger genetic network. Future investigations examining the effects of injury heterogeneity, expanding the region of interest to include additional CpG sites, examining whether *BDNF* genotype interacts with DNAm in affecting outcomes, and exploring the epigenetic effects of other candidate genes are warranted. Although, to our knowledge, this is the largest clinical study of DNAm following TBI to date, the sample size was relatively small. Relatedly, the interpretability of the results is limited by the large number of tests conducted. It is imperative that this study be replicated in a larger sample to confirm these findings. Finally, race was self-reported by study participants and use of this variable limited the ability to stringently control for population substructure. Moreover, our sample was homogeneous with all participants

reporting White race. Thus, the results may not generalize to individuals with non-White ancestries. Future efforts are needed to replicate these findings in diverse samples.

Conclusion

The investigation of epigenetic biomarkers has the potential to explain outcome heterogeneity following TBI. Results of the present exploratory pilot study suggest that patients' trajectory of *BDNF*DNAm in CSF over the first five days following severe TBI may be associated with short- and long-term outcomes over and above traditional models accounting for age, sex, and injury severity. While the present results require replication in larger and more racially diverse independent samples, *BDNF*DNAm from accessible biosamples may serve as an early postinjury biomarker that more accurately predicts TBI outcomes, thereby allowing for earlier identification of patients at greatest risk for poor recovery and the earlier provision of targeted intervention.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Iaccarino C, Carretta A, Nicolosi F, Morselli C. Epidemiology of severe traumatic brain injury. *J Neurosurg Sci*. Published online 2018. doi:10.23736/S0390-5616.18.04532-0
2. Dewan MC, Rattani A, Gupta S, et al. Estimating the global incidence of traumatic brain injury. *J Neurosurg*. Published online 2019. doi:10.3171/2017.10.JNS17352
3. Maas AIR, Lingsma HF, Roozenbeek B. Predicting outcome after traumatic brain injury. *Handb Clin Neurol*. 2015;128:455–474. <http://www.ncbi.nlm.nih.gov/pubmed/25701901> [PubMed: 25701901]
4. Saatman KE, Duhaime A-C, Bullock R, et al. Classification of traumatic brain injury for targeted therapies. *J Neurotrauma*. 2008;25(7):719–738. doi:10.1089/neu.2008.0586 [PubMed: 18627252]
5. Hawryluk GWJ, Manley GT. Classification of traumatic brain injury: past, present, and future. *Handb Clin Neurol*. 2015;127:15–21. doi:10.1016/B978-0-444-52892-6.00002-7 [PubMed: 25702207]
6. Egger G, Liang G, Aparicio A, Jones PA. Epigenetics in human disease and prospects for epigenetic therapy. *Nature*. 2004;429(6990):457–463. doi:10.1038/nature02625 [PubMed: 15164071]
7. Zhang Z-YZ, Zhang Z, Fauser U, Schluesener HJ. Global hypomethylation defines a sub-population of reactive microglia/macrophages in experimental traumatic brain injury. *Neurosci Lett*. 2007;429(1):1–6. doi:10.1016/j.neulet.2007.09.061 [PubMed: 17996371]
8. Sagarkar S, Bhamburkar T, Shelkar G, Choudhary A, Kokare DM, Sakharkar AJ. Minimal traumatic brain injury causes persistent changes in DNA methylation at *BDNF* gene promoters in rat amygdala: A possible role in anxiety-like behaviors. *Neurobiol Dis*. Published online 2017. doi:10.1016/j.nbd.2017.06.016

9. Schober ME, Ke X, Xing B, et al. Traumatic brain injury increased IGF-1B mRNA and altered IGF-1 exon 5 and promoter region epigenetic characteristics in the rat pup hippocampus. *J Neurotrauma*. Published online 2012. doi:10.1089/neu.2011.2276
10. Meng Q, Zhuang Y, Ying Z, Agrawal R, Yang X, Gomez-Pinilla F. Traumatic Brain Injury Induces Genome-Wide Transcriptomic, Methyloomic, and Network Perturbations in Brain and Blood Predicting Neurological Disorders. *EBioMedicine*. Published online 2017. doi:10.1016/j.ebiom.2017.01.046
11. Gao W-M, Chadha MS, Kline AE, et al. Immunohistochemical analysis of histone H3 acetylation and methylation--evidence for altered epigenetic signaling following traumatic brain injury in immature rats. *Brain Res*. 2006;1070(1):31–34. doi:10.1016/j.brainres.2005.11.038 [PubMed: 16406269]
12. Haghighi F, Ge Y, Chen S, et al. Neuronal DNA Methylation Profiling of Blast-Related Traumatic Brain Injury. *J Neurotrauma*. Published online 2015. doi:10.1089/neu.2014.3640
13. Debski KJ, Pitkanen A, Puhakka N, et al. Etiology matters-Genomic DNA Methylation Patterns in Three Rat Models of Acquired Epilepsy. *Sci Rep*. Published online 2016. doi:10.1038/srep25668
14. Lee R, Kermani P, Teng KK, Hempstead BL. Regulation of cell survival by secreted proneurotrophins. *Science* (80-). Published online 2001. doi:10.1126/science.1065057
15. Lubin FD, Roth TL, Sweatt JD. Epigenetic regulation of BDNF gene transcription in the consolidation of fear memory. *J Neurosci*. 2008;28(42):10576–10586. doi:10.1523/JNEUROSCI.1786-08.2008 [PubMed: 18923034]
16. Martinowich K, Hattori D, Wu H, et al. DNA Methylation – Related Chromatin Remodeling in Activity- Dependent Bdnf Gene Regulation. *Science* (80-). 2003;302(5646):890–894.
17. Failla MD, Kumar RG, Peitzman AB, Conley YP, Ferrell RE, Wagner AK. Variation in the BDNF gene interacts with age to predict mortality in a prospective, longitudinal cohort with severe TBI. *Neurorehabil Neural Repair*. 2015;29(3):234–246. doi:10.1177/1545968314542617 [PubMed: 25063686]
18. Failla MD, Conley YP, Wagner AK. Brain-Derived Neurotrophic Factor (BDNF) in traumatic brain injury-related mortality: interrelationships between genetics and acute systemic and central nervous system BDNF profiles. *Neurorehabil Neural Repair*. 2016;30(1):83–93. doi:10.1177/1545968315586465 [PubMed: 25979196]
19. Prasher D, Greenway SC, Singh RB. The impact of epigenetics on cardiovascular disease. *Biochem Cell Biol*. Published online 2020. doi:10.1139/bcb-2019-0045
20. Arockiaraj AI, Liu D, Shaffer JR, et al. Methylation Data Processing Protocol and Comparison of Blood and Cerebral Spinal Fluid Following Aneurysmal Subarachnoid Hemorrhage. *Front Genet*. Published online 2020. doi:10.3389/fgene.2020.00671
21. Aryee MJ, Jaffe AE, Corrada-Bravo H, et al. Minfi: A flexible and comprehensive Bioconductor package for the analysis of Infinium DNA methylation microarrays. *Bioinformatics*. Published online 2014. doi:10.1093/bioinformatics/btu049
22. Xu Z, Niu L, Li L, Taylor JA. ENmix: A novel background correction method for Illumina HumanMethylation450 BeadChip. *Nucleic Acids Res*. Published online 2016. doi:10.1093/nar/gkv907
23. Oros Klein K, Grinek S, Bernatsky S, et al. FuntooNorm: An R package for normalization of DNA methylation data when there are multiple cell or tissue types. *Bioinformatics*. Published online 2016. doi:10.1093/bioinformatics/btv615
24. McMillan T, Wilson L, Ponsford J, Levin H, Teasdale G, Bond M. The Glasgow Outcome Scale-40 years of application and refinement. *Nat Rev Neurol*. Published online 2016. doi:10.1038/nrneurol.2016.89
25. McCauley SR, Levin HS, Vanier M, et al. The neurobehavioural rating scale-revised: Sensitivity and validity in closed head injury assessment. *J Neurol Neurosurg Psychiatry*. Published online 2001. doi:10.1136/jnnp.71.5.643
26. Conley YP, Okonkwo DO, Deslouches S, et al. Mitochondrial polymorphisms impact outcomes after severe traumatic brain injury. *J Neurotrauma*. Published online 2014. doi:10.1089/neu.2013.2855

27. McHugh GS, Butcher I, Steyerberg EW, et al. Statistical approaches to the univariate prognostic analysis of the IMPACT database on traumatic brain injury. *J Neurotrauma*. Published online 2007. doi:10.1089/neu.2006.0026
28. Osier ND, Bales JW, Pugh B, et al. Variation in PPP3CC genotype is associated with long-term recovery after severe brain injury. *J Neurotrauma*. Published online 2017. doi:10.1089/neu.2015.4343
29. Bendinelli C, Ku D, King KL, Nebauer S, Balogh ZJ. Trauma patients with prehospital Glasgow Coma Scale less than nine: not a homogenous group. *Eur J Trauma Emerg Surg*. Published online 2020. doi:10.1007/s00068-019-01139-9
30. Jones BL, Nagin DS, Roeder K. A SAS Procedure Based on Mixture Models for Estimating Developmental Trajectories. *Sociol Methods Res*. 2001;29(3):374–393. doi:10.1177/0049124101029003005
31. Jones BL, Nagin DS. Advances in group-based trajectory modeling and an SAS procedure for estimating them. *Sociol Methods Res*. Published online 2007. doi:10.1177/0049124106292364
32. Heinsberg LW, Arockiaraj AI, Crago EA, et al. Genetic Variability and Trajectories of DNA Methylation May Support a Role for HAMP in Patient Outcomes After Aneurysmal Subarachnoid Hemorrhage. *Neurocrit Care*. Published online 2020. doi:10.1007/s12028-019-00787-4
33. Titus AJ, Gallimore RM, Salas LA, Christensen BC. Cell-type deconvolution from DNA methylation: A review of recent applications. *Hum Mol Genet*. Published online 2017. doi:10.1093/hmg/ddx275
34. Houseman EA, Molitor J, Marsit CJ. Reference-free cell mixture adjustments in analysis of DNA methylation data. *Bioinformatics*. Published online 2014. doi:10.1093/bioinformatics/btu029
35. Failla MD, Juengst SB, Arenth PM, Wagner AK. Preliminary Associations Between Brain-Derived Neurotrophic Factor, Memory Impairment, Functional Cognition, and Depressive Symptoms Following Severe TBI. *Neurorehabil Neural Repair*. 2016;30(5):419–430. doi:10.1177/1545968315600525 [PubMed: 26276123]
36. Korley FK, Diaz-Arrastia R, Wu AHB, et al. Circulating Brain-Derived Neurotrophic Factor Has Diagnostic and Prognostic Value in Traumatic Brain Injury. *J Neurotrauma*. 2016;33(2):215–225. doi:10.1089/neu.2015.3949 [PubMed: 26159676]
37. Schuster A, Treble-Barna A, Dekman M, Wagner AK. Chronic serum BDNF associations with global outcome and disability post-TBI. *J Neurotrauma*. 2018;35(16):A275.
38. Treble-Barna A, Schuster A, Dekman M, Wagner AK. Serum BDNF at 6 months following TBI in adults predicts chronic functional cognitive impairment and post-traumatic depression. *J Neurotrauma*. 2018;35(16):A273.
39. Barbey AK, Colom R, Paul E, et al. Preservation of general intelligence following traumatic brain injury: contributions of the Met66 brain-derived neurotrophic factor. *PLoS One*. 2014;9(2):e88733. doi:10.1371/journal.pone.0088733 [PubMed: 24586380]
40. Krueger F, Pardini M, Huey ED, et al. The role of the met66 brain-derived neurotrophic factor allele in the recovery of executive functioning after combat-related traumatic brain injury. *J Neurosci*. Published online 2011. doi:10.1523/JNEUROSCI.1399-10.2011
41. Islam F, Mulsant BH, Voineskos AN, Rajji TK. Brain-Derived Neurotrophic Factor Expression in Individuals With Schizophrenia and Healthy Aging: Testing the Accelerated Aging Hypothesis of Schizophrenia. *Curr Psychiatry Rep*. Published online 2017. doi:10.1007/s11920-017-0794-6
42. Finan JD, Udani SV, Patel V, Bailes JE. The Influence of the Val66Met Polymorphism of Brain-Derived Neurotrophic Factor on Neurological Function after Traumatic Brain Injury. *J Alzheimer's Dis*. Published online 2018. doi:10.3233/JAD-180585
43. Tapia-Arancibia L, Aliaga E, Silhol M, Arancibia S. New insights into brain BDNF function in normal aging and Alzheimer disease. *Brain Res Rev*. Published online 2008. doi:10.1016/j.brainresrev.2008.07.007
44. Egan MF, Kojima M, Callicott JH, et al. The BDNF val66met polymorphism affects activity-dependent secretion of BDNF and human memory and hippocampal function. *Cell*. 2003;112(2):257–269. doi:10.1016/S0092-8674(03)00035-7 [PubMed: 12553913]

45. Hariri AR, Goldberg TE, Mattay VS, et al. Brain-derived neurotrophic factor val66met polymorphism affects human memory-related hippocampal activity and predicts memory performance. *J Neurosci*. Published online 2003. doi:10.1523/JNEUROSCI.23-17-06690.2003
46. Duan S, Liu Y, Jiang D, et al. Elevation of Peripheral BDNF Promoter Methylation Links to the Risk of Alzheimer's Disease. *PLoS One*. 2014;9(11):e110773. doi:10.1371/journal.pone.0110773 [PubMed: 25364831]
47. Xie B, Liu Z, Liu W, et al. DNA Methylation and Tag SNPs of the BDNF Gene in Conversion of Amnesic Mild Cognitive Impairment into Alzheimer's Disease: A Cross-Sectional Cohort Study. *J Alzheimer's Dis*. 2017;58(1):263–274. doi:10.3233/JAD-170007 [PubMed: 28387675]
48. Ikegame T, Bundo M, Murata Y, Kasai K, Kato T, Iwamoto K. DNA methylation of the BDNF gene and its relevance to psychiatric disorders. *J Hum Genet*. 2013;58(7):434–438. doi:10.1038/jhg.2013.65 [PubMed: 23739121]
49. Pezawas L, Verchinski BA, Mattay VS, et al. The brain-derived neurotrophic factor val66met polymorphism and variation in human cortical morphology. *J Neurosci*. Published online 2004. doi:10.1523/JNEUROSCI.2680-04.2004
50. Kambeitz JP, Bhattacharyya S, Kambeitz-Ilankovic LM, Valli I, Collier DA, McGuire P. Effect of BDNF val66met polymorphism on declarative memory and its neural substrate: A meta-analysis. *Neurosci Biobehav Rev*. Published online 2012. doi:10.1016/j.neubiorev.2012.07.002
51. Ho BC, Milev P, O'Leary DS, Librant A, Andreasen NC, Wassink TH. Cognitive and magnetic resonance imaging brain morphometric correlates of brain-derived neurotrophic factor Val66Met gene polymorphism in patients with schizophrenia and healthy volunteers. *Arch Gen Psychiatry*. Published online 2006. doi:10.1001/archpsyc.63.7.731
52. Brown DT, Vickers JC, Stuart KE, Cechova K, Ward DD. The BDNF Val66Met polymorphism modulates resilience of neurological functioning to brain ageing and dementia: A narrative review. *Brain Sci*. Published online 2020. doi:10.3390/brainsci10040195
53. Carlberg L, Scheibelreiter J, Hassler MR, et al. Brain-derived neurotrophic factor (BDNF) - Epigenetic regulation in unipolar and bipolar affective disorder. *J Affect Disord*. Published online 2014. doi:10.1016/j.jad.2014.07.022
54. Na K-S, Won E, Kang J, et al. Brain-derived neurotrophic factor promoter methylation and cortical thickness in recurrent major depressive disorder. *Sci Rep*. 2016;6:21089. doi:10.1038/srep21089 [PubMed: 26876488]
55. Dell'Osso B, D'Addario C, Carlotta Palazzo M, et al. Epigenetic modulation of BDNF gene: Differences in DNA methylation between unipolar and bipolar patients. *J Affect Disord*. 2014;166:330–333. doi:10.1016/j.jad.2014.05.020 [PubMed: 25012449]
56. Roy B, Shelton RC, Dwivedi Y. DNA methylation and expression of stress related genes in PBMC of MDD patients with and without serious suicidal ideation. *J Psychiatr Res*. 2017;89:115–124. doi:10.1016/j.jpsychires.2017.02.005 [PubMed: 28246044]
57. Rostami E, Krueger F, Plantman S, et al. Alteration in BDNF and its receptors, full-length and truncated TrkB and p75NTR following penetrating traumatic brain injury. *Brain Res*. 2014;1542:195–205. doi:10.1016/j.brainres.2013.10.047 [PubMed: 24192075]
58. Saito T, Braun PR, Daniel S, et al. The relationship between DNA methylation in neurotrophic genes and age as evidenced from three independent cohorts: differences by delirium status. *Neurobiol Aging*. Published online 2020. doi:10.1016/j.neurobiolaging.2020.06.003
59. Fransquet PD, Lacaze P, Saffery R, et al. DNA methylation analysis of candidate genes associated with dementia in peripheral blood. *Epigenomics*. 2020;12(23):2109–2123. doi:10.2217/epi-2020-0236 [PubMed: 33300824]
60. Kertes DA, Bhatt SS, Kamin HS, Hughes DA, Rodney NC, Mulligan CJ. BDNF methylation in mothers and newborns is associated with maternal exposure to war trauma. *Clin Epigenetics*. Published online 2017. doi:10.1186/s13148-017-0367-x
61. KhorshidAhmad T, Acosta C, Cortes C, Lakowski TM, Gangadaran S, Namaka M. Transcriptional Regulation of Brain-Derived Neurotrophic Factor (BDNF) by Methyl CpG Binding Protein 2 (MeCP2): a Novel Mechanism for Re-Myelination and/or Myelin Repair Involved in the Treatment of Multiple Sclerosis (MS). *Mol Neurobiol*. Published online 2016. doi:10.1007/s12035-014-9074-1

62. Liu BL, Cheng JX, Zhang W, et al. Quantitative detection of multiple gene promoter hypermethylation in tumor tissue, serum, and cerebrospinal fluid predicts prognosis of malignant gliomas. *Neuro Oncol*. Published online 2010. doi:10.1093/neuonc/nop064
63. Masliah E, Dumaop W, Galasko D, Desplats P. Distinctive patterns of DNA methylation associated with Parkinson disease: Identification of concordant epigenetic changes in brain and peripheral blood leukocytes. *Epigenetics*. Published online 2013. doi:10.4161/epi.25865
64. Davies MN, Volta M, Pidsley R, et al. Functional annotation of the human brain methylome identifies tissue-specific epigenetic variation across brain and blood. *Genome Biol*. 2012;13(6):R43. doi:10.1186/gb-2012-13-6-r43 [PubMed: 22703893]
65. Hing B, Sathyaputri L, Potash JB. A comprehensive review of genetic and epigenetic mechanisms that regulate BDNF expression and function with relevance to major depressive disorder. *Am J Med Genet Part B Neuropsychiatr Genet*. Published online 2017. doi:10.1002/ajmg.b.32616
66. Stenz L, Zewdie S, Laforge-Escarra T, et al. BDNF promoter I methylation correlates between post-mortem human peripheral and brain tissues. *Neurosci Res*. Published online 2015. doi:10.1016/j.neures.2014.10.003
67. Kim JM, Stewart R, Park MS, et al. Associations of BDNF Genotype and Promoter Methylation with Acute and Long-Term Stroke Outcomes in an East Asian Cohort. *PLoS One*. 2012;7(12):1–9. doi:10.1371/journal.pone.0051280
68. Ursini G, Cavalleri T, Fazio L, et al. BDNF rs6265 methylation and genotype interact on risk for schizophrenia. *Epigenetics*. 2016;11(1):11–23. doi:10.1080/15592294.2015.1117736 [PubMed: 26889735]
69. Kang HJ, Kim JM, Kim SY, et al. A longitudinal study of BDNF promoter methylation and depression in breast cancer. *Psychiatry Investig*. 2015;12(4):523–531. doi:10.4306/pi.2015.12.4.523

Figure 1.

Trajectory plots for CpG sites cg26057780 and cg25328597 unadjusted for cell-type heterogeneity

Note: CSF, cerebrospinal fluid; TBI, traumatic brain injury; (A) cg26057780; (B) cg25328597

Figure 2.

Trajectory plots for CpG site cg25962210 unadjusted for cell-type heterogeneity

Note: CSF, cerebrospinal fluid; TBI, traumatic brain injury

Figure 3.

Age x DNA methylation interaction plot for NRS at 12 months at CpG site cg25962210

Note: NRS-R, Neurobehavioral Rating Scale-Revised (square root transformed)

Table 1.

Sample characteristics

Variable	Sample (n=112)
Age, mean years ($\pm$ SD)	37.8 ($\pm$ 16.9)
Sex, male, n (%)	89 (79.5%)
Glasgow Coma Scale, n (%)	
3–5	37 (33.0%)
6–8	75 (67.0%)
Primary hemorrhage pattern, ^I n (%)	
Epidural	10 (8.9%)
Subdural	42 (37.5%)
SAH	14 (12.5%)
ICH	35 (31.3%)
IVH	1 (0.9%)
DAI	10 (8.9%)
NRS-R-3, mean score (SD)	40.7 ($\pm$ 8.5)
NRS-R-12, mean score (SD)	39.7 ($\pm$ 8.0)
DRS-3, n (%)	
No disability (0)	3 (2.7%)
Mild disability (1–11)	61 (54.5%)
Severe disability (12–29)	25 (22.3%)
Death (30)	23 (20.5%)
Unknown	0 (0%)
DRS-12, n (%)	
No disability (0)	18 (16.1%)
Mild disability (1–11)	46 (41.1%)
Severe disability (12–29)	8 (7.1%)
Death (30)	26 (23.2%)
Unknown	14 (12.5%)
GOS-3, n (%)	
Good (4–5)	28 (25.0%)
Poor (1–3)	84 (75.0%)
Unknown	0 (0%)
GOS-12, n (%)	
Good (4–5)	49 (43.8%)
Poor (1–3)	51 (45.5%)
Unknown	12 (10.7%)
Death-3, n (%)	
No	89 (79.5%)
Yes	23 (20.5%)
Unknown	0 (0%)
Death-12, n (%)	

Variable	Sample (n=112)
No	74 (66.1%)
Yes	26 (23.2%)
Unknown	12 (10.7%)

Note: SD, Standard Deviation; SAH, Subarachnoid Hemorrhage; ICH, Intracranial Hemorrhage; IVH, Intraventricular Hemorrhage; DAI, Diffuse Axonal Injury; NRS-R-3, Neurobehavioral Rating Scale at 3 months (untransformed, raw data); NRS-R-12, Neurobehavioral Rating Scale at 12 months (untransformed, raw data); DRS3; Disability Rating Scale at 3 months; DRS-12, Disability Rating Scale at 12 months; GOS-3, Glasgow Outcome Scale at 3 months; GOS-12, Glasgow Outcome Scale at 12 months; Death-3, Death at 3 months; Death-12, Death at 12 month

¹Secondary hemorrhage patterns were present for many participants but information was not available for this sample

Table 2.

Trajectory group characteristics for cg26057780 and cg25328597

CpG Site	Variable	Low DNA Methylation	High DNA Methylation	<i>p</i>
cg26057780	Group membership, n (%)	105 (93.8)	7 (6.2)	
	Age, mean (SD) years	36.6 (16.2)	56.6 (17.7)	0.02 ^a
	Sex, male, n (%)	83 (79.0)	6 (85.7)	0.99 ^b
	GCS, n (%)			
	3–5	37 (35.2)	0	0.09 ^b
	6–8	68 (64.8)	7 (100)	
cg25328597	Group membership, n (%)	98 (87.5)	14 (12.5)	
	Age, mean (SD)	36.3 (16.0)	48.6 (19.7)	0.04 ^a
	Sex, male, n(%)	78 (80.6)	11 (78.6)	0.98 ^b
	GCS, n (%)			
	3–5	33 (33.7)	4 (28.6)	0.77 ^b
	6–8	65 (66.3)	10 (71.4)	

Note: SD, Standard Deviation; GCS, Glasgow Coma Scale;

^aT-test;^bFisher's exact test; trajectory groups correspond to Figure 1 and Table 3

Table 3.

Results of trajectory group-patient outcome association tests while controlling for age, sex, and dichotomized Glasgow Coma Score

Outcome	n	cg26057780			cg25328597		
		Est	95% CI	<i>p</i>	Est	95% CI	<i>p</i>
NRS-R-3 ^a	49	−0.72	−1.54 to −0.10	0.04 [*]	−0.37	−0.94 to 0.19	0.19
NRS-R-12 ^a	58	−0.38	−1.08 to 0.31	0.27	−0.05	−0.53 to 0.43	0.84
		OR	95% CI	<i>P</i>	OR	95% CI	<i>p</i>
DRS-3 ^b	112	0.43	0.06 to 2.33	0.35	0.22	0.06 to 0.74	0.02 [*]
DRS-12 ^b	98	0.10	0.02 to 0.60	0.01 [*]	0.11	0.03 to 0.38	0.001 [*]
GOS-3 ^c	112	0.53	0.09 to 4.24	0.50	0.38	0.11 to 1.45	0.14
GOS-12 ^c	100	0.30	0.04 to 2.11	0.22	0.21	0.05 to 0.81	0.03 [*]
Death-3 ^c	112	0.14	0.01 to 1.29	0.12	0.04	0.002 to 0.32	0.01 [*]
Death-12 ^c	100	0.09	0.01 to 0.95	0.04 [*]	0.02	0.001 to 0.19	0.003 [*]

Note: n, Sample size; Est, Unstandardized estimate from linear regression; OR, Odds Ratio from ordinal and binary logistic regression; CI, Confidence Interval; *p*, *p*-value for trajectory group (reference, low DNA methylation) based on an alpha of 0.05; NRS-R-3, Neurobehavioral Rating Scale at 3 months (with square root transformation applied as described in the text); NRS-R-12, Neurobehavioral Rating Scale at 12 months (with square root transformation applied as described in the text); DRS-3; Disability Rating Scale at 3 months (treated as ordinal as described in the text); DRS-12, Disability Rating Scale at 12 months (treated as ordinal as described in the text); GOS-3, Glasgow Outcome Scale at 3 months (treated as binary as described in the text); GOS-12, Glasgow Outcome Scale at 12 months (treated as binary as described in the text); Death-3, Death at 3 months; Death-12, Death at 12 month;

^aLinear regression;

^bOrdinal logistic regression;

^cBinary logistic regression;

^{*}Suggestive association (*p*-value <0.05).

Table 4.

Participant characteristics by DNA methylation trajectory group for cg25962210

Variable	Low DNA Methylation	High DNA Methylation	<i>p</i>
Group membership, n (%)	75 (67.0)	37 (33.0)	
Age, mean (SD) years	37.6 (17.3)	38.3 (16.3)	0.84 ^a
Sex, male, n (%)	58 (77.3)	31 (83.8)	0.43 ^b
GCS, n (%)			
3–5	26 (34.7)	11 (29.7)	0.60 ^b
6–8	49 (65.3)	26 (70.3)	

Note: SD, Standard Deviation; GCS, Glasgow Coma Scale;

^aT-test;^bChi-square test; trajectory groups correspond to Figure 2 and Table 4

Table 5.

Results of age-trajectory group interaction analyses while controlling for age, sex, DNA methylation trajectory group, and dichotomized Glasgow Coma Score

Interaction Test Statistics				
Outcome	n	Est	95% CI	p
NRS-R-3 ^a	49	-0.01	-0.03 to 0.03	0.65
NRS-R-12 ^a	58	-0.03	-0.05 to -0.01	0.004 [*]
		OR	95% CI	P
DRS-3 ^b	112	0.93	0.88 to 0.98	0.01 [*]
DRS-12 ^b	98	0.92	0.87 to 0.97	0.002 [*]
GOS-3 ^c	112	0.96	0.90 to 1.02	0.18
GOS-12 ^c	100	0.93	0.88 to 0.99	0.02 [*]
Death-3 ^c	112	0.96	0.90 to 1.04	0.34
Death-12 ^c	100	0.94	0.87 to 1.01	0.09

Note: n, sample size; Est, Unstandardized estimate from linear regression; OR, Odds Ratio from ordinal and binary logistic regression; 95% CI, 95% Confidence Interval; *p*, *p*-value for trajectory group-age interaction term based on an alpha of 0.05; NRS-R-3, Neurobehavioral Rating Scale at 3 months (with square root transformation applied as described in the text); NRS-R-12, Neurobehavioral Rating Scale at 12 months (with square root transformation applied as described in the text); DRS-3; Disability Rating Scale at 3 months (treated as ordinal as described in the text); DRS-12, Disability Rating Scale at 12 months (treated as ordinal as described in the text); GOS-3, Glasgow Outcome Scale at 3 months (treated as binary as described in the text); GOS-12, Glasgow Outcome Scale at 12 months (treated as binary as described in the text); Death-3, Death at 3 months; Death-12, Death at 12 month;

^a Linear regression;

^b Ordinal logistic regression;

^c Binary logistic regression;

^{*} Suggestive association (*p*-value <0.05).