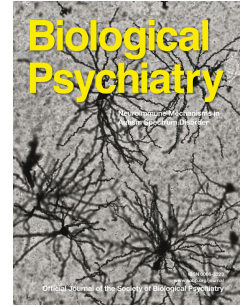


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## Additive effects of stress and alcohol exposure on accelerated epigenetic aging in Alcohol Use Disorder

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**Short title: Effects of Stress and Alcohol on Aging**

**Abstract****Background:**

Stress contributes to premature aging and susceptibility to alcohol use disorder (AUD) and AUD itself is a factor in premature aging; however, the interrelationships of stress, AUD and premature aging are poorly understood.

**Methods:**

We constructed a composite score of stress (CSS) from thirteen stress-related outcomes in a discovery cohort of 317 individuals with AUD and controls. We then developed a novel methylation score of stress (MS Stress) as a proxy of CSS comprising 211 CpGs selected by a penalized regression model. The effects of MS Stress on health outcomes and epigenetic aging were assessed in a sample of 615 AUD patients and controls using epigenetic clocks and DNAm telomere length (DNAmTL). Statistical analysis with an additive model using MS Stress and a methylation score for alcohol consumption (MS Alcohol) were conducted. Results were replicated in two independent cohorts (Generation Scotland GS n=7028 and the Grady Trauma Project GTP n=795).

**Results:**

CSS and MS Stress were strongly associated with heavy alcohol consumption, trauma experience, epigenetic age acceleration (EAA) and shortened DNAmTL in AUD. Together, MS Stress and MS Alcohol additively showed strong stepwise increases in EAA. Replication analyses showed robust association between MS Stress and EAA in the GS and GTP cohort.

**Conclusion:**

A methylation-derived score tracking stress exposure is associated with various stress-related phenotypes and EAA. Stress and alcohol have additive effects on aging, offering new insights into the pathophysiology of premature aging in AUD, and potentially, other aspects of gene dysregulation in this disorder.

**Keywords:** Alcohol, Stress, DNA Methylation, Aging, Epigenetics

## Introduction

Chronic and excessive stress have negative health consequences and are linked to cardiovascular, autoimmune, chronic inflammatory, psychiatric and substance use disorders (1-5). Stress triggers drinking behavior and has been suggested to play a critical role in all phases of alcohol abuse and addiction, including drinking initiation, duration, and relapse (6-8). In addition, there is strong evidence that stress contributes to premature aging and overall mortality (9-11), however the underlying mechanisms remain unclear and the interactions between stress and other environmental exposures, including alcohol, are largely unexplored.

Stress is defined as any stimulus/change that causes physical, emotional, or psychological strain or demand (12). The sources and types of stress are heterogeneous, both in terms of the nature of the stressor, stress exposure duration (acute versus chronic), timing of exposure (developmental versus later in life) and severity. Large inter-individual differences in perception of stress (13, 14) do not necessarily reflect the physiologic impact of stress, and currently most assessments of stress are based on self-report questionnaires dependent on memory recall and that may overestimate or underestimate the impact of the same event on different people. To better understand the biological mechanism by which stress affects risks for substance use, accelerated aging and ultimately mortality, robust biological markers are needed.

The classical pathway of human stress response is the hypothalamic-pituitary-adrenal (HPA) axis which includes the production of stress hormones by the adrenal glands calibrated by release of adrenocorticotrophic hormone (ACTH) by the anterior pituitary, and in turn prompting physiologic changes throughout the body (15). The HPA axis has been extensively studied with regard to stress-response and direct measures of HPA function at one or more timepoints including measures of cortisol and ACTH at various points in the diurnal cycle or following stressful cues or pituitary suppression have overall offered mixed results with regard to

predictive validity and/or diagnostic usefulness as a biomarker (16). An alternative, and hypothesis-free way of studying the long-term effects of stress on the body is by studying changes in the epigenome and transcriptome. While certain epigenetic signatures are stable during the lifetime, other epigenetic components dynamically respond to environmental exposures such as stress, and therefore might offer an opportunity to measure stress exposure. DNA methylation of cytosine nucleotides in the context of phosphate guanine (CpG) dinucleotides can show a dynamic pattern that correlates with environmental exposures, including childhood trauma, smoking and alcohol use (17, 18). Remarkably, DNA methylation patterns are strongly correlated with age/aging which has led to the development of several epigenetic clocks.

Early-stage DNA methylation age (DNAm Age) clocks such as Horvath's and Hannum's clocks were designed to predict chronological age (19-21). Recently, newer generation epigenetic clocks also incorporate other age-related indicators. DNAm PhenoAge was designed as a composite estimate of phenotypic age using both physiological biomarkers of mortality and morbidity as well as chronological age (22), and DNAm GrimAge aggregates DNAm proxies for seven plasma protein biomarkers and DNAm smoking pack-years (23). Similarly, DNAm telomere length (DNAmTL) is a proxy for leukocyte telomere length which can index cellular aging and predict certain clinical outcomes and lifespan (24). Each clock captures different characteristics of biological aging (25) but little is known about the interaction of stress and alcohol on cellular aging as captured by these different indices.

To address the unmet clinical need of identifying biological markers of stress that can guide early intervention strategies and identification of underlying molecular mechanisms for many chronic age-related disorders, and to address the gaps in our understanding of the

interaction between stress and alcohol on aging, we conducted a multi-level investigation of epigenetic biomarkers for stress. We first aimed to construct a composite score of stress (CSS) using 13 stress-related domains ranging from a physiological biomarker to neuropsychological variables in a sample of healthy controls (HC) and individuals with AUD. We then developed a novel epigenetic prediction of stress, which we termed Methylation Score of Stress (MS Stress) as a predictor of CSS. Moreover, we aimed to study the interplay between MS Stress, alcohol, and epigenetic age acceleration (EAA), replicating findings in independent large cohorts and ethnic groups.

## **Materials and Methods**

### ***Study participants***

We used two nested cohorts to develop CSS and MS Stress and two independent cohorts to validate and replicate our findings (Figure 1). A detailed description of the study participants and methods is provided in the Supplementary Methods S1.

**NIAAA Discovery Stress Cohort:** The sample consisted of 317 participants, 166 AUD cases and 151 HC (Table 1, Methods S1).

**NIAAA Epigenomic Cohort:** The epigenetic cohort consisted of 615 participants (372 AUD and 243 HC, Methods S1). All study participants provided written informed consent in accordance with the Declaration of Helsinki and were compensated for their time.

### ***DNA methylation measurements***

Whole blood DNA methylation was quantitated with Infinium MethylationEPIC BeadChip microarrays (Illumina Inc., San Diego, California). Detailed descriptions of the data process and a robust strategy to minimize the batch effects can be found in the Supplementary

Methods S2. The final methylome dataset consisted of  $\beta$ -values for 835,928 CpG sites for all 615 participants.

### ***Factor analysis for a composite score of stress in a discovery stress cohort***

Factor analysis (FA) was performed on thirteen stress-associated measures (Table 1) extracting maximum variance with the first factor and then extracting variance attributable to successive factors. The top three factors, all with eigenvalues  $\geq 1$ , captured 70% of the total variance (see Figure S1). Varimax rotation was applied to yield the most easily interpretable factors. The loading scores of variables onto factors were computed. Finally, the three independent homogeneous factor scores for each participant were added to construct a composite score of stress (CSS).

### ***Estimation of MS Stress and MS Alcohol consumption***

For the larger epigenomic cohort in which some stress-related variables (i.e. morning cortisol) were not measured for some participants, we developed a stress prediction model estimated by DNA methylation profiling. We employed a penalized regression approach based on an Elastic Net models, combined with bootstrap approaches (26). We then selected CpGs when they were presented in more than half of all 1000 bootstraps and included the 211 selected CpGs in the final model to regress them on CSS and estimate weighted coefficients of the 211 CpG sites. MS Stress was then calculated by the weighted sum of linear combinations of the selected CpG sites at the individual level. A detailed description of all procedures is available in the Supplementary Methods S3.



### ***Calculating DNAm Age and Telomere Length***

Six epigenetic clocks including DNA-methylation predictor of Pace-of-Aging (named DunedinPoAm) were estimated by the weighted averages of selected CpG sites (19, 20, 22-24, 27, 28). Detailed descriptions of these epigenetic clocks are in the Supplementary Methods S4 and Table S1. A measure of epigenetic age acceleration (EAA) was defined by taking the residual resulting from regressing DNAm age on chronological age to remove inter-individual variance of chronological age (19, 20, 22, 28). All epigenetic clocks with exception of DunedinPoAm were calculated using the Horvath epigenetic age calculator software (<http://dnamage.genetics.ucla.edu/>).

### ***Multivariate models***

A linear regression model was used to examine the effect of stress (i.e., CSS) on EAA as a dependent variable and stress as an independent variable with adjustment for covariates. A basic model was defined by adjustment for sex, race, AUD, five blood cell types compositions (CD8T, CD4T, NK, Bcell, Mon) by the variance inflation factor analysis. The full model included additional covariates, smoking status and body mass index (BMI). Detailed statistical analyses are available in the Supplementary Methods S5.

### ***Replication Studies***

#### **Generation Scotland cohort (GS, Set 1: n=2578, Set 2: n=4,450)**

DNA methylation from whole blood was assessed using the Infinium MethylationEPIC BeadChip arrays. Detailed cohort descriptions are provided in the Supplementary Methods S6.

#### **Grady Trauma Project (GTP, n=795)**

The Grady Trauma Project (GTP) (29, 30) sample includes 795 participants with DNA methylation profiles (MethylationEPIC BeadChip) and a subset of the sample (n=268) was used to validate accuracy of CSS and MS Stress generation. More details are provided in the Supplementary Methods S6.

## Results

Detailed demographic characteristics of the cohorts can be found in Table 1, Table S2, and the Supplementary Results S1. The exploratory factor analysis revealed three independent homogeneous factors. All three factors were evenly correlated with CSS and MS Stress respectively (50-60%,  $p < 0.0001$ , Figure S1-S2A-B). Finally, the correlation between MS Stress and CSS was 98.8% (Figure S2A-B) and was replicated in a subset of the GTP cohort ( $R = 92.7%$ ,  $p < 2.2E-16$ , Figure S3B), suggesting that our prediction model for MS Stress was highly accurate. Further analysis showed the correlation between CSS and MS Stress in males and females was not different and there was no difference between AUD and HC (Figure S2C-D,  $p = 0.68$ ).

### Association of Stress Scores with Clinical Phenotypes

In the NIAAA epigenomic cohort, increased MS Stress was associated with chronic heavy drinking measured by Total Drinks, Number of Drinking Days, Average Drinks per Day, and Heavy Drinking Days ( $p < 0.001$ , Table 2). These significant associations were also observed when analyzed with AUD cases only (Table S3). The findings were replicated in the two datasets of GS in which MS Stress correlated with weekly alcohol use ( $p < 0.02$ , Table 2) in this cohort that was not ascertained based on AUD. In the GTP, MS Stress was significantly higher in participants with PTSD symptoms within 30 days compared to participants without PTSD

symptoms ( $\beta=0.18$ ,  $p=0.02$ , Table S6). However, we did not observe any association with Trauma Events Inventory total score and moderate and severe childhood trauma assessed by CTQ.

### Association of Stress with Epigenetic Age Acceleration

**EAA in AUD:** EAA derived from GrimAge was 3.2 ( $SE=0.66$ ) years higher in AUD compared to HC ( $p=2.5E-06$ ) after additionally adjusting for CSS in the basic model. The EAA difference in AUD and HC remained significant in the full model (Table 3,  $\beta=2.53$ ,  $SE=0.59$ ,  $p=2.3E-05$ ).

**Composite Score of Stress:** We observed that with every one-unit increase in CSS, GrimAge was accelerated by 0.62-years ( $SE=0.17$ ,  $p=0.0003$ ) and PhenoAge by 0.75-years ( $SE=0.21$ ,  $p=0.0005$ ) respectively after additional adjustment for the effect of AUD in the basic model (Table 3, Figure 2A-B). We did not observe any significant correlation of CSS with Horvath and Hannum clocks (Table 3). Furthermore, GrimAge was accelerated by 5.7-years ( $SE=0.71$ ,  $p=4.55E-13$ ) and PhenoAge by 4.5-years ( $SE=0.94$ ,  $p=4.1E-06$ ) in the highest CSS quartile, compared to the lowest quartile (Figure 2C-D). These results indicate that severe stress remarkably accelerates epigenetic aging. Two stress variables, morning cortisol level and PSS, were not associated with any epigenetic clocks after additionally controlling for AUD, while CTQ total score was associated with PhenoAge and Horvath's clocks (Table S7A-C).

**Methylation Score of Stress:** In the NIAAA epigenome cohort, increased MS Stress was associated with accelerated GrimAge; each one-unit increase in MS Stress accelerating GrimAge by 1.18-years ( $SE=0.19$ ,  $p=1.5E-09$ ) in AUD cases in the basic model (Figure 3A-B, Table 4) and was still observed, although attenuated, in the full model ( $\beta=0.40$ ,  $SE=0.18$ ,  $p=0.03$ ). We observed no significant association between GrimAge and MS Stress in HC. Additional

adjustment for comorbid psychiatric disorders such as either/both MDD or drug dependences did not change our findings of the association between MS Stress and EAAs (Table S4).

DunedinPoAm had an increase pace-of-aging of 0.02 ( $SE=0.003$ ,  $p=2.2E-13$ ) with every one-unit increase of MS Stress in AUD, but no significant increase in HC ( $\beta=0.006$ ,  $p=0.05$ , Figure 3C).

We found no correlation of MS Stress to the Horvath or Hannum clocks. Furthermore, individuals (including AUD patients) in the highest MS Stress quartile were 6.5-years higher in GrimAge ( $SE=0.51$ ,  $p<2E-16$ ) and 4.8-years in PhenoAge ( $SE=0.70$ ,  $p=3.3E-11$ ) compared to those in the lowest quartile (Figure 3D-E). DunedinPoAm aging rate in the highest quartile was 0.11 ( $SE=0.008$ ,  $p<2E-16$ ) faster than that in the lowest. In AUD cases showing biological age acceleration, we observed the prominent effects of MS Stress on EAA (Figure 3G-H). Notably, AUD cases in the severe MS Stress exhibited accelerated GrimAge by 4.7-years ( $SE=0.70$ ,  $p=3.0E-10$ ) and PhenoAge by 3.6-years ( $SE=0.88$ ,  $p=6.1E-05$ ) relative to the lowest stress in the basic model. In the full model, GrimAge was accelerated by 2.42-years in the highest MS Stress quartile ( $SE=0.65$ ,  $p=0.0002$ ). DunedinPoAm aging rate in the highest stress had a 0.08 acceleration ( $p=1.4E-12$ ) in the basic model. In contrast to MS Stress, MS Alcohol had stronger association with PhenoAge than GrimAge: It accelerated PhenoAge by 4.12-years and GrimAge by 1.91-years in both models ( $ps<0.0001$ , Table 4). Detailed descriptions of the relationship between MS Alcohol and EAA is provided in the Supplementary Results S2.

**Additive effect of MS Stress and MS Alcohol:** Our joint analysis in AUD cases revealed that stress and alcohol consumption additively contribute to EAA. In comparison with individuals with low MS Stress and low MS Alcohol as a reference group, GrimAge and PhenoAge were step-wise increased across groups by high stress or high alcohol use, or both (Figure 4A-B). Notably, the group with high stress/high alcohol use exhibited a 3.86-year

increase in GrimAge ( $SE=0.63$ ,  $p=3.2E-09$ ) and those with high stress/low alcohol exhibited a 2.2-year acceleration ( $SE=0.68$ ,  $p=0.001$ ). Similarly, individuals with both high levels of stress and alcohol use had a 4.0-years increases in PhenoAge ( $SE=0.78$ ,  $p=4.7E-07$ ), and those with low stress/high alcohol use had a 2.44-years increase ( $p=0.006$ ). The additive effect of stress and alcohol consumption on EAA was replicated on the GTP cohort where GrimAge was accelerated by 4.57 ( $SE=0.83$ ,  $p<2E-16$ ), 4.14 ( $SE=0.57$ ,  $p=1.2E-12$ ), and 2.16 ( $SE=0.37$ ,  $p=8.2E-09$ )-years in the high stress/high alcohol, high stress/low alcohol, and low stress/high alcohol groups, respectively (Figure 4C-D).

**DNAm Telomere Length:** Age-adjusted DNAmTL was 0.12-kilobases/yr shorter in AUD cases compared to HC in the basic model ( $SE=0.016$ ,  $p=3.6E-14$ ) and the accelerated decline in DNAmTL in AUD cases persisted after controlling for the effect of MS Stress in the basic model ( $\beta=-0.08$ ,  $p=1.3E-05$ ). Figure 5A-B shows the negative correlations of DNAmTL with PhenoAge in both AUD ( $\beta=-14.3$ ,  $p<2E-16$ ) and HC ( $\beta=-10.8$ ,  $p=6.8E-09$ ) but GrimAge more strongly predicted telomere shortening in AUD cases ( $\beta=-13.6$ ,  $p<2E-16$ ) than that in HC ( $\beta=-2.5$ ,  $p=0.05$ ). Moreover, DNAmTL decreased 0.03-kilobase/yr with every one-unit increase in MS Stress in AUD ( $p=9.6E-05$ ), while it decreased 0.10-kilobase/yr in MS Alcohol ( $p=6.5E-08$ ) (Table 4, Figure 5C-D). The relationship between MS Alcohol and DNAmTL shortening remained significant in the full model ( $\beta=-0.01$ ,  $p=5.5E-08$ , Table 4). These findings suggested that alcohol use impacted DNAmTL shortening even more dramatically than stress, although both were associated with DNAmTL shortening in AUD.

### Replication of the effect of MS Stress on EAA in two independent cohorts

We replicated the findings that MS Stress accelerates biological aging in three independent datasets: in the GTP PTSD cohort (Table S5, GrimAge was accelerated by 1.55-years ( $SE=0.16$ ,  $p<2E-16$ ) and PhenoAge by 0.90-years ( $SE=0.24$ ,  $p=0.0002$ ) and DNAmTL decreased by 0.03-kb/yr ( $SE=0.007$ ,  $p=3.6E-05$ ) with MS Stress in the basic model. These findings were also replicated in the two GS datasets, in which MS Stress correlated with accelerated GrimAge ( $\beta>0.06$ ,  $p<2E-16$ ), accelerated PhenoAge ( $\beta>0.02$ ,  $p<0.001$ ), and DNAmTL shortening ( $\beta=-0.001$ ,  $p<1.0E-15$ ). The effect size of these three EAA by MS Stress in the full model were reduced but remained significant (Table S5).

### **Functional annotation of 211 CpGs underlying MS Stress**

The 211 CpGs underlying the MS Stress methylation index were annotated to 151 genes, and the remainder to regions lacking annotation (Table S10, Results S3, Supplementary Discussion). We used the Genomic Regions Enrichment of Annotations Tool (GREAT) to assign potential biological meaning to CpGs (31). Using the default settings (5kb upstream, 1kb downstream, up to 1000kb expansion), 342 genes were associated with the 211 CpGs. Enrichment analysis revealed gene sets related to three categories of GO terms (32) and the 342 genes showed enrichment for cell cycles, regulation of cell death and junction, and neurogenesis in the GO pathways (Table S8, FDR p-value<10E-05).

### **Discussion**

This study uses a novel methylome-based stress score to understand the effect of stress and its interaction with alcohol use on biological aging in AUD. It was performed in a deeply phenotyped sample with replication of the effects of stress on DNA methylation age and

telomere length in two independent cohorts. We constructed a composite score of stress (CSS) that broadly combined stress exposure and responses, including a physiological measurement of cortisol level. We then developed a methylome based MS Stress index that accurately predicted CSS having strong correlations with all thirteen stress-related variables and investigated the relationship between MS Stress and epigenetic age acceleration (EAA), focusing on DNAm PhenoAge and GrimAge, which showed strong correlations with CSS. Our studies revealed that both CSS and MS Stress had similar patterns; increased stress was associated with accelerated epigenetic aging in AUD (Table 3 and 4,  $p < 0.001$ ) and individuals in the highest quartile of stress showing the most pronounced EAA (Figure 2-3). Furthermore, the epigenetic signature of stress was associated with DNAmTL shortening ( $p < 0.0001$ ) and increased aging rate of DunedinPoAm ( $p < 0.0001$ ) in AUD after additional adjustment for the stronger effect of AUD in the basic model ( $\beta = -0.03$ ,  $p = 4.7E-05$ ).

Our sequential analyses revealed a dramatic 4-5 year epigenetic age acceleration among AUD cases in the highest MS Stress quartile (Figure 3,  $p < 0.0001$ ). In addition to MS Stress, MS Alcohol was associated with both acceleration in epigenetic aging and DNAmTL shortening (Table 4, Figure S3, Figure 5D). More importantly, we further showed that in AUD epigenetic age acceleration was additively rather than synergistically increased by stress and alcohol use (Figure 4). Surprisingly, drinking by participants who did not have AUD did not appear to accelerate cellular aging, either alone or additively with stress. In this same vein, we were able to dissociate stress and alcohol exposure using MS Stress and MS Alcohol high/low categories and observed a greater effect when both were at the “worst”. This additive effect is clinically important since AUD is often inherently tied to stress-related disorders and comorbidities such as PTSD, which commonly results in worse treatment outcomes and prognosis (33, 34). We

replicated the impact of our newly developed biological signature of stress on the epigenetic clocks using a stress-enriched sample comprising African American individuals as well as a European population-based cohort (Table S5). These replications further suggest that our findings are detecting stress that can be measured in other general populations as well as populations with severe trauma experience.

Interestingly, we observed main effects for the second-generation epigenetic clocks, (DNAm PhenoAge and GrimAge) as they might capture more pronounced biological aging processes including factors such as stress and alcohol exposure but did not find associations with the first-generation epigenetic clock which are mainly influenced by chronological age. Furthermore, Levine's PhenoAge clock has been found to be more strongly associated with alcohol intake, while GrimAge was methodologically designed to consider smoking-associated effects in contrast to the first-generation clocks (35). Interestingly, those findings are in line with our observation that GrimAge had a stronger correlation with stress than PhenoAge, while PhenoAge had a stronger correlation with alcohol consumption than GrimAge and confirms that the epigenetic clocks of the second generation reflect manifest aspects of stress- or alcohol-related aging process. Additional discussion regarding the effects of smoking on the clocks can be found in the Supplementary Discussion.

Clinically our findings illuminate the potential roles of a stress-related epigenetic signature on biological aging and health complications in AUD, with an aggregative, independently additive, effect of alcohol consumption. There are several reasons why the epigenetic effects of alcohol and stress might have been sub-additive, or super-additive. Alcohol is acutely anxiolytic, alleviating stress but on the other hand, and more profoundly, alcohol itself serves as a stressor activating the HPA axis (36, 37) and via longer term effects such as alcohol



withdrawal and social, medical, and legal problems triggered by alcohol vastly increases anxiety and dysphoria. Although we could not examine the role of alcohol abuse in the stress-alcohol mechanism directly, our findings that heavy drinking and stress additively accelerate biological age may have profound implications for reduced life expectancy and widespread organ damage observed in AUD.

AUD and stress-related disorders may share similar biological pathways (38) including similar regulatory epigenetic mechanisms which may lead to epigenetic age acceleration (39). The 211 stress-related CpGs we identified are co-localized to genes showing enrichment for cell cycle, regulation of cell death and junction, and cancer in the GO pathways (Supplementary Table S8, FDR p-value <10E-05). Cells respond to stress in various ways ranging from activation of pathways promoting survival to the initiation of cell death eliminating damaged cells and it is known that cell cycle is involved in recovery of stress (40). Furthermore, alcohol exposure alters cell cycle and disrupts growth factor related cell cycle progression (41). Therefore, stress and alcohol consumption might share the common pathways signaling that is involved in regulation of cell cycle and biological age acceleration. Moreover, our top six highest weighted CpGs are in *CDKN2C*, *FBXO42*, *TBRG4*, *FKBP11*, *FAM115A*, *ANAPC11* (see Supplementary Discussion).

There are several strengths of our study including the largest sample cohort with comprehensive stress measurements in AUD populations to date, providing accurate prediction to develop methylation driven stress and adequate statistical power to detect significant effects of stress and alcohol consumption on age acceleration. Furthermore, the availability of thirteen stress domains increase power to carry out the relationship with health complication rather than using individual stress variable. Finally, the validation from stress-enriched population to general

healthy populations supported our broad applicability of MS Stress. We also note some weaknesses that should be considered when interpreting our results. First, like measuring stress, the current standard is self-reports which induced recall bias and might not accurately, or fully capture stress exposure. Furthermore, the effect of the identical event can differ dramatically from one person to the next, depending on attachment to the lost object, concurrent events, personality, genotype, and culture. Analogously, alcohol assessments are mostly self-reported and more accurate measures for alcohol exposure are needed (42). In that regard, we would point out that the AUD diagnosis itself is highly reliable, having a very high kappa coefficient in interview/re-interview studies, and the diagnosis even being captured with very high ROC AUC (>.95) sensitivity/specificity with simple questionnaires such as the AUDIT. All our cases and controls were diagnosed by psychiatric interview. However, quantitation of lifetime alcohol exposure is less accurate. Using morning cortisol level as a single physiological stress measure can be problematic, as it is not correlated consistently with other stress domains (43-45). In this regard, both stress methylome and alcohol methylome indices can offer an improved understanding of the severity of exposure over a lifetime, especially when combined with other measures of exposure such as a CTQ childhood trauma/neglect questionnaire.

Although we replicated and confirmed the contribution of stress to EAA in independent populations, our study does not identify a causal relationship between stress and biological aging. A limitation of our cross-sectional study was that it included individuals of different chronological age at one time point. Future studies may collect methylome data longitudinally at multiple time points to better understand how stress and heavy drinking together accelerate epigenetic aging across the life-span. It would be beneficial to determine whether EAA can be a

biomarker that tracks changes in stress-related alcohol use over time and whether the prevention of harmful stress such as childhood abuse can decelerate aging.

MS Stress developed in peripheral blood should be followed by studies in various tissues and cells, and especially to uncover organ-specific pathoetiology. Even though epigenetic aging in peripheral blood and tissues has been shown to be highly correlated (20, 46), future studies should confirm the effect of biological stress on health outcomes in the various tissues and cells. In addition, our findings showed that DNA methylation differences in stress and long-term alcohol use are additively associated with EAA, but we could not determine the directions such as stress stimulates heavy alcohol use or alcohol compensates stress by HPA axis.

In conclusion, our study demonstrates that a methylation derived score tracking stress exposure is associated with various stress-related phenotypes and EAA. We find that stress and alcohol have additive effects on aging, offering new insights into the complex pathophysiology of AUD. Stress seems to affect methylation patterns of cell-cycle sensitive genes providing important new insights and targets for better understanding of the biology of stress.

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### **Author Contributions**

Conception and design of the work: JJ, FWL

Acquisition, analysis, or interpretation of data: JJ, DM, FWL

FWL, JJ drafted and revised the paper. DG provided critical revision of the paper for important intellectual content.

All authors critically reviewed content and approved the final version for publication.

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## Figure Descriptions

### Figure 1: Flowchart of datasets, phenotypes, and analyses

Four cohorts were assessed. These include the Discover Cohort with all stress-associated biomarkers, the Epigenomic Cohort to develop MS Stress, and two replication cohorts. The clinical phenotypes are listed under each cohort. MS Stress: methylation score of stress, MS Alcohol: methylation score of alcohol consumption, EAA: epigenetic age acceleration, DNAmTL: DNA methylation-based telomere length, PTSD: Posttraumatic Stress Disorder.

### Figure 2: Relationship of composite scores of stress to epigenetic age acceleration in the discovery cohort

**A-B:** The scatter plots describe two epigenetic age accelerations (EAA) versus composite score of stress (CSS) and the line in which DNA methylation age was regressed on CSS in AUD and HC respectively. R is a Pearson correlation with 95% confidence interval in parenthesis and p-value of the correlation in the legend. The scatter plot of PhenoAge versus CSS showed in the basic model there was no difference in EAA between AUD and HC ( $p=0.14$ ) but with each one-unit change in CSS, PhenoAge was increased by 0.75 years ( $SE=0.21$ ,  $p=0.0005$ ) in both AUD and HC together; The scatter plot of GrimAge versus CSS demonstrated that there was significant EAA difference between AUD and HC (3.16-yr in AUD,  $SE=0.66$ ,  $p=2.5E-06$ ). Every one-unit increases in CSS was associated with 0.62 years ( $SE=0.17$ ) age acceleration in



GrimAge ( $p=0.0003$ ). **C-D:** The bar plots show estimated means of EAA and standard error (SE) after adjusting for sex, race, and blood cell composition. Individuals with the highest CSS quartile (top 25 percentile) were compared to individuals with the lowest quartile (bottom 25 percentile). The PhenoAge and GrimAge were accelerated by 4.5 years ( $SE=0.94$ ,  $p=4.1E-06$ ) and 5.66 years ( $SE=0.71$ ,  $p=4.55E-13$ ) respectively in the highest quartile of CSS compared to the lowest quartile.

### Figure 3: Relationship of MS Stress to epigenetic age acceleration in the epigenome cohort

**A-B:** The scatter plots describe two epigenetic age accelerations (EAA) versus methylation score (MS) Stress and the line in which DNA methylation age was regressed on MS Stress in AUD and HC respectively, R is a Pearson correlation with 95% confidence interval in parenthesis and p-value of the correlation in the legend. In the basic model, AUD cases show a 0.89-years ( $SE=0.24$ ) acceleration in PhenoAge ( $p=0.0002$ ) and 1.18-years advance in GrimAge ( $SE=0.19$ ,  $p=1.52E-09$ ) for every one-unit increase of MS Stress, while HC showed no significant GrimAge acceleration ( $\beta=0.32$ ,  $SE=0.19$ ,  $p=0.22$ ) and a nominal significant PhenoAge ( $\beta=0.60$ ,  $SE=0.29$ ,  $p=0.04$ ). **C:** the scatter plot with two regression lines describes the DunedinPoAm versus MS Stress in AUD and HC. AUD had a pace-of-aging of 0.02 ( $SE=0.003$ ,  $p=2.2E-13$ ), while HC had 0.006 of aging rate ( $SE=0.003$ ,  $p=0.05$ ). **D-F:** The bar plots show estimated means of EAA and DunedinPoAm in the basic model. Individuals with the highest quartile (top 25%) of MS Stress were compared to individuals with the lowest quartile (bottom 25%). EAA and DunedinPoAm differed significantly between the highest and lowest quartiles of MS Stress. PhenoAge and GrimAge was accelerated by 4.8 ( $SE=0.70$ ,  $p=3.3E-11$ ) and 6.5 years ( $SE=0.51$ ,  $p<2E-16$ ) respectively in the highest quartile compared to the lowest. The aging rate is 0.11 ( $SE=0.008$ ,  $p<2E-16$ ) times faster in individuals with the highest quartile of MS Stress comparing the lowest quartile. **G-I:** AUD cases with the highest quartile of MS Stress were compared to AUD individuals with the lowest quartile. GrimAge was accelerates by 4.7 years ( $SE=0.70$ ,  $p=3.0E-10$ ) and PhenoAge increased by 3.6 years ( $SE=0.88$ ,  $p=6.1E-05$ ) in the highest comparing to the lowest quartiles of MS Stress. Aging rate of DunedinPoAm is 0.08 ( $SE=0.01$ ,  $p=1.4E-12$ ) faster in the highest to lowest quartile.

### Figure 4: Additive effects of MS Stress and MS Alcohol on Epigenetic Age Acceleration in AUD

The bar plots show the estimated means of EAA in the basic model across four groups classified by median splits of MS Stress and MS Alcohol use. High/High indicates individuals with above the median value of MS Stress and MS Alcohol; High/Low with above the median of MS Stress and below the median of MS Alcohol use, etc. **A-B: The estimated means of EAA of PhenoAge and GrimAge over the four groups in NIAAA.** Comparing to AUD participants with low stress/low alcohol as a reference group, MS Stress and MS Alcohol had an additive effect on both epigenetic clocks; GrimAge had 3.86 ( $SE=0.63$ )-years acceleration in high stress/high alcohol ( $p=3.2E-09$ ), 2.2-years ( $SE=0.68$ ,  $p=0.001$ ) in high stress/low alcohol, 1.42 years ( $p=0.05$ ) in low stress/high alcohol, while PhenoAge was advanced by 4.0 years ( $SE=0.78$ ,  $p=4.7E-07$ ) in high stress/high alcohol, 1.77 years ( $SE=0.83$ ,  $p=0.03$ ) in high stress/low alcohol,

2.44 years ( $SE=0.88$ ,  $p=0.006$ ) in low stress/high alcohol group respectively. Furthermore, a linear trend test (an additive effect) using the four groups (0=low/low, 1=low/high, 2=high/low, 3=high/high) shows a linear trend ( $\beta=1.29$ ,  $SE=0.25$ ,  $p=5.5E-07$  for PhenoAge,  $\beta=1.19$ ,  $SE=0.21$ ,  $p=3.02E-08$  for GrimAge) in the basic model. **C-D: The estimated means of EAA of PhenoAge and GrimAge over the four groups in GTP.** Similarly, GrimAge was associated with 4.57 years acceleration ( $SE=0.48$ ,  $p<2E-16$ ) in high stress/high alcohol, 4.14 years ( $SE=0.57$ ,  $p=1.2E-12$ ) in high stress/low alcohol, 2.16 years ( $SE=0.37$ ,  $p=8.2E-09$ ) in low stress/high alcohol group. PhenoAge had 2.07 years advance ( $SE=0.71$ ,  $p=0.003$ ) in high stress/high alcohol group, but it was not significantly accelerated in the other two groups (1.40,  $SE=0.85$  and 0.94,  $SE=0.55$  years,  $ps >0.05$ ).

### Figure 5: DNAm Telomere Length and MS Stress in the epigenome Cohort

The scatter plots show two EAAs versus Age-adjusted DNAm Telomere Length (DNAmTL). R is a Pearson correlation with 95% confidence interval in parenthesis and p-value of the correlation in the legend. **A-B:** The plots describe that in the basic model, PhenoAge has a negative correlation with DNAmTL in both AUD cases ( $\beta=-14.3$ ,  $SE=1.5$ ,  $p<2E-16$ ) and HC ( $\beta=-10.8$ ,  $SE=1.8$ ,  $p=6.8E-09$ ). GrimAge also has a negative association with DNAm TL in only AUD ( $\beta=-13.6$ ,  $SE=1.2$ ,  $p<2E-16$ ) but not in HC ( $\beta=-2.5$ ,  $SE=1.3$ ,  $p=0.05$ ). **C-D:** MS Stress was associated with DNAmTL shortening in AUD ( $\beta=-0.03$ ,  $SE=0.007$ ,  $p=9.6E-05$ ) but not in HC ( $\beta=-0.016$ ,  $SE=0.01$ ,  $p=0.10$ ). MS Alcohol had a significant negative correlation with shortened DNAm TL in AUD ( $\beta=-0.10$ ,  $SE=0.02$ ,  $p=6.5E-08$ ), but not in HC ( $\beta=-0.05$ ,  $SE=0.03$ ,  $p=0.08$ ).



**Key Resource Table**

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**Table 1. Sociodemographic characteristics of discovery stress cohort**

	HC (n=151)		AUD (n=166)		p-value
	N	%	N	%	
Sex					0.014
Male	73	48.34	103	62.05	
Race					0.28
White	72	47.68	92	55.42	
Black	72	47.68	70	42.17	
Others	7	4.64	4	2.41	
Smoking status	8	5.3	92	55.42	<0.0001
	Mean	SD	Mean	SD	
Age	37.59	13.47	46.10	11.23	<0.0001
BMI	26.82	4.62	27.36	5.73	0.35
Multiple Stress domains					
Morning Cortisol	9.92	4.25	12.89	5.19	<0.0001
Perceived Stress Score	8.87	5.67	20.32	7.61	<0.0001
Anxiety (BSA)	0.83	1.88	10.87	7.99	<0.0001
Anxiety (STAIT)	27.46	6.48	47.01	12.70	<0.0001
Depression (MADRS)	1.03	2.40	14.69	10.14	<0.0001
Early Life Stress (ELSQ) total score	2.59	2.86	3.22	3.05	0.07
Childhood Trauma Questionnaire (CTQ) Total Score	34.39	12.61	43.71	20.36	<0.0001
Emotional Abuse score	7.21	4.09	9.92	5.65	<0.0001
Physical Abuse score	6.57	2.70	8.04	4.45	0.0004
Sexual Abuse score	6.11	3.66	7.81	5.60	0.001
Emotional Neglect score	8.34	4.26	10.37	5.55	0.0003
Physical Neglect score	6.42	2.38	7.47	3.90	0.004
<b>Composite Score of Stress</b>	-1.20	0.96	1.15	1.46	<0.0001

**Table 2. Association of MS Stress to alcohol consumptions in the NIAAA and Generation Scotland samples**

Samples	Alcohol Consumption	$\beta$	<i>SE</i>	<i>STAT</i>	<i>P-value</i>
NIAAA Epigenome cohort (n=615)	Total Drinks	0.214	0.043	4.92	<b>9.59E-07</b>
	Drinking Days	0.107	0.033	3.2	<b>0.0015</b>
	Average Drinks Per Day	0.084	0.018	4.66	<b>5.33E-06</b>
	Heavy Drinking Days	0.163	0.031	5.27	<b>2.54E-07</b>
GS					
Data set1 (n=1501)	Standard Drinks/wk	0.075	0.023	3.19	<b>0.001</b>
Data set 2 (n=2717)	Standard Drinks/wk	0.035	0.016	2.25	<b>0.024</b>

*Note:* GS stands for Generation Scotland cohort and NIAAA stands for National Institute of Alcohol Abuse and Alcoholism. The p-values for NIAAA sample were from a linear regression model with natural log transformation of alcohol consumption variables and adjustment for age, sex, race, and AUD. Boldface indicates significance. The p-value for GS were from a linear model with original unit of weekly alcohol use and adjustment for age, sex.

**Table 3. Association of AUD diagnosis and composite score of stress on Epigenetic Age Acceleration in discovery cohort (N=317)**

EAA	AUD			Composite Score of Stress		
	$\beta$	SE	P-value	$\beta$	SE	P-value
GrimAge						
Basic model	3.16	0.66	<b>2.54E-06</b>	0.62	0.17	<b>0.0003</b>
Full model	2.53	0.59	<b>2.3E-05</b>	-0.1	0.17	0.57
PhenoAge						
Basic model	1.2	0.82	0.14	0.75	0.21	<b>0.0005</b>
Full model	0.66	0.81	0.42	0.58	0.23	<b>0.01</b>
Hannum						
Basic model	1.0	0.55	0.07	0.20	0.14	0.16
Full model	0.90	0.56	0.11	0.17	0.16	0.28
Horvath 2013						
Basic model	0.01	0.60	0.99	0.20	0.16	0.20
Full model	-0.10	0.60	0.87	0.30	0.17	0.08
Horvath 2018						
Basic model	-0.36	0.52	0.48	0.17	0.13	0.21
Full model	-0.44	0.52	0.40	0.15	0.15	0.33
DNAmTL						
Basic model	-0.09	0.03	<b>0.003</b>	-0.01	0.008	0.09
Full model	-0.08	0.03	<b>0.008</b>	0.00003	0.008	0.99

*Note:* Adjusted for sex, race, five blood-cell type compositions (CD8T, CD4T, NK, Bcell, Mon) in the basic model, and additionally adjusted for smoking status and body mass index in the full model for all EAA variables. Boldface indicates significance. Effect of AUD was obtained by additionally adjustments of stress (CSS) from a basic or full model; an effect of stress was obtained by additional adjustment by AUD diagnosis from a basic or full model.



**Table 4. Associations between EAA and MS Stress and MS Alcohol in the epigenome cohort (n=615)**

<b>Methylation Score of Stress (MS Stress)</b>							
<b>EAA</b>	AUD cases			Healthy Controls			AUD vs HC
	$\beta$	<i>SE</i>	<i>p-value</i>	$\beta$	<i>SE</i>	<i>p-value</i>	<i>p-value</i>
<b>GrimAge</b>							
Basic model	1.18	0.19	<b>1.52E-09</b>	0.24	0.19	0.22	<b>0.0003</b>
Full model	0.40	0.18	<b>0.03</b>	-0.03	0.18	0.85	<b>0.03</b>
<b>PhenoAge</b>							
Basic model	0.89	0.24	<b>0.0002</b>	0.60	0.29	<b>0.04</b>	0.23
Full model	0.61	0.26	<b>0.018</b>	0.65	0.29	<b>0.03</b>	0.27
<b>DNAmTL</b>							
Basic model	-0.03	0.007	<b>9.6E-05</b>	-0.016	0.01	0.09	0.09
Full model	-0.011	0.008	0.15	-0.015	0.01	0.14	0.39
<b>DunedinPoAm</b>							
Basic model	0.02	0.003	<b>2.2E-13</b>	0.006	0.003	0.05	<b>0.0001</b>
Full model	0.01	0.003	<b>9.5E-05</b>	0.002	0.003	0.48	<b>0.01</b>
<b>Methylation Score of Alcohol Consumption (MS Alcohol)</b>							
<b>GrimAge</b>							
Basic model	1.91	0.50	<b>0.0002</b>	0.61	0.59	0.30	0.06
Full model	1.77	0.42	<b>2.9E-05</b>	0.05	0.56	0.93	<b>0.008</b>
<b>PhenoAge</b>							
Basic model	4.12	0.58	<b>7.3E-12</b>	-0.06	0.90	0.95	<b>0.0005</b>
Full model	4.14	0.57	<b>2.2E-12</b>	0.02	0.90	0.98	<b>0.0003</b>
<b>DNAmTL</b>							
Basic model	-0.103	0.018	<b>6.5E-08</b>	-0.054	0.03	0.08	0.16
Full model	-0.098	0.018	<b>5.5E-08</b>	-0.052	0.03	0.09	0.09
<b>DunedinPoAm</b>							
Basic model	0.03	0.008	<b>0.0008</b>	0.002	0.01	0.85	<b>0.018</b>
Full model	0.025	0.007	<b>0.0003</b>	-0.007	0.01	0.46	<b>0.002</b>



**NIAAA: Discovery cohort**  
(AUD/HC, N=317)

Factor  
analysis

**Composite Score of Stress**  
• Epigenetic age acceleration



**NIAAA: Epigenomic cohort**  
(AUD/HC, N=615)

Elastic net  
regression

**Methylation Score of Stress**

- MS Alcohol
- Alcohol consumption
- Epigenetic age acceleration
- DNAm telomere length
- DunedinPoAm aging rate



**MS Stress**

*REPLICATION*

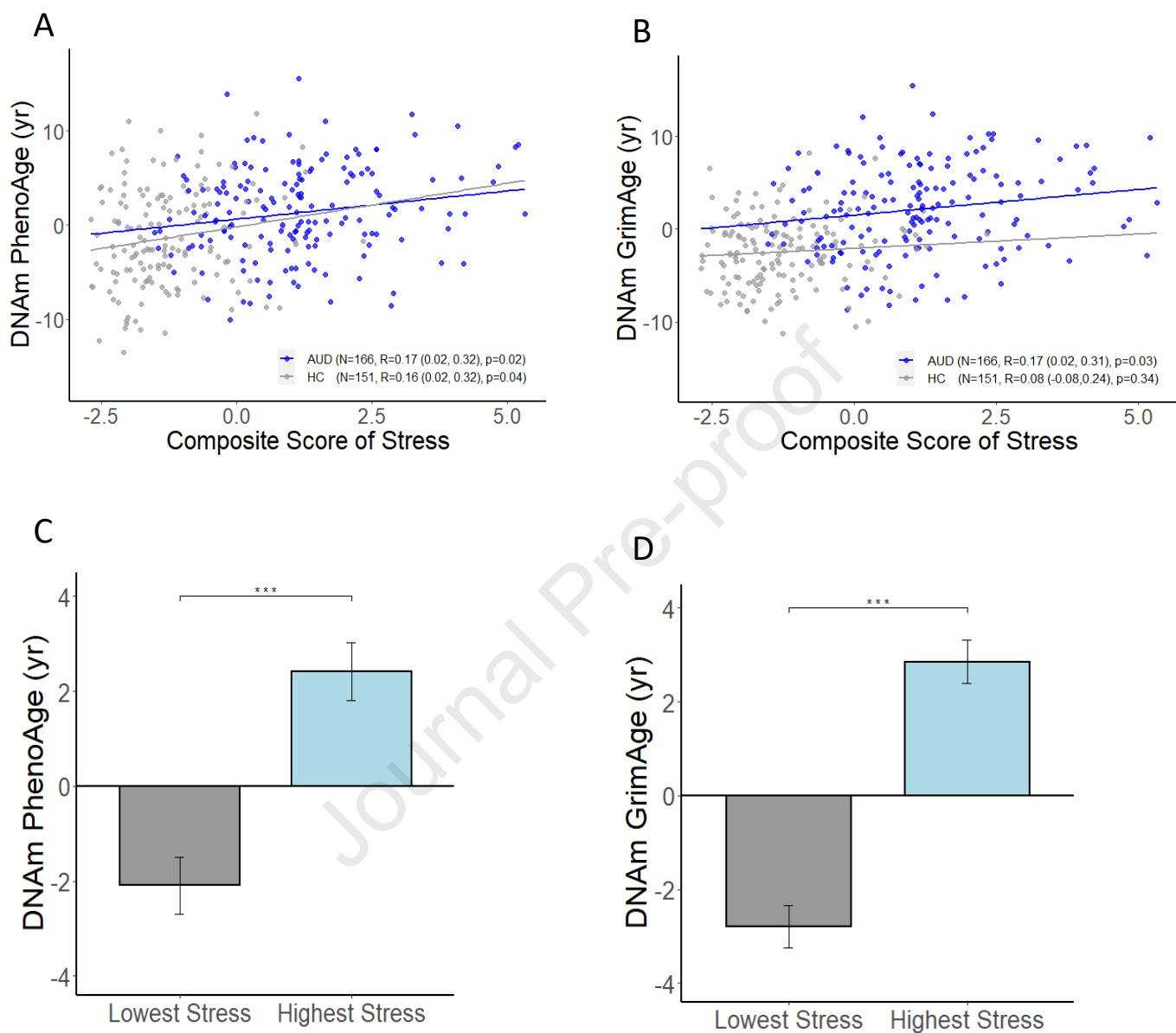
**Generation Scotland (N>7000)**

Data set 1 & 2

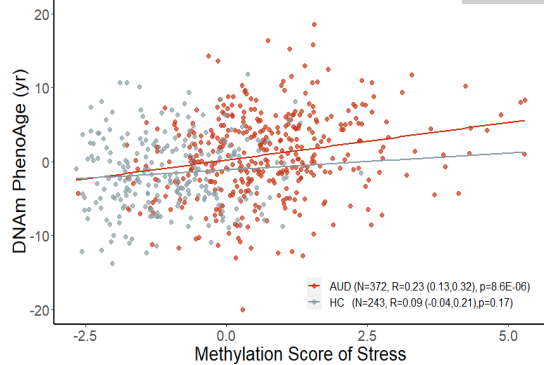
- Epigenetic age acceleration
- DNAmTL
- Alcohol consumption

**Grady Trauma Project (N=795)**

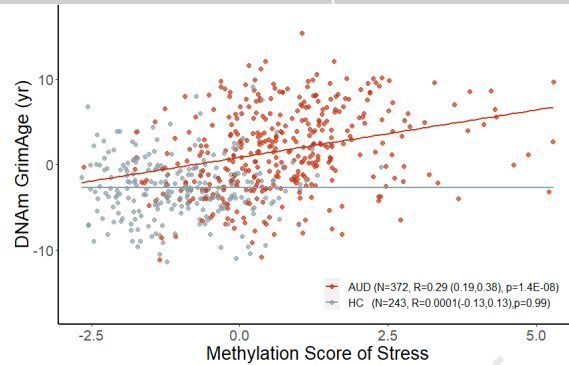
- Epigenetic age acceleration
- DNAmTL
- PTSD
- MS Alcohol



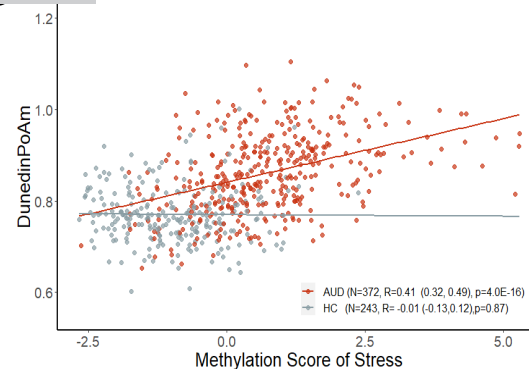
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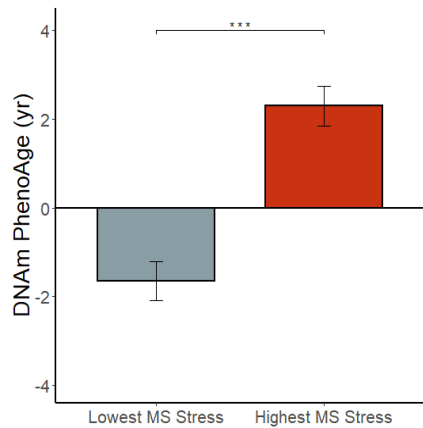
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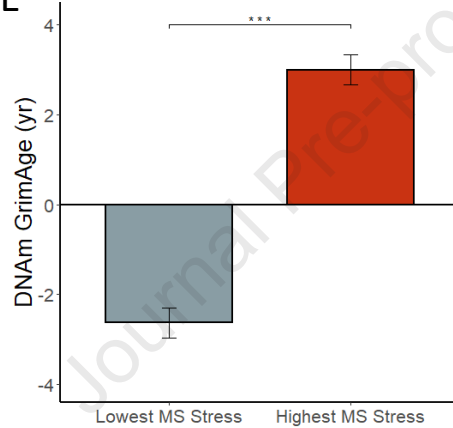
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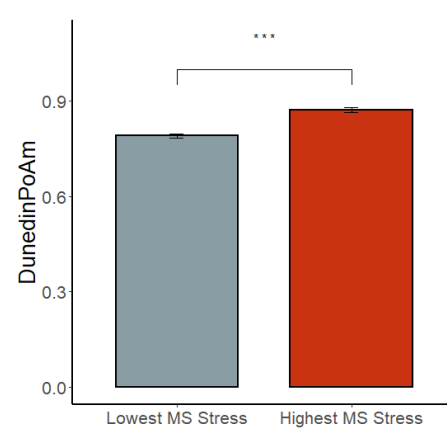
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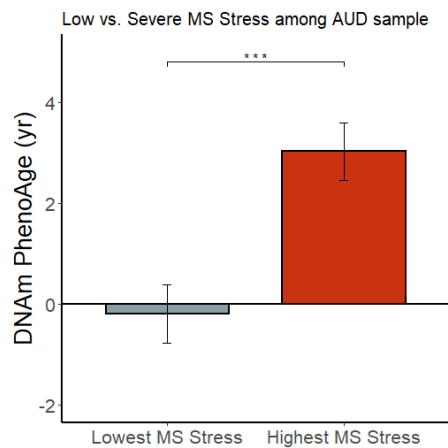
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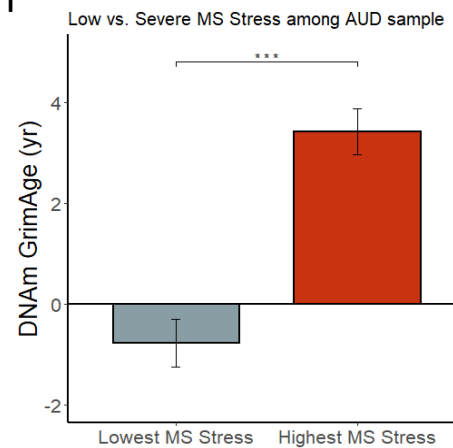
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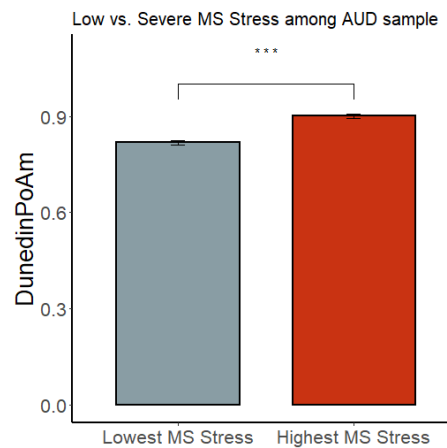
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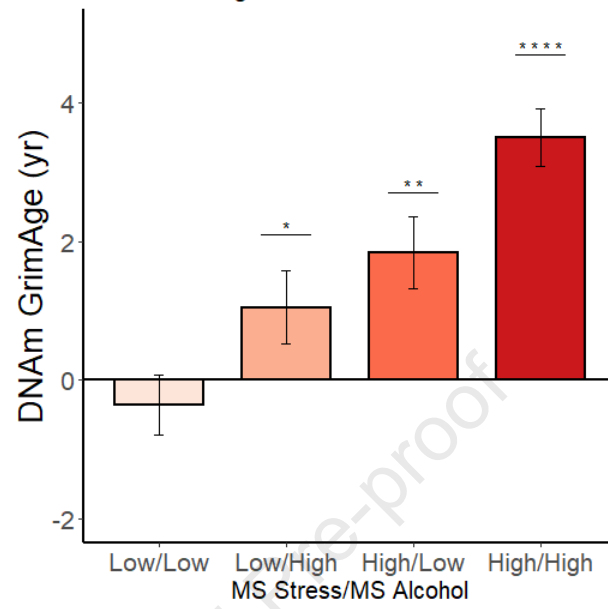
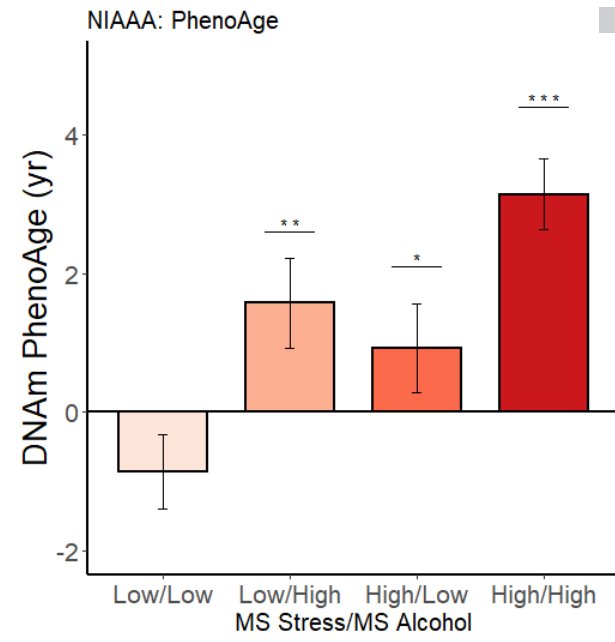
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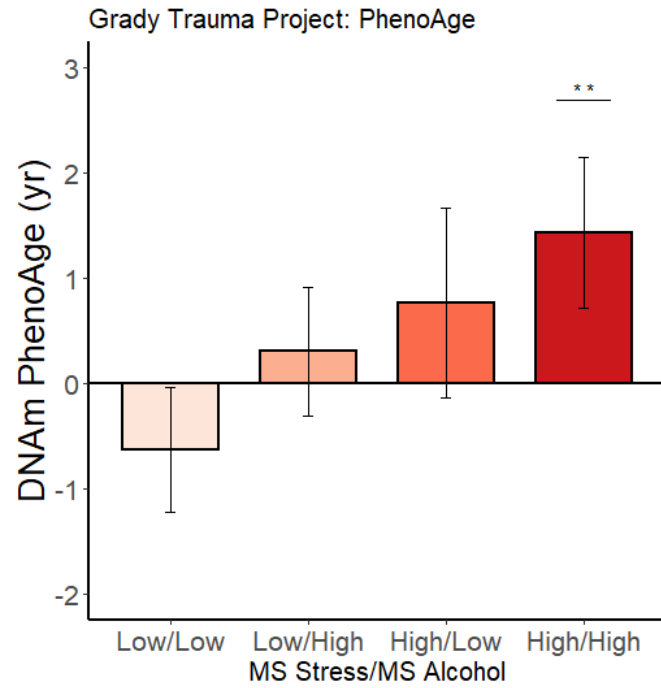
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A



C



D

