Abstract

DNA methylation patterns at specific cytosine-phosphate-guanine (CpG) sites predictably change with age and can be used to derive “epigenetic age”, an indicator of biological age, as opposed to merely chronological age. A relatively new estimator, called “DNAm GrimAge”, is notable for its superior predictive ability in older populations regarding numerous age-related metrics like time-to-death, time-to-coronary heart disease, and time-to-cancer. PTSD is associated with premature mortality and frequently has comorbid physical illnesses suggestive of accelerated biological aging. This is the first study to assess DNAm GrimAge in PTSD patients. We investigated the acceleration of GrimAge relative to chronological age, denoted “AgeAccelGrim” in combat trauma-exposed male veterans with and without PTSD using cross-sectional and longitudinal data from two independent well-characterized veteran cohorts. In both cohorts, AgeAccelGrim was significantly higher in the PTSD group compared to the control group (N = 162, 1.26 vs −0.57, p = 0.001 and N = 53, 0.93 vs −1.60 Years, p = 0.008), suggesting accelerated biological aging in both cohorts with PTSD. In 3-year follow-up study of individuals initially diagnosed with PTSD (N = 26), changes in PTSD symptom severity were correlated with AgeAccelGrim changes (r = 0.39, p = 0.049). In addition, the loss of CD28 cell surface markers on CD8+ T cells, an indicator of T-cell senescence/exhaustion that is associated with biological aging, was positively correlated with AgeAccelGrim, suggesting an immunological contribution to the accelerated biological aging. Overall, our findings delineate cellular correlates of biological aging in combat-related PTSD, which may help explain the increased medical morbidity and mortality seen in this disease.

Introduction

Post-traumatic stress disorder (PTSD) is associated with premature death and an increased prevalence of comorbid medical conditions associated with aging, such as cardiovascular, pulmonary and inflammatory diseases, as well as metabolic syndrome [1–4]. There is no universally accepted explanation for the increased prevalence of these conditions, and there are few, if any, early biomarkers to predict risk. One emerging hypothesis is that PTSD is a condition of “accelerated biological aging” [5, 6]. Chronological age is a measure of the actual time an individual has been alive, whereas biological age is defined based on physiological parameters and cellular health status, and as such, is more closely associated with disease processes and mortality. Accelerated biological age is inferred when the estimated biological age exceeds the chronological age. Evidence in
support of accelerated biological aging in PTSD includes not only premature development of age-related health conditions and premature mortality, but also shortened telomeres in leukocytes [7] and immunosenescence [8], which are both associated with biological aging [5, 9]. In addition, a study using population-based data of adults investigating the distribution of T-cell phenotypes in PTSD reported a higher ratio of late-differentiated effector CD8+ CD28− to naïve CD8+ CD28+ T-cell populations, one of the most prominent markers associated with aging [8]. It has been hypothesized that aging-associated accumulation of CD28− T cells is a result of the prominent role of epigenetics (i.e. DNA methylation) in fate decisions during T-cell differentiation [10]. Overall, there is a growing body of evidence suggesting accelerated biological aging in PTSD.

Recent investigations have applied “epigenetic clocks” to assess biological aging in healthy individuals, as well as individuals with PTSD and other illnesses [11]. Using machine learning and large independent datasets, DNA methylation site information can be trained to predict chronological age, clinical outcomes or other age-associated biological states (e.g., time-to-death, time-to-illness) [12–15]. For example, Horvath’s Clock leverages patterns of DNA methylation (DNAm) changes at specific cytosine-phosphate-guanine (CpG) sites that correlate remarkably well with chronological age (correlation coefficients generally >0.90) [13]: this is an intrinsic marker of cell aging that is independent of cell type composition. Other examples of epigenetic clocks of biological aging include the Hannum clock, which is an extrinsic marker of cell age that integrates information about age-related blood cell type changes [12] and the skin and blood clock [16]. Similarly, DNAm PhenoAge was developed from whole blood, but is trained on phenotypic age-related variables [14]. While each clock differs in the type of information the epigenetic data are trained on, and, in some cases, the types of cells and tissues sampled, they all measure certain aspects of biological aging and are all correlated with chronological age, although they show varying degrees of correlation among themselves [15, 17], suggesting that “biological aging” is not a unitary process.

“DNAm GrimAge” is a DNA methylation clock developed in 2019 by Lu et al. [15]. It differs from prior clocks in having superior predictive ability for lifespan, time-to-death, time-to-coronary heart disease and time-to-cancer, as well as exhibiting a strong relationship with visceral adiposity/fatty liver and a general medical comorbidity index [15]. Adjusting DNAm GrimAge for chronological age generates a measure of epigenetic age acceleration, termed AgeAccelGrim, such that positive AgeAccelGrim values represent accelerated aging, and negative ones represent decelerated aging relative to that expected from chronological age alone. DNAm GrimAge was trained on age-related biochemical markers rather than on only chronological age, yielding, not surprisingly, a better correlation with age-related health status than other clocks [15]. Specifically, DNAm GrimAge is a composite biomarker consisting of DNAm surrogate biomarkers of seven plasma proteins that are associated with various age-related conditions (described in the Methods section, Support Datasets 1) plus a DNAm surrogate that is associated with tobacco smoking pack-years (DNAmPACK-YRS), while each of these individual biomarkers is based on dozens, if not hundreds, of CpGs that are located across the genome. AgeAccelGrim is strongly associated with age-related conditions and traits such as congestive heart failure, hypertension, diabetes, and general physical functioning levels [15]. AgeAccelGrim has not yet been investigated in PTSD, even though PTSD may represent a condition of accelerated biological aging that is associated with excess medical morbidity and mortality [2]. Here we report results from the first investigation of epigenetic aging in PTSD using AgeAccelGrim, with both cross-sectional and longitudinal data from two independent well-characterized male Operation Iraqi Freedom/Operation Enduring Freedom (OIF/OEF) veteran cohorts from the PTSD Systems Biology Consortium (SBC) [18]. The results indicate PTSD is associated with higher AgeAccelGrim.

Result

AgeAccelGrim in PTSD and relationship with symptom severity

In our primary analysis, combat trauma-exposed male veterans with PTSD (hereafter referred to as the PTSD group), compared to combat trauma-exposed male veterans of similar chronological age but without PTSD (hereafter referred to as the Control group), and showed significantly higher AgeAccelGrim values, indicating relatively accelerated epigenetic aging. These statistically significant results were demonstrated in two independently recruited cohorts (refer to “Cohorts and Study Design” in “Methods”), which we termed the Discovery [N = 162; (mean ± SD [Years]), 1.26 ± 3.93 vs −0.57 ± 3.38; t = −3.184; p = 0.001] and the Validation [N = 53; 0.93 ± 3.73 vs −1.60 ± 2.96; t = −2.725; p = 0.008] (Fig. 1a) cohorts.

AgeAccelGrim has a significant positive correlation with Clinician Administered PTSD Scale CAPS score total (CAPS-IV) among all participants (r = 0.236, N = 271, p < 0.001). However, AgeAccelGrim was not, significantly correlated with current PTSD symptom severity in either the PTSD or the Control cohorts individually, or across the combined Discovery and Validation groups (Fig. 1b).
Correlations of AgeAccelGrim with BMI, smoking status, inflammation and major depression comorbidity

Although PTSD is often comorbid with major depressive disorder (MDD) [19], no significant difference in AgeAccelGrim was found between PTSD subjects with (N = 63) and without (N = 20) MDD (t = −0.561, p = 0.578). Moreover, across all subjects from both Discovery and Validation cohorts, AgeAccelGrim was not significantly associated with ethnicity, sleep, alcohol abuse or early life trauma (see Supplementary information Appendix, Table S1) and had no significant correlation with circulating inflammatory factors, Interferon (IFN) gamma, IL6, IL8, IL10, C-Reactive Protein (CRP), and Tumor Necrosis Factor (TNF) alpha (see Supplementary information Appendix, Table S2). On the other hand, the PTSD group had significantly higher BMI and percentage of smokers than the control group (t = −2.766, p = 0.006, and $X^2 = 16.749$, p = 0.0001, respectively). Across all subjects, higher BMI and the presence of smoking were associated with greater AgeAccelGrim ($r = 0.149$, p = 0.031, and $t = −5.761$, p < 0.0001, respectively). Nonetheless, based on a multivariate regression analysis covarying BMI, CD8+ CD28− (discussed below), smoking status as determined by plasma cotinine levels (Metabolon Inc.) (Supporting Datasets 2), and estimated granulocyte, monocyte, natural killer, CD4 T-cell percentages (by Houseman method) [20], the between-group difference in AgeAccelGrim remained significant (N = 175, $t = 2.251$, p = 0.026). If self-report smoking status was used instead of plasma cotinine levels, the between-group difference in AgeAccelGrim remained in the same direction but was attenuated (N = 152, $t = 1.928$, p = 0.056), likely due to more missing data.

Since the DNAm GrimAge measure is partially based on a surrogate marker of smoking history, we further assessed AgeAccelGrim differences between the PTSD and control groups after excluding the surrogate variable DNAmPACKYRS from the AgeAccelGrim formula (see “Methods”) and applied the modified AgeAccelGrim formula on all subjects. After removing DNAmPACKYRS from the AgeAccelGrim formula and adjusting
for the same covariates, the PTSD group still had significantly higher AgeAccelGrim than the control group ($N = 177, t = 2.499, p = 0.0134$). These findings indicate that the group difference in AgeAccelGrim was not fully explained by BMI, smoking history, cell compositions or MDD comorbidity.

**Longitudinal association of AgeAccelGrim with PTSD symptom severity progression**

Next, we analyzed changes in AgeAccelGrim in relation to changes in PTSD symptom severity in individuals from the Recall group. The Recall PTSD group is comprised of individuals of the Discovery cohort who were diagnosed with PTSD at baseline (T1) and who returned for longitudinal follow-up approximately three years later (T2) ($N = 26$ with initial diagnoses of PTSD). Longitudinal changes in AgeAccelGrim were positively correlated with longitudinal changes in PTSD symptom severity defined by CAPS total ($r = 0.391, p = 0.049$) (Fig. 1c, see Supplementary information Appendix, Table S3). Specifically, longitudinal increases or decreases in PTSD severity ratings were paralleled by increases or decreases (respectively) in AgeAccelGrim. In an exploratory analysis, we examined correlations of changes in specific symptom clusters of the CAPS ratings (e.g., CAPS-B re-experiencing, CAPS-C avoidance, and CAPS-D hyperarousal) with changes in AgeAccelGrim. Across all subjects (whether initially diagnosed with PTSD or not), longitudinal changes in AgeAccelGrim were directly correlated with changes in the CAPS hyperarousal symptom complex ($N = 55, r = 0.284, p = 0.036$) (see Supplementary information Appendix, Table S3).

**AgeAccelGrim is associated with CD8$^+$ CD28$^-$ T cells**

T-cell distributions, were determined by flow cytometry (see Supplementary information). The CD8$^+$ CD28$^-$ T (i.e., CD8 T cells lacking the costimulatory surface molecule, CD28) cell proportions were positively correlated with GrimAge ($r = 0.249, p = 0.001$) and chronological age ($r = 0.183, p = 0.012$), which is in agreement with prior findings in the aging literature [10, 21]. We also found that the percentage of CD8$^+$ CD28$^-$ T cells was positively correlated with AgeAccelGrim across all subjects ($r = 0.179, p = 0.014$) (Fig. 2). We then conducted an ANOVA to evaluate the association of AgeAccelGrim and PTSD status while controlling for CD8$^+$ CD28$^-$ T-cell proportions. The PTSD group continued to have significantly higher AgeAccelGrim than the control group ($F = 17.252, p < 0.001$), indicating that the greater AgeAccelGrim in the PTSD group was not fully explained by increases in immunosenescence as indicated by their higher proportion of CD8$^+$ CD28$^-$ T cells.

**Four DNAm surrogate biomarkers of AgeAccelGrim are associated with PTSD status**

AgeAccelGrim was modeled using eight DNAm surrogate biomarkers based on 1030 CpGs to predict biological age acceleration. Each DNAm surrogate biomarker is an estimate of a particular plasma protein that has its own implications in aging-related mechanisms and health. The DNAm surrogate biomarkers (age-adjusted) exhibited high inter-correlations in our combined cross-sectional data (see Supplementary information Appendix, Fig. S1, Table S4). When comparing each DNAm surrogate biomarker individually between the PTSD and control groups, the PTSD subjects had significantly higher DNAm values for cystatin C ($t = -2.620, p = 0.010$), plasminogen activator inhibitor 1 (PAI-1) ($t = -2.316, p = 0.022$), tissue inhibitor metalloproteinase 1 (TIMP1) ($t = -2.687, p = 0.008$) and smoking pack-years ($t = -3.645, p < 0.001$) (see Supplementary information Appendix, Table S5).

**Relationship between GrimAge and other DNAm clocks and biological age markers**

Consistent with the findings of Lu et al. [15], we found significant correlations of AgeAccelGrim with AgeAccelPheno ($r = 0.35, p < 1e^{-6}$), an epigenetic clock trained on aging-related clinical laboratory values [14], but AgeAccelPheno was not significantly different between the PTSD
and Control groups \( (t = -0.089, p = 0.929) \) (see Supplementary information Appendix, Table S6). AgeAccelGrim also significantly correlated with AgeAccelHannum and Extrinsic Epigenetic Age Acceleration (EEAA), marginally with AgeAccelSkinBloodClock, but not with Intrinsic Epigenetic Age Acceleration (IEAA), IEAA, Hannum (IEAA based on Hannum clock), and AgeAccelHorvath (Fig. 3; see Supplementary information Appendix, Table S7). Horvath’s Clock (AgeAccelHorvath), but not AgeAccelHannum, showed significant differences between the PTSD and control groups. We previously reported that both “PTSD-positive” and “PTSD-negative” combat trauma-exposed males showed accelerated epigenetic age per Horvath’s clock (AgeAccelHorvath) [22], but the “PTSD-positive” men showed significantly lower AgeAccelHorvath than the “PTSD-negative” men, and we postulated that the PTSD-positive group may have had an attenuated AgeAccelHorvath due to increased telomerase or BDNF activity [22] Kang et al, 2019, pers. comm.). Notably, in the present study, results with the AgeAccelGrim were in the opposite direction of the previous AgeAccelHorvath results [22] (i.e., combat-PTSD subjects had higher AgeAccelGrim and lower AgeAccelHorvath than the combat-exposed control group (see Supplementary information Appendix, Table S6), suggesting that methylation at Cpg sites known to be associated with chronological aging convey different information, or may be affected by different factors, than do methylation changes associated with disease-related proteins (and smoking-related) changes in PTSD [15].

**Discussion**

This is the first study to investigate epigenetic aging in PTSD using AgeAccelGrim, which has been reported to outperform the first generation of DNAm age estimators in predicting all-cause mortality and time to onset of several serious illnesses, at least in older populations [15]. We analyzed two independent cohorts of young to middle aged combat trauma-exposed male war veterans cross-sectionally, as well as longitudinally. The major findings in this study were: (1) AgeAccelGrim was significantly increased in combat trauma-exposed men with PTSD compared to trauma-exposed PTSD-negative controls, assessed cross-sectionally; and this difference was not accounted for by differences in BMI, smoking, cell count distribution, MDD comorbidity or early life trauma (as measured by Early Trauma Inventory) (see Supplementary information Appendix, Table S1) (2) Across all subjects, AgeAccelGrim was positively correlated with PTSD symptom severity ratings; (3) AgeAccelGrim was significantly positively correlated with the proportion of CD8+CD28− T cells, which is a measure of T-cell exhaustion associated immunosenescence; (4) Longitudinal changes in AgeAccelGrim tracked changes in PTSD symptom severity, (5) Four individual components of AgeAccelGrim were significantly associated with PTSD (DNAm surrogate values for cystatin C, PAI-1, TIMP1 and smoking pack-years, a marker highly correlated with cotinine levels but also related to other factors, discussed below).

The significant correlation we found between CAPS-Total scores and AgeAccelGrim across all subjects (PTSD and controls) could indicate a true association of AgeAccelGrim with symptom severity considered as a continuous rather than a dichotomous (diagnosis-based) variable. Alternatively, it could simply reflect the finding of a higher AgeAccelGrim and a higher CAPS-Total score in PTSD when the diagnostic grouping is assessed as a dichotomous variable (a “dumbbell effect” or “Simpson’s paradox” resulting from correlating two variables with distinct groupings on both variables [23]). The lack of a significant correlation between PTSD symptom severity and AgeAccelGrim within each separate group (PTSD and controls separately) favors the latter interpretation.

The observation that PTSD is associated with premature development of certain comorbid physical health conditions, ranging from metabolic and cardiovascular diseases, to cognitive decline and premature death, has received great attention in recent years [4, 24–26]. Over time, epigenetic alterations, including age-associated DNA methylation

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**Fig. 3 Correlations of AgeAccelGrim with other epigenetic age clocks.** The heatmap plot visualizes the inter-correlations between eight DNAm clocks. The deeper color indicates stronger correlations. The dendrogram shows the similarity between the clocks based on hierarchical clustering on Euclidean distance of 1 - correlation. AgeAccelGrim was relatively independent to other clocks, while AgeAccelPheno was highly associated with the acceleration of Horvath, Hannum, and skinblood clocks.
changes occur and may be related to these health outcomes, although that remains speculative and requires longitudinal study over longer periods of time. This complex and multidimensional nature of biological aging, to some extent, was captured by DNA methylation age, namely “GrimAge” in the current study. Prior to the present study, several papers reported mixed findings in PTSD based on the Horvath and Hannum epigenetic clocks, which were trained largely on chronological age. Verhoeven et al. found Horvath age was accelerated in PTSD and non-PTSD combat-exposed veterans, but this was significantly attenuated in the PTSD-positive subjects [22], while Wolf et al. showed Hannum age was more accelerated [27], respectively. In contrast, DNAm GrimAge changes in surrogate markers of proteins that are strongly associated with lifespan (in addition to smoking pack-years), at least in older populations, as opposed to being trained primarily on chronological age, and therefore, may be better conceptualized as a “health clock”, and be better suited for assessing age-related decline of tissue performance, changes in cell composition, inflammation, and immunosenescence [15]. Notably, the first study referenced above, using the Horvath clock [22], was conducted on a partially overlapping sample of subjects as in the present study using the GrimAge clock. The different results obtained using these two different biological clocks, which were trained on different outcome measures (chronological age vs. methylation proxies of health and longevity-related proteins) reinforces the importance of interpreting each separate clock appropriately.

An additional point to consider is that the PTSD-negative subjects in the present study had GrimAge values actually lower than that expected from their age. (mean = −0.843, \( p = 0.009 \)). This unexpected result may be due to miscalibration of the GrimAge formula when applied to young healthy individuals, since the GrimAge formula was trained on older individuals, e.g., from the Framingham study [15]. Alternatively, the PTSD-negative subjects in the present study may have represented an especially resilient population, since they had been exposed to significant combat trauma, yet failed to develop PTSD.

Findings from our longitudinal cohort indicate that AgeAccelGrim changes over time are directly related to changes PTSD subjects’ symptom severity (Fig. 1c). In particular, AgeAccelGrim changes were positively associated with changes in the hyperarousal symptom cluster. The small sample size of this cohort, however, necessitates replication.

Aging-related declines in CD28 expression on CD8+ T cells are among the most prominent features of immunosenescence [21], and denote increased susceptibility to infections, reduced effectiveness of vaccinations and a higher incidence of diseases associated with immune dysfunction [10, 28]. Biologically, CD28− T cells exhibit reduced antigen receptor diversity, defective antigen-induced proliferation, increased cytotoxicity, increased proinflammatory cytokine release, shorter telomeres and a shorter replicative lifespan [10, 28]. In the current study, we found that AgeAccelGrim was positively associated with immunosenescence, as measured by an increased proportion of CD8+ CD28− T cells, but not with proinflammatory cytokines (see Supplementary information Appendix, Tables S2, 8). This positive correlation of AgeAccelGrim with CD28− expression aligns with findings in population-based data with AgeAccelGrim [15]. Similarly, Aiello et al. [8] reported an increased ratio of late-differentiated effector T cells (i.e., CD28−) to naïve T cells (i.e., CD28+ ) in PTSD. Together, the PTSD-associated accelerated biological aging (as indexed by AgeAccelGrim) is associated with declines in certain, but not all, aspects of immune function that are typically seen with advancing age or with chronic infection. Nonetheless, the significant between-group differences we report with AgeAccelGrim did not entirely rely on immunological aging or changes in CD8+ CD28− cell proportions, as substantiated by the significantly greater AgeAccelGrim in PTSD even after controlling for differences in CD28 expression.

These findings may also be relevant for informing and investigating the causes of physical illnesses and mortality in PTSD, since the epigenetic data were modeled on physiological and biochemical data, rather than on chronological age alone. While AgeAccelGrim purposely captures the overall epigenetic age profile associated with the health span of an individual (at least in older populations), each of the seven DNAm-based surrogate biomarkers represents its own biological functional entity. These plasma protein levels are strongly linked to various age-related conditions such as fibrinolysis, kidney function, and aspects of cardiac function, inflammation, and mitochondrial dysfunction [15, 29]. DNAm-based surrogate biomarkers of plasma protein levels provide an informative epigenetic-protein interaction network on biological aging processes. Our findings with the individual component DNAm surrogates suggest potential applications of these plasma proteins in identifying the mechanisms of epigenetic aging in PTSD (Fig. S2). There were three DNAm surrogate biomarkers that were significantly different between PTSD and control groups: Cystatin C, PAI-1, and TIMP1. Cystatin C is primarily used as a biomarker for kidney function [30], but was recently linked to cardiovascular diseases as well [31]. Robust evidence suggests a critical role of PAI-1 in numerous age-related clinical and subclinical conditions (e.g., inflammation, atherosclerosis, thrombosis, insulin resistance, obesity, liver disease) [29]. TIMP-1 is a known inhibitor of apoptosis in mammalian cells and has been implicated in the cancer, arthritis, inflammatory pain,
wound healing, tissue remodeling, neuroprotection and other processes and conditions [32–34]. Notably, DNAm-based surrogates are predictions of plasma protein levels, and further experiments are required to confirm the actual protein levels.

Importantly, DNAmPACKYRS, one component of DNAm GrimAge, is nominally a smoking estimator, but it likely also informs on various non-smoking-related aging mechanisms, since AgeAccelGrim remains a highly significant predictor of lifespan even in non (never)-smokers [15]. This observation suggests that the methylation sites that are nominally associated with smoking are also associated with additional aspects of aging [15]. For example, methylation of one of the genes associated with this surrogate, cg05575921, which codes for aryl hydrocarbon receptor repressor (AHRR), is not solely a function of smoking, but also reflects exposure to various environmental exposures and xenobiotics, including polycyclic hydrocarbons, airborne particulate matter and certain pharmaceuticals [35]. Of potential importance, the aryl hydrocarbon receptor also plays important roles in inflammation, cell differentiation and cell cycle control [36].

Our study has several strengths. This is the first study to examine DNAm GrimAge and AgeAccelGrim in PTSD. We recruited a sample of well-phenotyped male veterans who were all exposed to combat trauma, limiting variability of trauma types. Our control group, despite exposure to combat-related trauma, never developed PTSD, helping tease out the impact of PTSD versus the experience of trauma per se. On the other hand, this could represent a liability, since combat trauma-exposed individuals who fail to develop PTSD may represent an especially resilient group. As another strength, we conducted sensitivity analyses to tease apart the effects of smoking. Specifically, we utilized a biological measure of cotinine to objectively assess cigarette use, reducing inaccurate assessments of nicotine exposure and various biases that may result from self-reporting. However, cotinine measurements only reflect nicotine exposure over the past 10 days, rather than reflecting lifetime exposure. Therefore, we repeated our analyses using self-report smoking data, although this latter analysis lacked sufficient power due to missing data. Importantly, we validated our initial cross-sectional findings in an independent sample of combat-exposed PTSD subjects and controls. This reduces the likelihood that our data constitute a false positive finding. We also assessed whether our findings were independent of comorbid MDD and BMI, which might, in themselves, be associated with aspects of accelerated biological aging [7]. Furthermore, we assessed immunologic changes in tandem with AgeAccelGrim and symptom severity estimates, providing leads for future investigations into immunological aspects (whether causal or not) of epigenetic/biological aging. Finally, our study analyzed both cross-sectional and longitudinal data to study how DNAm GrimAge and PTSD symptoms jointly change over time.

Limitations of our study include the following: (1) we studied only males and only combat-related PTSD, limiting generalizability to other populations; (2) the longitudinal data only included two discrete points in time, separated by approximately 3 years apart; (3) we did not have a healthy control group without trauma exposure; (4) the sample size of our Recall group was relatively small, thus requiring replication in the future using larger samples; and (5) the participants in this study were younger than those in the Framingham Heart Study cohort, upon which the GrimAge was trained, and the specific relationship between GrimAge in young and middle-aged populations and age-related morbidity and mortality has yet to be determined. Nonetheless, this does not change the relative difference in GrimAge between our two age-matched groups.

Overall, our findings are consistent with other emerging data of accelerated biological aging in PTSD, and they point to alterations in the expression of specific proteins and immune factors, and, to some extent, smoking history. Our study links objective biological measures with accelerated biological aging in PTSD and provides a foundation for future mechanistic studies. Our finding that longitudinally determined changes in AgeAccelGrim parallel changes in PTSD symptom severity, although found in a relative smaller sample size (N = 26), raise the possibility that GrimAge may prove clinically useful in monitoring underlying disease progression. The original population-based report on AgeAccelGrim found it to be highly predictive of lifespan, disease processes and time-to-death [15], although as noted, the applicability of this predictive ability in the age range studied here is not known. It will be important in future prospective studies to determine whether the AgeAccelGrim changes we observed. Similarly predict future medical morbidity and mortality in PTSD, and whether effective treatment can attenuate this risk.

**Material and methods**

Sample characteristics are presented in Table S9 (see Supplementary information Appendix). Detailed information on recruitment, inclusion and exclusion criteria, and biological assays are listed in Supplementary information.

**Cohorts and study design**

A set of two independent cohorts (with longitudinal repeat assessments in a subset of the first) totaling 215 male veterans from Operation Enduring Freedom (OEF) and/or Operation Iraqi Freedom (OIF) conflicts were recruited as
part of our large SBC study [18, 37] designed to identify biomarkers for PTSD diagnosis. Participants were recruited in two distinct cohorts, called the Discovery and Validation cohorts. A subgroup of the Discovery cohort returned for longitudinal assessment and formed the Recall cohort. For the current study, the Discovery cohort consists of 80 combat trauma-exposed PTSD and 82 combat trauma-exposed control participants who met the inclusion and exclusion criteria (described in Supplementary information) and who had DNA methylation data. Participants from the Discovery cohort were invited back for clinical re-evaluation and a blood draw approximately three years (range: 1–5 years) after their initial evaluation. Fifty-nine participants from the Discovery cohort responded to, and participated in, the second evaluation (i.e., Recall cohort) and no significant difference in age, BMI, and CAPS between the selected and the entire Discovery cohort were found. Of the 59, we only included a total of 55 participants as they had DNA methylation data at both time points in our longitudinal analysis. The 55 Recall participants included 14 who were PTSD-positive at both time points, 12 were PTSD-positive at T1 and PTSD subthreshold or negative at T2, 27 who were PTSD-negative at both time points and 2 subjects who were PTSD-negative at T1 then PTSD-positive at T2. There was no apparent bias in selecting the PTSD-positive subjects who participated in the follow-up (CAPS of all PTSD-positive subjects vs Recalled, t = 0.365, p = 0.717). A second cohort, the Validation cohort, was an independent group of 26 PTSD-positive and 27 control participants. A summary of the cohort characteristics is shown in Supplement Table S9 (see Supplementary information Appendix). Subjects were similarly diagnosed using the DSM-4 diagnosis and SCID interview, and CAPS scores with the same exclusion criteria as the OIF/OEF veteran cohorts.

The protocol for all studied cohorts were approved by the respective Institutional Review Board at each study site and Human Research Protection Office (HRPO) from the Department of Defense. The written informed consent was obtained from all of the participants.

**DNA methylation and GrimAge**

Blood was drawn by venipuncture in the morning after a night of fasting. Blood draws were collected in PAXgene™ DNA tubes (Preanalytix) and frozen at −80 °C until the DNA extraction. Genomic DNA was extracted from peripheral blood using the PAXgene Blood DNA Kit (Qiagen, Germantown, MD, USA) according to the manufacturer’s instructions followed by quality check using a Tapestation (Agilent). Genomic DNA (500 ng) was treated with sodium bisulfite using the Zymo EZ96 DNA Methylation Kit (Zymo Research, Orange, CA, USA), and genome-wide DNA methylation patterns were profiled using the Infinium HumanMethylation450 BeadChip array (Illumina, Inc., San Diego, CA, USA). BeadChips were washed, single-base extension labeled and stained with multiple layers of fluorescence followed by scanning using the Illumina iScan system (Illumina Inc, CA). The samples from three cohorts were randomized and processed in seven plates. No visual batch effects were found between the plates. IDAT files containing the raw intensity signals were generated using Illumina’s iControl software. The methylation level is called a “β value” and for each CpG site it corresponds to the ratio between the fluorescence signal of the methylated allele (C) and the sum of the fluorescent signals of the methylated (C) and unmethylated (T) alleles. Three PTSD and one control sample did not have enough DNA to assay, thus yielding 80 PTSD and 82 control subjects for the Discovery baseline cohort. Noob background correction [38] was used to preprocess the data prior to submitting it to the DNAm age website https://dnamage.genetics.ucla.edu for analysis. All samples passed quality control and mean absolute difference between samples and the “gold standard”, which is the mean of a large whole-blood DNAm cohort, are all lower than 0.08. Complete Blood Counts (CBC) were performed in the CLIA-certified laboratory at the Icahn School of Medicine at Mount Sinai, and estimated cell composition from DNAm were highly correlated to the CBC counts [22]. We performed technical replication on 20 samples, and compared the difference between the four types of age estimators we used (e.g., Horvath, Hannum, Phenage, and Grimage). The GrimAge has lowest percentage change (Estimation_Repeat–Estimation_Original /Chronological age), and lowest standard deviation of change (Horvath Age: 8.9%, 3.09; Hannum Age: 5.8%, 1.84; Phenage 12.2%, 4.71; GrimAge 4.3%, 1.69) (see Supplementary information Appendix, Table S10). The high repeatability supports the validity of the analysis of the longitudinal study.

**Data analysis**

Statistical analyses were performed using R v 3.3.0 and the Statistical Package for the Social Sciences (SPSS), version 25.0 (SPSS Inc., Chicago, IL, USA). For variables with non-normal distribution, normal score transformation with the Blom method [39] was conducted. Descriptive statistics were calculated for socio-demographic characteristics, clinical assessments and biological markers where continuous variables were summarized with means ± standard deviations (SD) and independent t-test and categorical variables were summarized with frequencies and χ² tests. Independent t-tests were conducted to evaluate group differences between PTSD and control groups. We controlled for any demographic or clinical characteristics that
were significantly correlated with AgeAccelGrim and different between PTSD and control groups in additional ANCOVA analyses to examine confounding effects. Paired t-tests were used to assess changes in symptom severity ratings over time. Pearson correlations were conducted to examine the relationships between quantitative variables such as AgeAccelGrim, symptom severity, and CD8 + CD28− proportions, as well as the delta values of these variables. The Cohen’s d effective size of AgeAccelGrim in this cohort is 0.538. With significance at alpha = 0.05 and power = 0.8, minimal 55 samples are required, while our three cohorts have 162, 53, and 55 samples. For all secondary analyses in addition to the initial group comparison, we combined the Discovery cohort and Validation cohort to increase sample size and power of the analyses.

**DNA methylation surrogates of AgeAccelGrim**

The AgeAccelGrim linear model is defined as
\[
\text{AgeAccelGrim} = 3.268 \cdot \sum_{i=1}^{8} \beta_i x_i,
\]
where \(x_i\) are the eight DNA methylation surrogates, and the coefficients \(\beta_i\) are listed in the Table 2 of Lu et al. [15], \(i = 1, \ldots, 8\).

**Logistic regression model of AgeAccelGrim, surrogates, CD4 Naïve, and plasmablast percentage**

Logistic regression analysis was conducted to estimate the probability of identifying an individual as PTSD-positive or negative, based on AgeAccelGrim, surrogates, and age-related cells as predictors. The DNA Methylation Age Calculator ([https://dnamage.genetics.ucla.edu](https://dnamage.genetics.ucla.edu)) provides DNA methylation estimates of the percentage of four aging-related cells, including CD8 Naïve, CD4 Naïve, Plasmablast, and CD8 + CD28−CD45RA− T cells. Using the combined Discovery Baseline and Validation dataset (N = 215), AgeAccelGrim was only significantly correlated with CD8 Naïve and CD8 + CD28−CD45RA− T cells (See SI Appendix, Table S11). The probability outcome of the logistic regression model was used to compute AUC (Area Under ROC curve). Sensitivity and specificity of the model were then calculated by Youden’s Index.

We trained two logistic models of AgeAccelGrim, four PTSD-related DNA methylation surrogates, as well as CD4 Naïve, and Plasmablast on Discovery baseline and follow-up Recall subjects to predict PTSD status:

- **Model 1**: PTSD status ~ AgeAccelGrim + PlasmablastAdjAge + CD4.NaïveAdjAge
- **Model 2**: PTSD status ~ DNAmPACKYRSAdjAge + DNAmCystatinCAdjAge + DNAmPAI1AdjAge + DNAmTIMP1AdjAge + CD4.NaïveAdjAge + PlasmablastAdjAge

We validated the predictive performance on the validation cohort. The coefficients of the models, and classification performance were listed in see Supplementary information Appendix, Table S12.

**Data availability**

All datasets for selected cohorts are available with permission through the SysBioCube, at [https://sysbiocube-abcc.ncifcrf.gov](https://sysbiocube-abcc.ncifcrf.gov).

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**Author contributions**

RY, GWYW, RH, OMW, and SHM designed research; all authors performed research and proofed or contributed to the manuscript; RY and GWYW analyzed data; and RY, GWYW, SHM, and OMW wrote the paper.

**Compliance with ethical standards**

**Conflict of interest**

The authors declare that they have no conflict of interest.

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**References**

1. McLeay SC, Harvey W, Romaniuk NM, Crawford D, Colquhoun DM, Young R, et al. Physical comorbidities of post-traumatic stress disorder in Australian Vietnam War veterans. Med J Aust. 2017;206:251–7.

2. Wolf EI, Schnurr PP. Posttraumatic stress disorder-related cardiovascular disease and accelerated cellular aging. Psychiatr Ann. 2016;46:527–32.

3. Cohen BE, Marmar C, Ren L, Bertenthal D, Seal KH. Association of cardiovascular risk factors with mental health diagnoses in Iraq and Afghanistan war veterans using VA health care. JAMA. 2009;302:489–92.

4. Schlenger WE, Corry NH, Williams CS, Kulka RA, Mulvaney-Day N, DeBakey S, et al. A prospective study of mortality and...
trauma-related risk factors among a nationally representative sample of vietnam veterans. Am J Epidemiol. 2015;182:980–90.

5. Lohr JB, Palmer BW, Eidt CA, Aalaboyina S, Maubsch BT, Wolkowitz OM, et al. Is post-traumatic stress disorder associated with premature senescence? A review of the literature. Am J Geriatr Psychiatry. 2015;23:709–25.

6. Wolf EJ, Morrison FG. Traumatic stress and accelerated cellular aging: from epigenetics to cardiometabolic disease. Curr Psychiatry Rep. 2017;19:75.

7. Darrow SM, Verhoeven JE, Rêvész D, Lindqvist D, Penninx BWJH, Delucchi KL, et al. The Association between psychiatric disorders and telomere length: a meta-analysis involving 14,827 persons. Psychosom Med. 2016;78:776–87.

8. Aiello AE, Dowd JB, Jayabalasingham B, Feinstein L, Uddin M, Simonek AM, et al. PTSD is associated with an increase in aged T cell phenotypes in adults living in Detroit. Psychoneuroendocrinology. 2016;77:133–41.

9. Jylhävä J, Pedersen NL, Hägg S. Biological age predictors. EBioMed. 2017;21:29–36.

10. Weng N-P, Akbar AN, Goronzy J. CD28(-) T cells: their role in Watanabe Kitamura, et al. Epigenetic age in male combat-exposed war veterans: associations with posttraumatic stress disorder status. Mol Neuropsychiatry. 2018;4:90–99.

11. Wolf EJ, Maniates H, Nugent N, Maihofer AX, Armstrong D, Hannum G, Guinney J, Zhao L, Zhang L, Hughes G, Sadda S, et al. Genome-wide methylation profiles reveal quantitative views of human aging rates. Mol Cell. 2013;49:359–67.

12. Hannum G, Guinney J, Zhao L, Zhang L, Hughes G, Sadda S, et al. Genome-wide methylation profiles reveal quantitative views of human aging rates. Mol Cell. 2013;49:359–67.

13. Horvath S. DNA methylation age of human tissues and cell types. Genome Biol. 2013;14:R115–R115.

14. Levine ME, Lu AT, Quach A, Chen BH, Assimes TL, Bandinelli S, et al. An epigenetic biomarker of aging for lifespan and healthspan. Aging. 2018;10:573–91.

15. Lu AT, Quach A, Wilson JG, Reiner AP, Aviv A, Raj K, et al. DNA methylation GrimAge strongly predicts lifespan and healthspan. Aging. 2019;11:303–27.

16. Harvath S, Oshima J, Martin GM, Lu AT, Quach A, Cohen H, et al. Epigenetic clock for skin and blood cells applied to Hutchinson Gilford Progeria Syndrome and ex vivo studies. Aging. 2018;10:1758–75.

17. Marioni RE, Shah S, McRae AF, Chen BH, Colicino E, Harris SE, et al. DNA methylation age predicts all-cause mortality in later life. Genome Biol. 2015;16:25.

18. Dean KR, Hammanieh R, Mellon SH, Abu-Amara D, Flor JD, Guffanti G, et al. Multi-omic biomarker identification and validation for diagnosing warzone-related post-traumatic stress disorder. Mol Psychiatry. 2019;2019:1–3.

19. Flor JD, Yehuda R. Comorbidity between post-traumatic stress disorder and major depressive disorder: alternative explanations and treatment considerations. Dialogues Clin Neurosci. 2015;17:141–50.

20. Houseman EA, Accamando WP, Koestler DC, Christensen BC, Marsit CJ, Nelson HH, et al. DNA methylation arrays as surrogate measures of cell mixture distribution. BMC Bioinforma. 2012;13:86.

21. Chen X, Liu Q, Xiang AP. CD8+CD28- T cells: not only age-related cells but a subset of regulatory T cells. Cell Mol Immunol. 2018;15:734–6.

22. Verhoeven JE, Yang R, Wolkowitz OM, Bersani FS, Lindqvist D, Mellon SH, et al. Epigenetic age in male combat-exposed war veterans: associations with posttraumatic stress disorder status. Mol Neuropsychiatry. 2018;4:90–99.

23. Kievit R, Frankenhuis WE, Waldorp L, Borsboom D, Simpson’s paradox in psychological science: a practical guide. Front Psychol. 2013;4:513.

24. Wolf EJ, Bovin MJ, Green JD, Mitchell KS, Stoop TB, Barretto KM, et al. Longitudinal associations between post-traumatic stress disorder and metabolic syndrome severity. Psychological Med. 2016;46:2215–26.

25. Ahmad N, Hajasadeghi F, Mirshkarlo HB, Budoff M, Yehuda R, Ebrahimi R. Post-traumatic stress disorder, coronary atherosclerosis, and mortality. Am J Cardiol. 2011;108:29–33.

26. Yaffe K, Vittinghoff E, Lindquist K, Barnes D, Covinsky KE, Neylan T, et al. Posttraumatic stress disorder and risk of dementia among US veterans. Arch Gen Psychiatry. 2010;67:608–13.

27. Wolf EJ, Logue MW, Stoop TB, Schichman SA, Stone A, Sadeh N, et al. Accelerated DNA methylation age: associations with PTSD and mortality. Psychosomatic Med. 2017;80:42–8.

28. Chou JP, Effros RB. T cell replicative senescence in human aging. Curr Pharm Des. 2013;19:1680–98.

29. Cesari M, Pahor M, Incalzi RA. REVIEW: plasminogen activator inhibitor-1 (PAI-1): a key factor linking fibrinolysis and age-related subclinical and clinical conditions. Cardiovascular Therapeutics. 2010;28:e72–e91.

30. Ferguson TW, Komenda P, Tangri N. Cystatin C as a biomarker for estimating glomerular filtration rate. Curr Opin Nephrol Hypertension. 2015:24:295–300.

31. Levin A, Lan JH. Cystatin C and cardiovascular disease. J Am Coll Cardiol. 2016;68:946.

32. Ashutosh, Chao C, Bergmann K, Brew K, Ghorpade A. Tissue inhibitor of metalloproteinases-1 protects human neurons from staurosporine and HIV-1-induced apoptosis: mechanisms and relevance to HIV-1-associated dementia. Cell Death Dis. 2012;3:e332.

33. Song G, Xu S, Zhang H, Wang Y, Xiao C, Jiang T, et al. TIMP1 is a prognostic marker for the progression and metastasis of colon cancer through FAK-Pi3K/AKT and MAPK pathway. J Exp Clin Cancer Res. 2016;35:148.

34. Rivera S, Tremblay E, Timsit S, Canals O, Ben-Ari Y, Khrestchatsky M. Tissue inhibitor of metalloproteinases-1 (TIMP-1) is differentially induced in neurons and astrocytes after seizures: evidence for developmental, immediate early gene, and lesion response. J Neurosci. 1997;17:4223.

35. Mills JA, Beach SRH, Dogan M, Simons RL, Gibbons FX, Long JD, et al. A direct comparison of the relationship of epigenetic aging and epigenetic substance consumption markers to mortality in the framingham heart study. Genes (Basel). 2019;10:51.

36. Stejskalova L, Dvorak Z, Pavek P. Endogenous and exogenous brinolysis and age-related risk factors among a nationally representative sample of vietnam veterans. Am J Epidemiol. 2015;182:980–90. R. Yang et al.
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A DNA methylation clock associated with age-related illnesses and mortality is accelerated in men with...