Additive effects of stress and alcohol exposure on accelerated epigenetic aging in alcohol use disorder

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Additive Effects of Stress and Alcohol Exposure on Accelerated Epigenetic Aging in Alcohol Use Disorder

Jeesun Jung, Daniel L. McCartney, Josephin Wagner, Joyce Yoo, Andrew S. Bell, Lucas A. Mavromatis, Daniel B. Rosoff, Colin A. Hodgkinson, Hui Sun, Melanie Schwandt, Nancy Diazgranados, Alicia K. Smith, Vasiliki Michopoulos, Abigail Powers, Jennifer Stevens, Bekh Bradley, Negar Fani, Rosie M. Walker, Archie Campbell, David J. Porteous, Andrew M. McIntosh, Steve Horvath, Riccardo E. Marioni, Kathryn L. Evans, David Goldman, and Falk W. Lohoff

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 from 13 stress-related outcomes in a discovery cohort of 317 individuals with AUD and control subjects. We then developed a novel methylation score of stress (MS stress) as a proxy of composite score of stress comprising 211 CpGs selected using a penalized regression model. The effects of MS stress on health outcomes and epigenetic aging were assessed in a sample of 615 patients with AUD and control subjects using epigenetic clocks and DNA methylation–based telomere length. Statistical analysis with an additive model using MS stress and a MS for alcohol consumption (MS alcohol) was conducted. Results were replicated in 2 independent cohorts (Generation Scotland, $N = 7028$ and the Grady Trauma Project, $N = 795$).

RESULTS: Composite score of stress and MS stress were strongly associated with heavy alcohol consumption, trauma experience, epigenetic age acceleration (EAA), and shortened DNA methylation–based telomere length 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 Generation Scotland and Grady Trauma Project cohorts.

CONCLUSIONS: 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.

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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 vs. chronic), timing of exposure (developmental vs. later in life), and severity. Large interindividual differences in perception of stress (13,14) do not necessarily reflect the physiological impact of stress, and currently most assessments of stress are based on self-report questionnaires dependent on memory recall 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 adrenocorticotropic hormone by the anterior pituitary and in turn prompting physiologic changes

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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 time points including measures of cortisol and adrenocorticotropic hormone 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 (DNAm) of CpG dinucleotides can show a dynamic pattern that correlates with environmental exposures, including childhood trauma, smoking, and alcohol use (17,18). Remarkably, DNAm patterns are strongly correlated with age/aging, which has led to the development of several epigenetic clocks.

Early-stage DNAm age (DNAm age) clocks such as Horvath and Hannum 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 physiological biomarkers of mortality and morbidity as well as chronological age (22), and DNAm GrimAge aggregates DNAm proxies for 7 plasma protein biomarkers and DNAm smoking pack-years (23). Similarly, DNAm-based telomere length (DNAmTL) is a proxy for leukocyte telomere length that 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 multilevel 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 control subjects (HCs) and individuals with alcohol use disorder (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.

METHODS AND MATERIALS

Study Participants

We used 2 nested cohorts from the National Institute on Alcohol Abuse and Alcoholism (NIAAA) to develop CSS and MS stress and 2 independent cohorts to validate and replicate our findings (Figure 1). A detailed description of the study participants and methods is provided in Supplemental Methods and Materials S1.

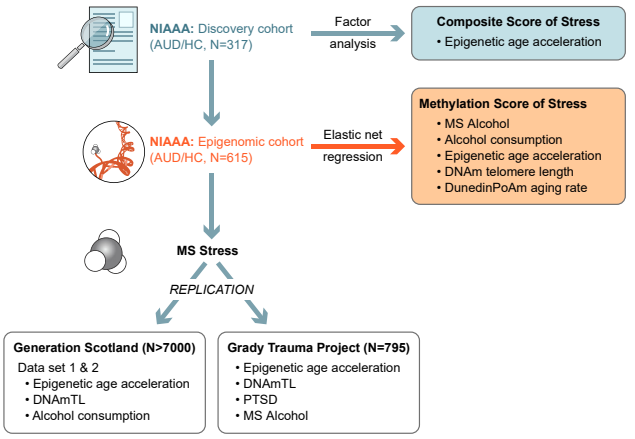

Figure 1. Flowchart of datasets, phenotypes, and analyses. Four cohorts were assessed. These include the discovery cohort with all stress-associated biomarkers, the epigenomic cohort to develop MS stress, and 2 replication cohorts. The clinical phenotypes are listed under each cohort. AUD, alcohol use disorder; DNAmTL, DNA methylation-based telomere length; DunedinPoAm, DNA-methylation predictor of pace-of-aging; HC, healthy control subjects; MS alcohol, methylation score for alcohol consumption; MS stress, methylation score of stress; NIAAA, National Institute on Alcohol Abuse and Alcoholism; PTSD, posttraumatic stress disorder.

NIAAA Discovery Stress Cohort. The sample consisted of 317 participants, 166 AUD cases and 151 HCs (Table 1; Supplemental Methods and Materials S1).

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

DNAm Measurements

Whole-blood DNAm was quantitated using Infinium MethylationEPIC BeadChip microarrays (Illumina, Inc.). Detailed descriptions of the data process and a robust strategy to minimize the batch effects can be found in Supplemental Methods and Materials S2. The final methylome dataset consisted of β values for 835,928 CpG sites for all 615 participants.

Factor Analysis for a CSS in a Discovery Stress Cohort

Factor analysis was performed on 13 stress-associated measures (Table 1) extracting maximum variance with the first factor and then extracting variance attributable to successive factors. The top 3 factors, all with eigenvalues ≥ 1, captured 70% of the total variance (Figure S1). Varimax rotation was applied to yield the most easily interpretable factors. The loading scores of variables onto factors were computed. Finally, the 3 independent homogeneous factor scores for each participant were added to construct a CSS.

Table 1. Sociodemographic Characteristics of Discovery Stress Cohort

Demographics and Stress-Related Measures	HC, *n* = 151	AUD, *n* = 166	*p* Value
Sex, Male	73 (48.34%)	103 (62.05%)	.014
Race	–	–	.28
Black	72 (47.68%)	70 (42.17%)	–
Others	7 (4.64%)	4 (2.41%)	–
White	72 (47.68%)	92 (55.42%)	–
Smoking Status (FTND)[a]	8 (5.3%)	92 (55.42%)	<.0001
Age, Years	37.59 ± 13.47	46.10 ± 11.23	<.0001
BMI	26.82 ± 4.62	27.36 ± 5.73	.35
Multiple Stress Domains			
Morning cortisol, μg/dL	9.92 ± 4.25	12.89 ± 5.19	<.0001
Perceived stress score	8.87 ± 5.67	20.32 ± 7.61	<.0001
Anxiety (BSA)	0.83 ± 1.88	10.87 ± 7.99	<.0001
Anxiety (STAIT)	27.46 ± 6.48	47.01 ± 12.70	<.0001
Depression (MADRS)	1.03 ± 2.40	14.69 ± 10.14	<.0001
Early-life stress (ELSQ) total score	2.59 ± 2.86	3.22 ± 3.05	.07
CTQ total score	34.39 ± 12.61	43.71 ± 20.36	<.0001
Emotional abuse score	7.21 ± 4.09	9.92 ± 5.65	<.0001
Physical abuse score	6.57 ± 2.70	8.04 ± 4.45	.0004
Sexual abuse score	6.11 ± 3.66	7.81 ± 5.60	.001
Emotional neglect score	8.34 ± 4.26	10.37 ± 5.55	.0003
Physical neglect score	6.42 ± 2.38	7.47 ± 3.90	.004
Composite Score of Stress	−1.20 ± 0.96	1.15 ± 1.46	<.0001

Data are presented as *n* (%) or mean ± SD.

AUD, alcohol use disorder; BMI, body mass index; BSA, Brief Scale for Anxiety; CTQ, Childhood Trauma Questionnaire; ELSQ, Early Life Stress Questionnaire; FTND, Fagerström Test for Nicotine Dependence; HC, healthy control subjects; MADRS, Montgomery–Åsberg Depression Rating Scale; STAIT, State-Trait Anxiety Inventory, Trait version.

[a]In the FTND a score of 0 indicates a nonsmoker and 1–10 indicates a smoker.

Estimation of MS Stress and MS Alcohol

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 through DNAm profiling. We used a penalized regression approach based on an elastic net model, 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 Supplemental Methods and Materials S3.

Calculating DNAm Age and Telomere Length

Six epigenetic clocks including DNAm 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 Supplemental Methods and Materials S4 and Table S1. A measure of EAA was defined by taking the residual resulting from regressing DNAm age on chronological age to remove interindividual variance of chronological age (19,20,22,28). All epigenetic clocks with the 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 adjusting for sex, race, AUD, 5 blood cell type compositions (CD8 T cell, CD4 T cell, natural killer cell, B cell, monocyte) using the variance inflation factor analysis. The full model included additional covariates, smoking status and body mass index. Detailed statistical analyses are available in Supplemental Methods and Materials S5.

Replication Studies

Generation Scotland Cohort (set 1: *N* = 2578, set 2: *N* = 4450). DNAm from whole blood was assessed using the Infinium MethylationEPIC BeadChip arrays. Detailed cohort descriptions are provided in Supplemental Methods and Materials S6.

Grady Trauma Project (*N* = 795). The Grady Trauma Project (GTP) (29,30) sample included 795 participants with DNAm 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 Supplemental Methods and Materials S6.

RESULTS

Detailed demographic characteristics of the cohorts can be found in Table 1, Table S2, and Supplemental Results S1. The exploratory factor analysis revealed 3 independent homogeneous factors. All 3 factors were evenly correlated with CSS and MS stress (50%–60%, $p < .0001$) (Figures S1 and 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 ($R = 92.7\%$, $p < 2.2 \times 10^{-16}$) (Figure S3B), suggesting that our prediction model for MS stress was highly accurate. Further analysis showed that the correlation between CSS and MS stress in males and females was not different, and there was no difference between AUD and HCs (Figure S2C, D) ($p = .68$).

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 ($ps < .001$) (Table 2). These significant associations were also observed when analyzed with AUD cases only (Table S3). The findings were replicated in the 2 datasets of Generation Scotland in which MS stress correlated with weekly alcohol use ($p < .02$) (Table 2), which was not ascertained based on AUD. In the GTP, MS stress was significantly higher in participants with posttraumatic stress disorder (PTSD) symptoms diagnosed in the past 30 days than

Table 2. Association of MS Stress to Alcohol Consumptions in the NIAAA and Generation Scotland Samples

Samples	Alcohol Consumption	β	SE	*t* Test	*p* Value
NIAAA Epigenome Cohort, *N* = 615	Total drinks	0.214	0.043	4.92	9.59×10^{-7}[a]
	Drinking days	0.107	0.033	3.2	.0015[a]
	Average drinks per day	0.084	0.018	4.66	5.33×10^{-6}[a]
	Heavy drinking days	0.163	0.031	5.27	2.54×10^{-7}[a]
GS Data Set 1, *N* = 1501	Standard drinks/wk	0.075	0.023	3.19	.001[a]
GS Data Set 2, *N* = 2717	Standard drinks/wk	0.035	0.016	2.25	.024[a]

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. The *p* value for GS were from a linear model with original unit of weekly alcohol use and adjustment for age, sex.

AUD, alcohol use disorder; GS, Generation Scotland cohort; MS Stress, methylation score of stress; NIAAA, National Institute on Alcohol Abuse and Alcoholism.

[a]Indicates significance.

in participants without PTSD symptoms (β = 0.18, *p* = .02) (Table S6). However, we did not observe any association with Trauma Events Inventory total score and moderate and severe childhood trauma assessed using the Childhood Trauma Questionnaire.

Association of Stress With EAA

EAA in AUD. EAA derived from GrimAge was 3.2 (SE = 0.66) years higher in AUD than in HCs ($p = 2.5 \times 10^{-6}$) after additionally adjusting for CSS in the basic model. The EAA difference between AUD and HCs remained significant in the full model (Table 3) (β = 2.53, SE = 0.59, $p = 2.3 \times 10^{-5}$).

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* = .0003) and PhenoAge by 0.75 years (SE = 0.21, *p* = .0005) 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, $p = 4.55 \times 10^{-13}$) and PhenoAge by 4.5 years (SE = 0.94, $p = 4.1 \times 10^{-6}$) in the highest CSS quartile compared with the lowest quartile (Figure 2C, D). These results indicate that severe stress remarkably accelerates epigenetic aging. Two stress variables, morning cortisol level and Perceived Stress Scale score, were not associated with any epigenetic clocks after additionally controlling for AUD, while the Childhood Trauma Questionnaire total score was associated with PhenoAge and Horvath 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, $p = 1.5 \times 10^{-9}$) in AUD cases in the basic model (Figure 3A, B; Table 4) and was still observed, although attenuated, in the full model (β = 0.40, SE = 0.18, *p* = .03). We observed no significant association between GrimAge and MS stress in HCs. Additional adjustment for comorbid psychiatric disorders such as major depressive disorder, drug dependence, or both 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, $p = 2.2 \times 10^{-13}$) with every one-unit increase of MS stress in AUD, but no significant increase in HCs (β = 0.006, *p* = .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, $p < 2 \times 10^{-16}$) and 4.8 years in PhenoAge (SE = 0.70, $p = 3.3 \times 10^{-11}$) than in the lowest quartile (Figure 3D, E). DunedinPoAm aging rate in the highest quartile was 0.11 (SE = 0.008, $p < 2 \times 10^{-16}$) faster than in the lowest. In AUD cases showing biological age acceleration, we observed 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, $p = 3.0 \times 10^{-10}$) and PhenoAge by 3.6 years (SE = 0.88, $p = 6.1 \times 10^{-5}$) 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* = .0002). DunedinPoAm aging rate in the highest stress had a 0.08 acceleration ($p = 1.4 \times 10^{-12}$) in the basic model. In contrast to MS

Table 3. Association of AUD Diagnosis and CSS on EAA in Discovery Cohort (*N* = 317)

	AUD			CSS		
EAA	β	SE	*p* Value	β	SE	*p* Value
GrimAge						
Basic model	3.16	0.66	2.54×10^{-6}[a]	0.62	0.17	.0003[a]
Full model	2.53	0.59	2.3×10^{-5}[a]	−0.1	0.17	.57
PhenoAge						
Basic model	1.2	0.82	.14	0.75	0.21	.0005[a]
Full model	0.66	0.81	.42	0.58	0.23	.01[a]
Hannum						
Basic model	1.0	0.55	.07	0.20	0.14	.16
Full model	0.90	0.56	.11	0.17	0.16	.28
Horvath 2013						
Basic model	0.01	0.60	.99	0.20	0.16	.20
Full model	−0.10	0.60	.87	0.30	0.17	.08
Horvath 2018						
Basic model	−0.36	0.52	.48	0.17	0.13	.21
Full model	−0.44	0.52	.40	0.15	0.15	.33
DNAmTL						
Basic model	−0.09	0.03	.003[a]	−0.01	0.008	.09
Full model	−0.08	0.03	.008[a]	0.00003	0.008	.99

Adjusted for sex, race, and 5 blood cell type compositions (CD8 T cells, CD4 T cells, natural killer cells, B cells, monocytes) in the basic model, and additionally adjusted for smoking status and body mass index in the full model for all EAA variables. Effect of AUD was obtained by additional adjustments for stress (CSS) from a basic or full model; an effect of stress was obtained by additional adjustment for AUD diagnosis from a basic or full model.

AUD, alcohol use disorder; CSS, composite score of stress; DNAmTL, DNA methylation-based telomere length; EAA, epigenetic age acceleration.

[a]Indicates significance.

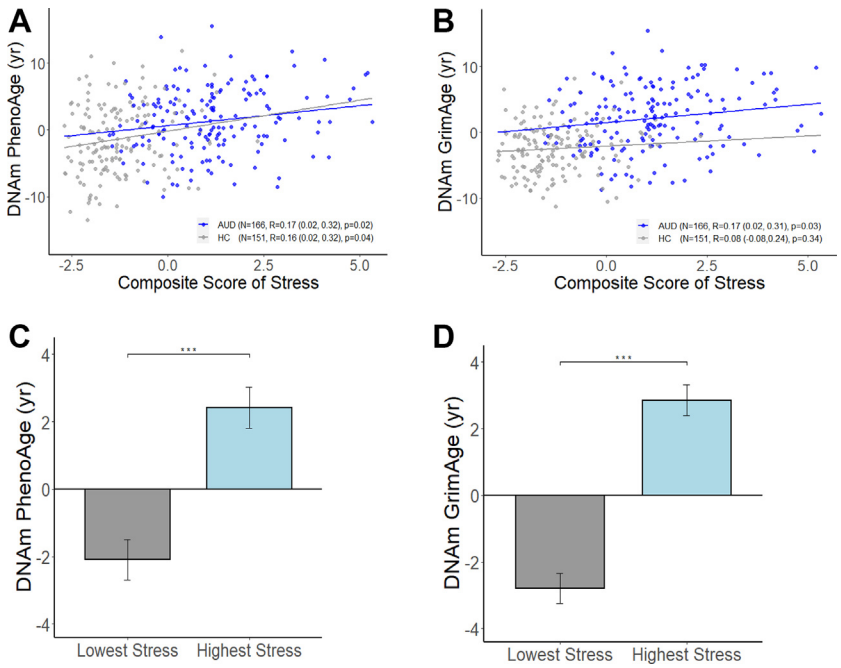

Figure 2. Relationship of CSS to EAA in the discovery cohort. **(A, B)** The scatter plots describe 2 EAAs vs. CSS and the line in which DNAm age was regressed on CSS in AUD and HCs, respectively. *R* is Pearson correlation with 95% confidence interval in parenthesis and *p* value of the correlation in the legend. In the scatter plot of PhenoAge vs. CSS shown in the basic model, there was no difference in EAA between AUD and HCs ($p = .14$) but with each one-unit change in CSS, PhenoAge was increased by 0.75 years (SE = 0.21, $p = .0005$) in both AUD and HCs together. The scatter plot of GrimAge vs. CSS shows that there was significant EAA difference between AUD and HCs (3.16-year in AUD, SE = 0.66, $p = 2.5 \times 10^{-6}$). Every one-unit increases in CSS was associated with 0.62 years (SE = 0.17) age acceleration in GrimAge ($p = .0003$). **(C, D)** The bar plots show estimated means of EAA and standard error after adjusting for sex, race, and blood-cell composition. Individuals with the highest CSS quartile (top 25 percentile) were compared with individuals with the lowest quartile (bottom 25 percentile). The PhenoAge and GrimAge were accelerated by 4.5 years (SE = 0.94, $p = 4.1 \times 10^{-6}$) and 5.66 years (SE = 0.71, $p = 4.55 \times 10^{-13}$), respectively, in the highest quartile of CSS compared with the lowest quartile. AUD, alcohol use disorder; CSS, composite score of stress; DNAm, DNA methylation; EAA, epigenetic age acceleration; HC, healthy control subject.

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 (*p*s < .0001) (Table 4). Detailed descriptions of the relationship between MS alcohol and EAA is provided in Supplemental 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 stepwise 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, $p = 3.2 \times 10^{-9}$) and those with high stress/low alcohol exhibited a 2.2-year acceleration (SE = 0.68, $p = .001$). Similarly, individuals with both high levels of stress and alcohol use had a 4.0-year increase in PhenoAge (SE = 0.78, $p = 4.7 \times 10^{-7}$), and those with low stress/high alcohol use had a 2.44-year increase ($p = .006$). The additive effect of stress and alcohol consumption on EAA was replicated in the GTP cohort in which GrimAge was accelerated by 4.57 (SE = 0.83, $p < 2 \times 10^{-16}$), 4.14 (SE = 0.57, $p = 1.2 \times 10^{-12}$), and 2.16 (SE = 0.37, $p = 8.2 \times 10^{-9}$) 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 kb/y shorter in AUD cases than in HCs in the basic model (SE = 0.016, $p = 3.6 \times 10^{-14}$), and the accelerated decline in DNAmTL in AUD cases persisted after controlling for the effect of MS stress in the basic model ($\beta = -0.08$, $p = 1.3 \times 10^{-5}$). Figure 5A, B shows the negative correlations of DNAmTL with PhenoAge in both AUD ($\beta = -14.3$, $p < 2 \times 10^{-16}$) and HCs ($\beta = -10.8$, $p = 6.8 \times 10^{-9}$), but GrimAge more strongly predicted telomere shortening in AUD cases ($\beta = -13.6$, $p < 2 \times 10^{-16}$) than in HCs ($\beta = -2.5$, $p = .05$). Moreover, DNAmTL decreased 0.03 kb/y with every one-unit increase in MS stress in AUD ($p = 9.6 \times 10^{-5}$), while it decreased 0.10 kb/y in MS alcohol ($p = 6.5 \times 10^{-8}$) (Table 4; Figure 5C, D). The relationship between MS alcohol and DNAmTL shortening remained significant in the full model ($\beta = -0.01$, $p = 5.5 \times 10^{-8}$) (Table 4). These findings suggested that alcohol use affected 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 2 Independent Cohorts

We replicated the findings that MS stress accelerates biological aging in 3 independent datasets; in the GTP PTSD cohort (Table S5), GrimAge was accelerated by 1.55 years (SE = 0.16, $p < 2 \times 10^{-16}$) and PhenoAge by 0.90 years (SE = 0.24, $p = .0002$), and DNAmTL decreased by 0.03 kb/y (SE = 0.007, $p = 3.6 \times 10^{-5}$) with MS stress in the basic model. These findings were also replicated in the 2 Generation Scotland datasets in which MS stress correlated with accelerated GrimAge ($\beta > 0.06$, $p < 2 \times 10^{-16}$), accelerated PhenoAge ($\beta > 0.02$, $p < .001$), and DNAmTL shortening ($\beta = -0.001$, $p < 1.0 \times 10^{-15}$). The effect size of these 3 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; Supplemental Results S3;

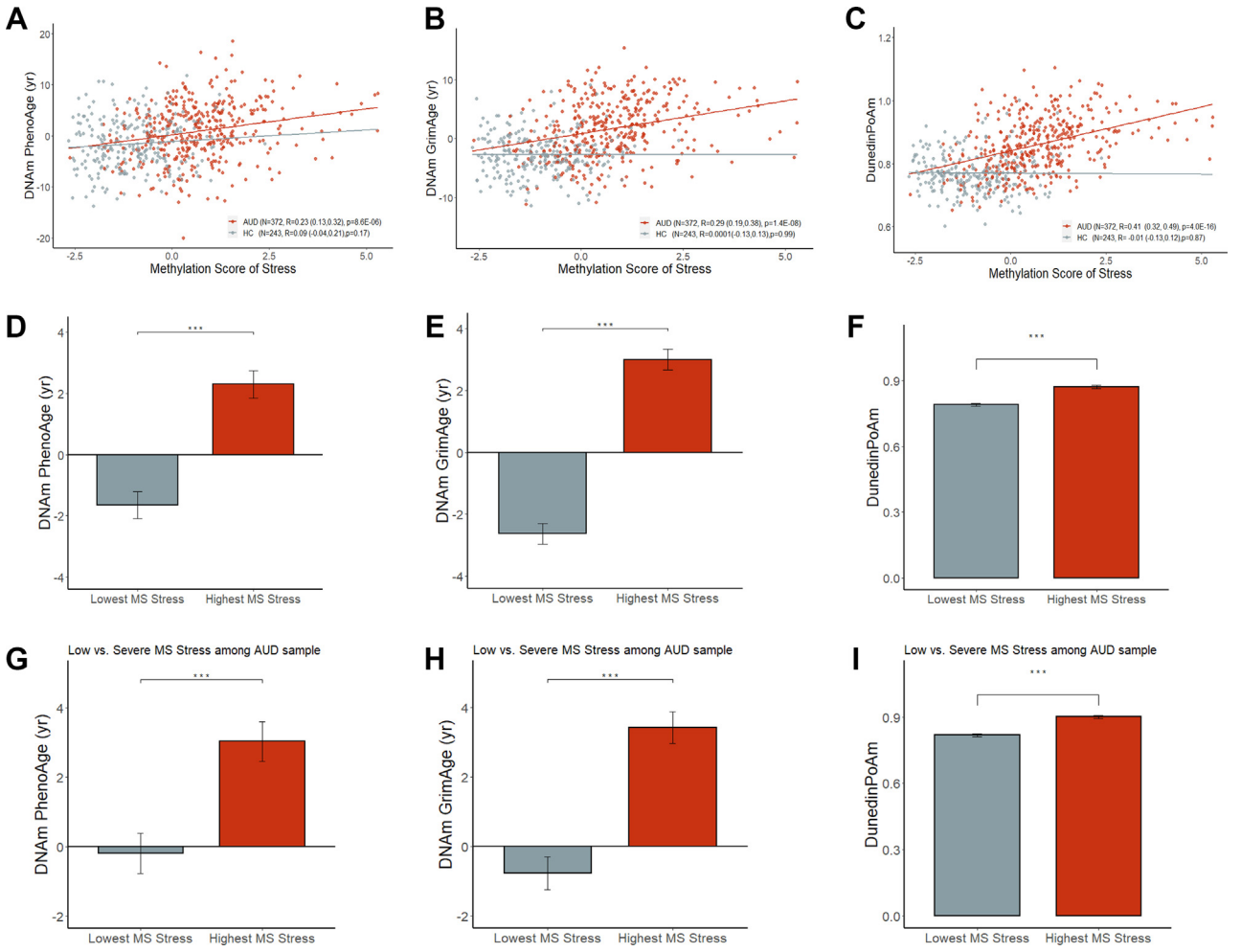

Figure 3. Relationship of MS stress with EAA in the epigenome cohort. **(A, B)** The scatter plots describe 2 EAAs vs. MS stress and the line in which DNAm age was regressed on MS stress in AUD and HCs, respectively. *R* is Pearson correlation with 95% confidence interval in parenthesis and *p* value of the correlation in the legend. In the basic model, AUD cases show a 0.89 years (SE = 0.24) acceleration in PhenoAge (p = .0002) and 1.18 years advance in GrimAge (SE = 0.19, $p = 1.52 \times 10^{-9}$) for every one-unit increase of MS stress, while HCs showed no significant GrimAge acceleration (β = 0.32, SE = 0.19, p = .22) and a nominal significant PhenoAge (β = 0.60, SE = 0.29, p = .04). **(C)** The scatter plot with 2 regression lines describes the DunedinPoAm vs. MS stress in AUD and HC. AUD had a pace-of-aging of 0.02 (SE = 0.003, $p = 2.2 \times 10^{-13}$), while HCs had 0.006 of aging rate (SE = 0.003, p = .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 with individuals with the lowest quartile (bottom 25%). EAA and DunedinPoAm differed significantly between the highest and lowest quartiles of MS stress. PhenoAge and GrimAge were accelerated by 4.8 (SE = 0.70, $p = 3.3 \times 10^{-11}$) and 6.5 years (SE = 0.51, $p < 2 \times 10^{-16}$), respectively, in the highest quartile compared with the lowest. The aging rate was 0.11 (SE = 0.008, $p < 2 \times 10^{-16}$) times faster in individuals with the highest quartile of MS stress when compared with the lowest quartile. **(G–I)** AUD cases with the highest quartile of MS stress were compared with AUD individuals with the lowest quartile. GrimAge was accelerated by 4.7 years (SE = 0.70, $p = 3.0 \times 10^{-10}$) and PhenoAge increased by 3.6 years (SE = 0.88, $p = 6.1 \times 10^{-5}$) in the highest quartiles when compared with the lowest quartiles of MS stress. Aging rate of DunedinPoAm was 0.08 (SE = 0.01, $p = 1.4 \times 10^{-12}$) times faster in the highest than the lowest quartile. AUD, alcohol use disorder; DNAm, DNA methylation; DunedinPoAm, DNA-methylation predictor of pace-of-aging; EAA, epigenetic age acceleration; HC, healthy control subject; MS stress, methylation score of stress.

Supplemental Discussion). We used the Genomic Regions Enrichment of Annotations Tool to assign potential biological meaning to CpGs (31). Using the default settings (5 kb upstream, 1 kb downstream, up to 1000-kb expansion), 342 genes were associated with the 211 CpGs. Enrichment analysis revealed gene sets related to 3 categories of Gene Ontology terms (32) and the 342 genes showed enrichment for cell cycles, regulation of cell death and junction, and neurogenesis in the Gene Ontology pathways (Table S8) (false discovery rate p value $< 10 \times 10^{-5}$).

DISCUSSION

This study used 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 DNAm age and telomere length in 2 independent cohorts. We constructed a 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

Table 4. Associations Between EAA and MS Stress and MS Alcohol in the Epigenome Cohort (*N* = 615)

	AUD Cases			Healthy Control Subjects			AUD vs. HC
EAA	β	SE	*p* Value	β	SE	*p* Value	*p* Value
MS Stress							
GrimAge							
Basic model	1.18	0.19	1.52×10^{-9}[a]	0.24	0.19	.22	.0003[a]
Full model	0.40	0.18	.03[a]	−0.03	0.18	.85	.03[a]
PhenoAge							
Basic model	0.89	0.24	.0002[a]	0.60	0.29	.04[a]	.23
Full model	0.61	0.26	.018[a]	0.65	0.29	.03[a]	.27
DNAmTL							
Basic model	−0.03	0.007	9.6×10^{-5}[a]	−0.016	0.01	.09	.09
Full model	−0.011	0.008	.15	−0.015	0.01	.14	.39
DunedinPoAm							
Basic model	0.02	0.003	2.2×10^{-13}[a]	0.006	0.003	.05	.0001[a]
Full model	0.01	0.003	9.5×10^{-5}[a]	0.002	0.003	.48	.01[a]
MS Alcohol							
GrimAge							
Basic model	1.91	0.50	.0002[a]	0.61	0.59	.30	.06
Full model	1.77	0.42	2.9×10^{-5}[a]	0.05	0.56	.93	.008[a]
PhenoAge							
Basic model	4.12	0.58	7.3×10^{-12}[a]	−0.06	0.90	.95	.0005[a]
Full model	4.14	0.57	2.2×10^{-12}[a]	0.02	0.90	.98	.0003[a]
DNAmTL							
Basic model	−0.103	0.018	6.5×10^{-8}[a]	−0.054	0.03	.08	.16
Full model	−0.098	0.018	5.5×10^{-8}[a]	−0.052	0.03	.09	.09
DunedinPoAm							
Basic model	0.03	0.008	.0008[a]	0.002	0.01	.85	.018[a]
Full model	0.025	0.007	.0003[a]	−0.007	0.01	.46	.002[a]

AUD, alcohol use disorder; DNAmTL, DNA methylation-based telomere length; DunedinPoAm, DNA-methylation predictor of pace-of-aging; EAA, epigenetic age acceleration; MS alcohol, methylation score for alcohol consumption; MS stress, methylation score of stress.
[a]Indicates significance.

all 13 stress-related variables and investigated the relationship between MS stress and 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 (Tables 3 and 4) (*p*s $<$ .001) and individuals in the highest quartile of stress showing the most pronounced EAA (Figures 2 and 3). Furthermore, the epigenetic signature of stress was associated with DNAmTL shortening (*p*s $<$ .0001) and increased aging rate of DunedinPoAm (*p*s $<$ .0001) in AUD after additional adjustment for the stronger effect of AUD in the basic model ($\beta = -0.03$, $p = 4.7 \times 10^{-5}$).

Our sequential analyses revealed a dramatic 4- to 5-year EAA among AUD cases in the highest MS stress quartile (Figure 3) (*p*s $<$ .0001). In addition to MS stress, MS alcohol was associated with both acceleration in epigenetic aging and DNAmTL shortening (Table 4 and Figure 5D; Figure S3). More importantly, we further showed that in AUD, EAA 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 because 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 effect 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 suggested that our findings were detecting stress that can be measured in other general populations as well as populations with severe trauma experiences.

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 clocks, 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

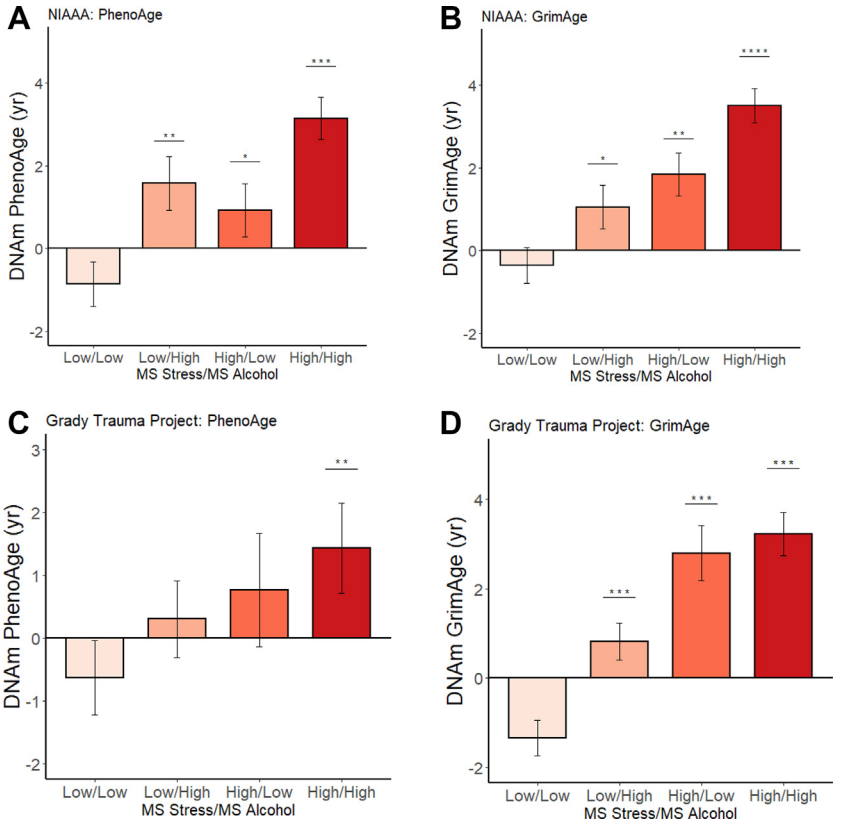

Figure 4. Additive effects of MS stress and MS alcohol on EAA in alcohol use disorder. The bar plots show the estimated means of EAA in the basic model across 4 groups classified by median splits of MS stress and MS alcohol. High/High indicates individuals with above the median value of MS stress and MS alcohol, High/Low indicates above the median of MS stress and below the median of MS alcohol, and so on. **(A, B)** The estimated means of EAA of PhenoAge and GrimAge over the 4 groups in NIAAA. Comparing participants with alcohol use disorder and low stress/low alcohol as a reference group, it was found that MS stress and MS alcohol had an additive effect on both epigenetic clocks; GrimAge had acceleration of 3.86 (SE = 0.63) years in high stress/high alcohol ($p = 3.2 \times 10^{-9}$), 2.2 years (SE = 0.68, $p = .001$) in high stress/low alcohol, 1.42 years ($p = .05$) in low stress/high alcohol; while PhenoAge was advanced by 4.0 years (SE = 0.78, $p = 4.7 \times 10^{-7}$) in high stress/high alcohol, 1.77 years (SE = 0.83, $p = .03$) in high stress/low alcohol, 2.44 years (SE = 0.88, $p = .006$) in low stress/high alcohol group. Furthermore, a linear trend test (an additive effect) using the 4 groups (0 = low/low, 1 = low/high, 2 = high/low, 3 = high/high) showed a linear trend ($\beta = 1.29$, SE = 0.25, $p = 5.5 \times 10^{-7}$ for PhenoAge; $\beta = 1.19$, SE = 0.21, $p = 3.02 \times 10^{-8}$ for GrimAge) in the basic model. **(C, D)** The estimated means of EAA of PhenoAge and GrimAge over the 4 groups in the Grady Trauma Project cohort. Similarly, GrimAge was associated with acceleration of 4.57 years (SE = 0.48, $p < 2 \times 10^{-16}$) in high stress/high alcohol, 4.14 years (SE = 0.57, $p = 1.2 \times 10^{-12}$) in high stress/low alcohol, 2.16 years (SE = 0.37, $p = 8.2 \times 10^{-9}$) in low stress/high alcohol group. PhenoAge had 2.07 years advance (SE = 0.71, $p = .003$) in high stress/high alcohol group, but it was not significantly accelerated in the other 2 groups (1.40, SE = 0.85 and 0.94, SE = 0.55 years, $ps > .05$). DNAm, DNA methylation; EAA, epigenetic age acceleration; MS alcohol, methylation score for alcohol consumption; MS stress, methylation score of stress; NIAAA, National Institute on Alcohol Abuse and Alcoholism.

was methodologically designed to consider smoking-associated effects in contrast to the first-generation clocks (35). 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 Supplemental 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 subadditive or superadditive. 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 EAA (39). The 211 stress-related CpGs that we identified are colocalized to genes showing enrichment for cell cycle, regulation of cell death and junction, and cancer in the Gene Ontology pathways (Table S8) (false discovery rate p value $< 10 \times 10^{-5}$). 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 signaling pathways that are involved in regulation of cell cycle and biological age acceleration. Moreover, our top 6 highest weighted CpGs are in *CDKN2C*, *FBXO42*, *TBRG4*, *FKBP11*, *FAM115A*, and *ANAPC11* (see Supplemental 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 13 stress domains increase power to carry out the analysis of relationship with health complication rather than

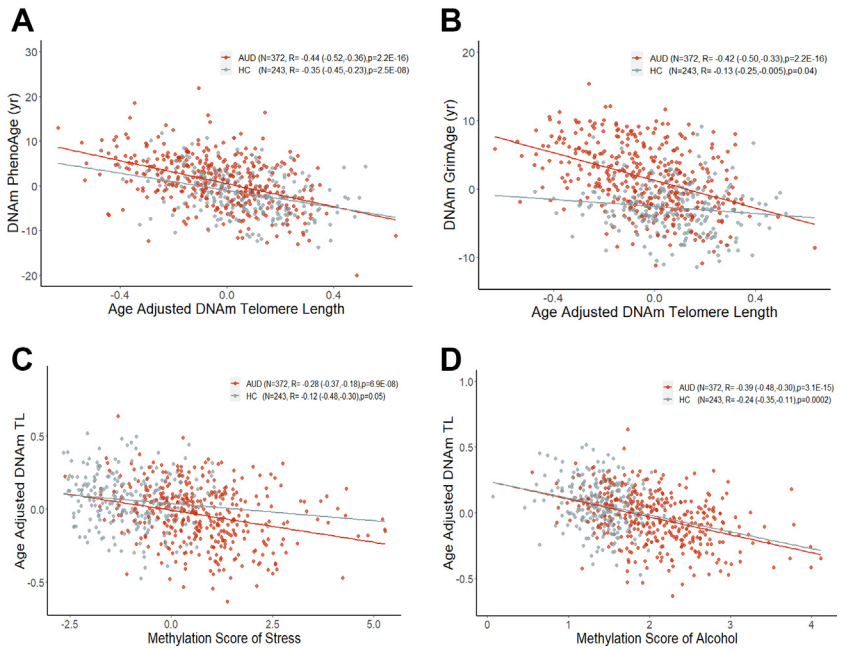

Figure 5. DNAmTL and MS stress in the epigenome cohort. The scatter plots show 2 epigenetic age accelerations vs. age-adjusted DNAmTL. *R* is Pearson correlation with 95% confidence interval in parenthesis and *p* value of the correlation in the legend. **(A, B)** The plots describe that in the basic model, PhenoAge has a negative correlation with DNAmTL in both AUD cases ($\beta = -14.3$, SE = 1.5, $p < 2 \times 10^{-16}$) and HCs ($\beta = -10.8$, SE = 1.8, $p = 6.8 \times 10^{-9}$). GrimAge also has a negative association with DNAmTL in only AUD ($\beta = -13.6$, SE = 1.2, $p < 2 \times 10^{-16}$) but not in HCs ($\beta = -2.5$, SE = 1.3, $p = .05$). **(C, D)** MS stress was associated with DNAmTL shortening in AUD ($\beta = -0.03$, SE = 0.007, $p = 9.6 \times 10^{-5}$) but not in HCs ($\beta = -0.016$, SE = 0.01, $p = .10$). MS alcohol had a significant negative correlation with shortened DNAmTL in AUD ($\beta = -0.10$, SE = 0.02, $p = 6.5 \times 10^{-8}$), but not in HCs ($\beta = -0.05$, SE = 0.03, $p = .08$). AUD, alcohol use disorder; DNAm, DNA methylation; DNAmTL, DNAm-based telomere length; HC, healthy control subject; MS alcohol, methylation score for alcohol consumption; MS stress, methylation score of stress.

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. For instance, for measuring stress, the current standard is self-reports, which induce recall bias and might not accurately or fully capture stress exposure. Furthermore, the effect of an 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/reinterview studies, and the diagnosis even being captured with very high area under the receiver operating characteristic curve (>0.95) sensitivity/specificity with simple questionnaires such as the Alcohol Use Disorders Identification Test. All our cases and controls were diagnosed through psychiatric interview. However, quantitation of lifetime alcohol exposure is less accurate. Using morning cortisol level as a single physiological stress measure can be problematic because 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 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 is 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 lifespan. 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 various tissues and cells. In addition, our findings showed that DNAm 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 through the HPA axis.

In conclusion, our study showed that a methylation-derived score tracking stress exposure is associated with various stress-related phenotypes and EAA. We found 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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JJ and FWL were responsible for conception and design of the work. JJ, DLM, and FWL were responsible for acquisition, analysis, or interpretation of data. FWL and JJ drafted and revised the paper. DG provided critical revision of the paper for important intellectual content. All authors critically reviewed the content and approved the final version for publication.

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ARTICLE INFORMATION

From the Section on Clinical Genomics and Experimental Therapeutics (JJ, JW, JY, ASB, LAM, DBR, FWL) and Laboratory of Neurogenetics (CAH, HS, MS, ND, DG), National Institute on Alcohol Abuse and Alcoholism, National Institutes of Health, Bethesda, Maryland; Centre for Genomic and Experimental Medicine (DLM, RMW, AC, DJP, AMM, REM, KLE), Institute of Genetics and Cancer, University of Edinburgh, Edinburgh, United Kingdom; Department of Gynecology & Obstetrics (AKS) and Department of Psychiatry and Behavioral Sciences (AKS, VM, AP, JS, BB, NF), Emory University School of Medicine, Atlanta, Georgia; and Department of Human Genetics (SH), David Geffen School of Medicine and Department of Biostatistics (SH), Fielding School of Public Health, University of California, Los Angeles, Los Angeles, California.

DG and FWL contributed equally to this work as joint senior authors.

Address correspondence to Falk W. Lohoff, M.D., at falk.lohoff@nih.gov.

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