Epigenetic age acceleration and cognitive function in African-American adults in midlife

Epigenetic Age Acceleration and Cognitive Function in African-American Adults in Midlife: The Atherosclerosis Risk in Communities Study

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Abstract

Methylation levels measured at defined sites across the genome have recently been shown to be correlated with an individual's chronological age. Age acceleration, or the difference between age estimated from DNA methylation status and chronological age, has been proposed as a novel biomarker of aging. In this study, the cross-sectional association between two different measures of age acceleration and cognitive function was investigated using whole blood samples from 2,157 African-American participants 47-70 years of age in the population-based Atherosclerosis Risk in Communities (ARIC) Study. Cognition was evaluated using three domain-specific tests. A significant inverse association between a one-year increase in age acceleration calculated using a blood-based age predictor and scores on the Word Fluency Test was found using a general linear model adjusted for chronological age, gender, and years of education (β = -0.140 words; p = 0.001) and after adding other potential confounding variables (β = -0.104 words, p = 0.023). The results were replicated in 1,670 European participants in the Generation Scotland: Scottish Family Health Study (fully adjusted model: β = -0.199 words; p = 0.034). A significant association was also identified in a trans-ethnic meta-analysis across cohorts that included an additional 708 European American ARIC study participants (fully adjusted model: β = -0.110 words, p = 0.003)**.** There were no associations found using an estimate of age acceleration derived from multiple tissues. These findings provide evidence that age acceleration is a correlate of performance on a test of verbal fluency in middle-aged adults.

Keywords: DNA methylation age, epigenetic age, neuropsychological tests, verbal fluency

Introduction

DNA methylation is a widely studied epigenetic mechanism in which methyl groups are covalently attached to cytosine residues, usually in the context of CpG dinucleotides, by DNA methyltransferases. Although the underlying DNA sequence is not altered, methylation at specific CpG sites correlates with both the activation and repression of gene expression. DNA methylation patterns across the genome have been shown to change with age and in response to environmental exposures and lifestyle factors that can impact the risk of age-related common diseases (1). Two independent groups of investigators have recently developed predictors of chronological age based on measurement of DNA methylation at defined sets of CpG sites. The Hannum et al. age predictor is based on levels of DNA methylation at 71 CpG sites in whole blood selected after assessing a single cohort of individuals, whereas the Horvath predictor incorporates information on methylation status for 51 healthy tissues and cell types and includes 353 CpG sites (2,3). Both predictors have been used to calculate age acceleration where predicted methylation age exceeds chronological age as a potential biomarker of biological aging. Age acceleration has previously been associated with all-cause mortality, exceptional longevity, frailty, Parkinson's disease, Werner's syndrome, and Alzheimer's disease neuropathology (4-10). A relationship between epigenetic age acceleration and cognitive function has been found in some but not all previous studies. A significant cross-sectional correlation between higher age acceleration using the Horvath estimate and lower measures of general cognitive ability was reported for 1,091 participants in the Lothian Birth Cohort 1936 who were approximately 70 years of age, and older epigenetic age using the Hannum but not the Horvath predictor was associated with lower cognitive function at 38 years of age in the Dunedin Study birth cohort (11,12). When the difference between the Horvath estimate of DNA

methylation age and chronological age was assessed in groups differing in maintenance of memory over a 15-year period, a smaller difference was found for those with sustained high performance compared to individuals with average or accelerated decline among 52 Swedish participants in the Betula study (13). However, there was no association found for epigenetic age acceleration using both the Horvath and Hannum et al. predictors and cross-sectional or longitudinal measures of composite cognitive ability when 486 pairs of middle-aged monozygotic twins were compared (14). Whereas all of these studies calculated DNA methylation age in blood, the association between measures of cognitive decline in individuals with and without Alzheimer's disease and epigenetic age based on the Horvath predictor was analyzed using methylation levels for postmortem samples of dorsolateral prefrontal cortex obtained from 700 participants in the Religious Order Study and the Rush Memory and Aging Project. Epigenetic age acceleration was associated with decline in global cognitive functioning, episodic memory, and working memory in the individuals with Alzheimer's disease but not in controls without dementia (10). Although the inconsistent results could be attributable to the use of a diverse set of cognitive measures and reliance on only the Horvath estimate of age acceleration in three of the studies, an additional factor may be that the study samples were not all adequately powered to detect small effects. The goal of this study is to evaluate the association of age acceleration and methylation age with scores on three tests of cognitive function in middle-aged African-American participants in the population-based Atherosclerosis Risk in Communities (ARIC) Study. Since several population-based studies carried out in multi-ethnic communities have previously shown that the prevalence of cognitive impairment and Alzheimer's disease is approximately two times higher in African-Americans than in whites, defining blood-based biomarkers that can be used to assess when the impact of controlling risk

factors such as hypertension and diabetes could be maximized would be particularly advantageous in this population that has not previously been represented in studies of the role of epigenetic aging in cognitive function and cognitive decline (15,16).

Methods

Atherosclerosis Risk in Communities (ARIC) Study

The ARIC study is a prospective longitudinal investigation of the development of atherosclerosis and its clinical sequelae in which 15,792 individuals aged 45 to 64 years were enrolled at baseline (1987-1989). A detailed description of the ARIC study has been reported previously (17). At the inception of the study, participants were selected by probability sampling from four communities in the United States: Forsyth County, North Carolina; Jackson, Mississippi (African-Americans only); suburban Minneapolis, Minnesota; and Washington County, Maryland. Four examinations were carried out at three-year intervals (exam 1, 1987-1989; exam 2, 1990-1992; exam 3, 1993-1995; exam 4, 1996-1998.) A fifth and sixth clinical examination were completed in 2011-2013 and 2016-2017, respectively, and a seventh is underway. Subjects were contacted annually to update their medical histories. Written informed consent was provided by all study participants, and the study design and methods were approved by institutional review boards at each of the collaborating medical institutions: University of Mississippi Medical Center Institutional Review Board (Jackson Field Center); Wake Forest University Health Sciences Institutional Review Board (Forsyth County Field Center); University of Minnesota Institutional Review Board (Minnesota Field Center); and Johns Hopkins University School of Public Health Institutional Review Board (Washington County Field Center).

Cognitive testing

Cognitive testing was performed in the entire cohort at visits 2 and 4. Cognitive function was assessed by three neuropsychological tests and has been described previously (18): 1) the Delayed Word Recall Test, a measure of verbal learning and recent memory in which the participant is required to use each of 10 common nouns in a sentence. After a 5-minute delay the participant is asked to recall the 10 nouns (19); 2) the Digit Symbol Substitution Test, a subtest of the Wechsler Adult Intelligence Scale-Revised, tests processing speed and requires timed translation of number to symbols using a key. The score is calculated as the number of correct translations within 90 seconds (20); the Word Fluency Test, a measure of phonemic verbal fluency that is one aspect of executive function. In three separate 1-minute trials, the participant is asked to generate as many words as possible beginning with the letters F, A, and S. The score is the combined total of correct words produced (21,22). The tests were administered by trained interviewers in a standardized order and were given in a single session. For all of the neuropsychological tests, lower scores indicate a lower measure of cognition. Six-year change in cognitive function was analyzed as the difference between the test score obtained at visit 4 and the test score obtained at visit 2 for each neuropsychological test. Participants selected for DNA methylation assays had to have at least 1 ug of DNA isolated at visit 2 available, no restrictions on the use of the DNA, and previous genome-wide genotyping using the Affymetrix Human SNP Array 6.0, Illumina Cardio-MetaboChip, or Illumina HumanExome BeadChip. After the application of quality control procedures for the DNA methylation data, there were 2,400 African-American study participants eligible to be entered in the analysis of baseline cognitive function; of these, individuals were excluded if they had a history of prevalent or unknown history of stroke at visit 2 (n = 84), if they were missing all three cognitive tests scores (n = 71),

or if missing information for any covariates (n = 88). DNA methylation data obtained at visit 2 at the same time as the test of cognitive function was available for 2,155 African-American study participants with Delayed Word Recall Test scores, for 2,135 participants with Digit Symbol Substitution Test scores, and for 2,149 participants with Word Fluency Test scores. There were 1,401 individuals included in the analyses of the change in scores on the Delayed Word Recall Test, 1,384 in the analyses of change in scores on the Digit Symbol Substitution Test, and 1,394 in the analyses of change in scores on the Word Fluency Test after exclusions were made for those who did not have scores for at least one of the tests at both visits 2 and 4 (n = 732), who had prevalent stroke or an unknown history of stroke at visit 2 (n = 84), who had an incident stroke between visits 2 and 4 (n = 125), or if missing any covariates (n = 56). There were also 759 European American ARIC study participants with methylation data obtained at visit 2 who could potentially be entered in the study to replicate results found in the African-American discovery sample. After application of the same exclusions described above for the African-American study participants (history of prevalent or unknown history of stroke at visit 2 (n = 6), missing information for any covariates (n =45)), there were 708 participants with Delayed Word Recall Test scores, 705 participants with Digit Symbol Substitution Test scores, and 708 participants with Word Fluency Test scores.

DNA methylation

Detailed information on the DNA methylation assay and quality control procedures using the wateRmelon R package have been reported previously (23,24). In brief, bisulfite converted DNA extracted from peripheral blood leukocytes collected at visit 2 was hybridized to the Illumina HumanMethylation450 BeadChip following the Illumina HD Methylation protocol (Illumina Inc., San Diego, CA). An average detection p-value <0.01 was used as a measure of performance

for each probe and was determined using an algorithm in the GenomeStudio® software module (Illumina, Inc.) to assess whether its methylation level was above background using negative control bead types included on the array. Individuals were excluded from the analyses if the pass rate for the DNA sample for the participant was < 95% (number of probes with a detection p-value <0.01/number of probes on the array), if there was possible gender mismatch based on principal component analysis, or if there was genotype mismatch based on 24 single nucleotide polymorphisms present on the array. At the target level, poor-quality CpG sites with average detection p-value > 0.01 were flagged, and the percentage of samples having detection p-value >0.01 for each autosomal and X chromosome CpG site was calculated. CpG sites were not analyzed in this study if more than 5% of the samples showed a detection p-value > 0.01, or if the average detection p-value was > 0.01 on the Y chromosome for males.

DNA methylation age and age acceleration

Two measures of DNA methylation age were estimated for each study participant from the background-subtracted beta values calculated in GenomeStudio® for each probe. The beta value is a continuous variable that ranges between 0 (completely unmethylated) and 1 (fully methylated) and is determined as the ratio of the fluorescence intensity of the methylated allele to the sum of the fluorescence intensities of the methylated and unmethylated alleles. Imputation of missing beta values and data normalization was performed using R codes provided by Horvath (3). The Horvath predicted methylation age is calculated based on 353 probes using data obtained across a variety of tissues and cell types, and was implemented using an online calculator (http:labs.genetics.ucla.edu.horvath/dnamage/). The Hannum predicted methylation age is derived from 71 probes using published coefficients reported for data generated from whole blood (2). DNA methylation age acceleration was calculated by regressing each of the

predicted methylation age estimates on chronological age and using the corresponding residual value in the linear regression models. A positive value of age acceleration indicates that the epigenetic age is higher than the chronological age.

Clinical and laboratory measurements

The clinical and laboratory measurements used for this study were obtained at visit 2. Body mass index (BMI) was calculated as weight in kilograms/(height in meters)^2. Information on cigarette smoking was acquired at visit 2 using an interviewer-administered questionnaire and was classified as current, former, or never. Blood pressure was measured three times while seated using a random-zero sphygmomanometer and the last two measurements were averaged for analysis. Hypertension was defined by diastolic blood pressure of $\geq$ 90 mm Hg, systolic blood pressure of $\geq$140 mm Hg, or use of antihypertensive medication. Serum glucose was measured by a standard hexokinase method on a Coulter DACOS chemistry analyzer (Coulter Instruments, Fullerton, CA). The prevalence of diabetes was defined using a fasting glucose level $\geq$ 7.0 mmol/L, a nonfasting glucose level $\geq$ 11.1 mmol/L, and/or self-reported physician diagnosis or treatment for diabetes. Plasma total cholesterol and triglycerides were measured by enzymatic methods (25,26). High density lipoprotein cholesterol was measured after dextran-magnesium precipitation of non-high density lipoprotein cholesterol (27). Low density lipoprotein cholesterol was calculated using the Friedewald method (28). Hypercholesterolemia was defined as low density lipoprotein cholesterol > 3.36 mmol/L (29). Since the various blood subpopulations have different methylation patterns and can confound DNA methylation association studies, the proportions of neutrophils, lymphocytes, monocytes, eosinophils, and basophils were imputed using the Houseman method based on the measured differential cell counts available for a subset of African-American ARIC participants at visit 2 (n = 175) and

Houseman cell types were estimated using the Horvath method for European American ARIC participants (3,30,31).

Genotyping

Genotyping of the apolipoprotein E (*APOE*) polymorphisms was performed using the TaqMan system (Applied Biosystems, Foster City, CA, USA). The sequences for primers and probes are available upon request. Since the TaqMan assay detects two alleles in each genotyping reaction, the *APOE* variants at codons 112 and 158 were measured separately. The data from these two codons were combined to generate the six *APOE* genotypes. Allele detection was performed using the ABI Prism 7700 Sequence Detection System (Applied Biosytems, Thermo Fisher Scientific, Foster City, CA). The genotype call rate, or the percentage of samples to which a genotype was assigned, was determined prior to exclusion of individuals from the analysis and was 95.7%.

Statistical analysis

Three general linear models were used to analyze the cross-sectional association of three cognitive test scores modeled as continuous variables and the Hannum and Horvath measures of age acceleration. The first model was adjusted for chronological age, sex, and years of education as an ordinal variable based on the highest level attained ($\leq$ 11 years; 12-16 years; > 16 years). A second model included the variables in the primary model and was further adjusted for *APOE* genotype (at least 1 ε4 allele), BMI, smoking status, hypertension, diabetes, and hypercholesterolemia. A third model was adjusted for estimated white blood cell proportions as well as the covariates included in the second model. Akaike's information criterion (AIC) was calculated for each of six nested statistical models for each neuropsychological test to examine their relative performance; the delta AIC was then calculated to compare each model relative to

the model with the lowest AIC (32,33). In addition, multivariable logistic regression was used to evaluate the risk of being in the quartile with the lowest cognitive test scores compared to the other three quartiles. The summary statistics for the cohort-specific analyses were combined by performing inverse-variance meta-analyses using the program METAL (34). All of the covariates included in the statistical models were assessed at visit 2 except for education which was assessed at visit 1. The analyses of the association of scores for each of the three cognitive tests and the two measures of age acceleration were performed using Stata 9 software (StataCorp, College Station, TX, USA). A two-sided p-value <0.05 was considered statistically significant.

Replication Study

Replication of results for African-Americans in the ARIC study was sought in both a sample of 708 European American ARIC participants who had both cognitive and methylation data available at visit 2, and in the Generation Scotland: Scottish Family Health Study (GS)**.** GS is a large, family-structured, population-based cohort of individuals of European ancestry that includes over 24,000 adults aged between 18 and 99 years who were recruited between 2006 and 2011 (35,36). All components of GS received ethical approval from the NHS Tayside Committee on Medical Research Ethics (REC Reference Number: 05/S1401/89)**.** Participants completed detailed cognitive, health, and lifestyle questionnaires. Here, cognitive test data included scores on the Verbal Fluency Test (combined score from three one-minute trials for the letters C, F, and L) and the Digit Symbol Substitution Task (21,37). Self-reported years of education was categorized into low ($\leq$11 years), medium (12-15 years), and high ($\geq$16 years). BMI was assessed as body mass (kg) divided by the square of height (meters). Self-reported disease information was provided for stroke, Alzheimer's disease, diabetes, and high blood

pressure. Self-reported smoking status was coded as ever smoked versus never smoked. The general linear models and logistic regression models were the same as those used for statistical analysis in the ARIC study except that hypercholesterolemia was not entered as a covariate in the second and third models.

The current analysis was restricted to 5,200 GS participants for whom Illumina HumanMethylation EPIC BeadChip DNA methylation data were available. After removing samples and probes that failed to meet quality control standards, 860,926 CpG sites were retained for 5,100 individuals (38). Epigenetic age estimates were calculated by multiplying the regression weights reported by Hannum et al. and Horvath as in the ARIC cohort analysis (2,3). Of the 353 and 71 CpG sites used in the Hannum and Horvath signatures, 335 and 65 were available in GS after quality control. White blood cell counts (CD4+ T cells, CD8+ T cells, B cells, natural killers cells and granulocytes) were estimated from the methylation beta values using the 'estimatesCellCounts' function in minfi, which implements a modified version of the algorithm used by Houseman et al (31,39). GCTA-GRM was used to derive a genetic relationship matrix on the 5,100 individuals in the methylation cohort (40). After filtering on a relatedness coefficient <0.05, there were 2,709 unrelated individuals in the cohort. Further filtering to remove individuals with a self-report for stroke (n = 45), and to restrict the age range between 45 and 70 years, left a sample of 1,868 individuals. Finally, filtering to remove individuals with any missing data gave a final analysis sample of 1,670.

Results

The demographic and clinical characteristics of the ARIC study participants included in the analysis of the association of cognitive function and epigenetic age acceleration are shown in Table 1. In this study population, the mean Horvath predicted age (55.5 $\pm$ 6.7 years) was lower

than the mean chronological age (56.0 $\pm$ 5.7 years), and the mean Hannum predicted age was higher (60.1 $\pm$ 7.8 years) than the mean chronological age. The correlation between chronological age and Horvath predicted age was 0.62 and was 0.71 for Hannum predicted age (eTable 1, eFigure 1, and eFigure2 in Supplementary data). The correlation between the Horvath and Hannum methylation-based predicted ages was 0.74. Since DNA methylation data was not available for all participants, the clinical and demographic characteristics of those with and without these measurements were compared (eTable 2, Supplementary data). Individuals with methylation profiles were significantly younger, had a higher BMI, and had higher scores on the test of verbal declarative memory than those who were not included in the analysis.

A significant inverse association between years of Hannum estimated age acceleration and scores on the Word Fluency Test was found after adjustment of the general linear models for chronological age, gender, and years of education (β = -0.140 words per year of age acceleration; p = 0.001) (Table 2); the association was attenuated but still significant after accounting for potential confounding variables and white blood cell counts (β = -0.104 words per year of age acceleration; p = 0.023). Scatterplots of the relationship between scores on the test of verbal fluency and Horvath and Hannum age acceleration are shown in eFigure 3 (Supplementary data). An alternative presentation of these results after standardization of the cognitive test scores to have a mean of zero and a standard deviation of one is shown in eTable 3 (Supplementary data). After rescaling the Word Fluency Test scores, β = -0.008 (p = 0.023) for a one-year increase in age acceleration after the addition of all covariates to the general linear model. Age acceleration based on the Hannum methylation age predictor was not associated with scores on either the Delayed Word Recall Test or the Digit Symbol Substitution Test, and age acceleration based on the Horvath methylation age predictor was not associated with performance on any of the

neurocognitive tests (Table 2). Although the covariates were selected *a priori* for inclusion in the three statistical models as established risk factors for interindividual variation in cognitive function**,** AIC values were calculated as a metric for comparison of six different nested regression models to examine their relative fit to the data (eTable 4, Supplementary data). The minimum AIC value was observed for both Horvath and Hannum age acceleration after chronological age, gender, years of education, BMI, smoking status, hypertension, diabetes, and hypercholesterolemia were included as independent variables in the analyses of test scores attained on the Digit Symbol Substitution Test and the Word Fluency Test (Model 4). For the Delayed Word Recall Test and the tests of association with both Horvath and Hannum age acceleration, the minimum AIC was found for the fully adjusted model (Model 6). When each of the models was ranked using delta AIC for each of the three cognitive tests, there was substantial evidence for Model 5 (delta AIC < 2) for both the Digit Symbol Substitution Test and the Word Fluency Test for estimation of the relationship with both the Horvath and Hannum measures of age acceleration, and for Model 6 for Horvath age acceleration and the Word Fluency Test (33). Model 5 differs from Model 4 by further adjustment for carrying at least one *APOE* ε4 allele, and includes all of the covariates entered in the fully adjusted model (Model 6) with the exception of white blood cell type proportions that are a known source of confounding of epigenome-wide association studies that use blood as the target tissue.

Replication of the results found for African-Americans in the ARIC study was attempted in a sample of 1,670 unrelated GS participants of European ancestry with a mean age of 55.7 $\pm$ 5.7 years. In contrast to the ARIC study, the mean Horvath predicted methylation age was higher than the mean chronological age, and the mean Hannum predicted methylation age was lower than the mean chronological age in this cohort. A description of the study participants is

provided in eTable 5 (Supplementary data). A significant association of scores on the test of verbal fluency and Hannum age acceleration (β = -0.199 words per year of age acceleration; p = 0.034) was observed using the fully adjusted model (eTable 6, Supplementary data). As in the ARIC study, there was no association observed for scores on the Verbal Fluency Test and Horvath age acceleration, or for either Hannum or Horvath age acceleration and Digit Symbol Substitution Task scores (eTable 6, Supplementary data). A sample of 708 European Americans from the ARIC study (mean age 58.5 $\pm$ 5.2 years) was also available for replication of study findings but no associations were found for performance on any of the cognitive tests and either measure of age acceleration (eTable 6, Supplementary data). The clinical and demographic characteristics of these participants are shown in eTable 7 (Supplementary data). The correlations between chronological age and the Horvath and Hannum predicted DNA methylation ages are displayed in eTable 1 (Supplementary data) and were similar to those found for the African-American study participants.

When the sample size was increased by combining the cohort-specific results for ARIC African-American participants, ARIC European American participants, and GS study participants using inverse-variance weighted meta-analysis, there was an increase in the strength of the association of verbal fluency and Hannum age acceleration for all three statistical models (fully adjusted model: β = -0.110 words per year of age acceleration; p = 0.003) (eTable 8, Supplementary data). Meta-analysis of the results for only the ARIC European American participants and the GS participants yielded a marginally significant association between Hannum age acceleration and scores on the tests of verbal fluency (fully adjusted model: β = -0.121 words per year of age acceleration; p = 0.051).

Logistic regression was then used to examine the association of age acceleration and the risk of being in the quartile with the lowest test scores compared to being in quartiles two through four combined (eTable 9, Supplementary data). Mean cognitive scores for each quartile for each test are shown in Table 1. The Hannum estimate of age acceleration was found to be associated with an increased risk for being in the top quartile for performance on the Word Fluency Test (odds ratio (OR) = 1.02 per year of age acceleration, 95% confidence interval (CI) = 1.00 – 1.04, p = 0.029) after adjustment for all covariates. A significant association between performance on the Digit Symbol Substitution Test and the Hannum estimate of age acceleration was observed after adjustment for age and gender but the association was no longer significant after the addition of other potential confounding variables and white blood cell counts to the models. In GS, neither Hannum nor Horvath age acceleration was associated with having the lowest scores for the test of verbal fluency. A significant association of Hannum age acceleration and being in the quartile with the lowest scores for processing speed as assessed by the Digit Symbol Substitution Task was found only for the fully adjusted model (OR = 1.04 per year of age acceleration, 95% CI = 1.00 – 1.08, p = 0.036) (eTable 10, Supplementary data). For European American participants in the ARIC study, an association of Horvath age acceleration and belonging to the quartile with the lowest scores on the Digit Symbol Substitution Test was observed using all three statistical models (fully adjusted model: OR = 1.04 per year of age acceleration, 95% CI = 1.00 – 1.08; p = 0.049) while there were no associations found for the tests of verbal memory or verbal fluency (eTable 10, Supplementary data).

In an exploratory analysis of early cognitive change, the association between age acceleration and change in neurocognitive test scores over a six-year period between examinations 2 and 4 was also evaluated in 1,401 African-American ARIC study participants but no significant

relationships were identified (Table 3). The clinical and demographic characteristics of these individuals are shown in eTable 11 (Supplementary data).

Discussion

Accelerated epigenetic aging which is operationalized as a DNA methylation-based measure of age that is higher than an individual's chronological age was associated with lower performance on the test of verbal fluency (β = -0.104 words per year of age acceleration; p = 0.023) using the predictor generated by Hannum et al. This association was independent of chronological age, gender, years of education, and potential confounding variables including diabetes and hypertension case status, BMI, LDL cholesterol level, current smoking and alcohol consumption, *APOE* genotype, and blood cell composition. Replication of these results in the independent GS study population, as well as the detection of a significant association when the cohort-specific results from all participants were meta-analyzed adds to the evidence that age acceleration may serve as a valid predictor of performance on tests of verbal fluency. However, since these analyses were cross-sectional it is not possible to determine whether age acceleration was the cause of the lower test scores or even whether having lower scores on a test of verbal fluency could lead to accelerated epigenetic aging through an as yet unknown mechanism. Neither the Hannum nor the Horvath measure of age acceleration was associated with 6-year change in scores for any of the cognitive tests in African-American ARIC study participants. In contrast to the cross-sectional analysis, epigenetic age acceleration was assessed at baseline and preceded the follow-up period for the longitudinal analyses of cognitive change. Use of a smaller and different study sample than for the cross-sectional analyses limits the ability to directly compare these results and further investigation is needed.

The Hannum et al. estimate of age acceleration was associated with interindividual variation in scores for the test of verbal fluency but not for verbal memory and processing speed, while the Horvath estimate of age acceleration was not associated with any of the three test scores. The differing results when the two methylation age predictors were used suggests that the association of verbal fluency with Hannum age acceleration in African-Americans may be capturing an effect of age-related methylation that is more prominent in blood than in other tissues. A stronger negative correlation with estimated naïve CD4+ and CD8+ T-cell counts for the Hannum measure of age acceleration than for the Horvath measure has previously been reported (4). The abundance of these T-cell subsets decreases with chronological age, and is a component of an altered adaptive immune response that has been linked to the development of chronic age-related diseases including atherosclerosis and Alzheimer's disease in which inflammation plays a role in pathogenesis (41,42). In addition, the Hannum estimate of age acceleration was also associated with verbal fluency in African-American participants in the ARIC study and with digit symbol coding in GS when cognitive test scores were modeled as a categorical variable.

The association of age acceleration and cognitive function was analyzed using three neuropsychological tests representing different cognitive domains that differ in the degree to which they decline with age. Whereas processing speed and verbal memory begin to decline in the third decade of life, tests of verbal fluency measure word retrieval as well as executive function and performance is reported to be lower in middle aged and older adults than in young adults (43-48). Notably, the age-related decrease in phonemic verbal fluency has been attributed to slower information processing speed and Hannum age acceleration was associated with both of these cognitive abilities in the GS cohort (46).

This study has considerable strengths including the deeply phenotyped ARIC and GS cohorts, and the availability of comparable methylation data and cognitive assessments that enabled the association of Hannum age acceleration and verbal fluency identified in African-Americans to be replicated in individuals of European descent in an independent cohort. There are also limitations. The analysis was carried out using blood as the target tissue for a brain-related trait. Although there are differences in methylation between blood and brain, a distinct set of differentially methylated probes associated with Alzheimer's disease was recently characterized in peripheral blood providing proof of principle for the utility of blood-based biomarkers to identify individuals affected with a neurological disorder (49,50). The study also employed a cross-sectional design for some of the tests of association that limits the ability to draw inferences about causality. Finally, the observed effect size of – 0.008 standard deviation units per year of epigenetic age acceleration for the association of the Hannum estimate and verbal fluency in African-Americans after adjusting for all covariates was modest but contributed almost as much as chronological age (β = - 0.014; p = <0.001). Further studies will be required to replicate and extend our findings to larger and more diverse populations, and to examine how the association reported here reflects biological mechanisms involved in cognitive aging.

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Author Contributions

Study design: M.F.; Acquisition of data: T.H.M., E.B., M.L.G., R.F.G., B.G.W., R.M.W., K.L.E., A.M.M., I.J.D.; Data analysis and statistical methods: M.F., J.B., R.X., W.G., R.E.M., R.M.W.; Interpretation of data and results: M.F., T.H.M., E.B., J.B., R.X., W.G., J.S.P., R.E.M., I.J.D.; Drafted the manuscript: J.B., R.E.M.; Critical review of manuscript for intellectual content: M.F., T.H.M., E.B., J.B., R.X., R.F.G., B.G.W., M.L.G., W.G., J.S.P., R.E.M., R.M.W., K.L.E., A.M.M., I.J.D.

Conflict of Interest

R.F.G. serves as Associated Editor for *Neurology*. B.G.W. serves on the Editorial Board of *The Journal of Gerontology: Medical Sciences*.

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Table 1. Clinical and Demographic Characteristics – ARIC Study (1990 – 1992)		
	N	
Age (years) (mean, SD)	2,157	56.0 (5.7)
Female (N, %)	2,157	1,392 (64.5)
Education (N, %)	2,157	
< High school		825 (38.3)
High school		615 (28.5)
> High school		717 (33.2)
Horvath predicted age (years) (mean, SD)	2,157	55.5 (6.7)
Horvath age acceleration (years) (mean, SD)	2,157	-3.0×10^{-9} (5.3)
Horvath age acceleration (years) (median)	2,157	-0.090
Horvath age acceleration (years)(Q1; Q3)	2,157	-3.39; 3.33
Hannum predicted age (years) (mean, SD)	2,157	60.1 (7.8)
Hannum age acceleration (years) (mean, SD)	2,157	1.7×10^{-8} (5.5)
Hannum age acceleration (years) (median)	2,157	-0.017
Hannum age acceleration (years) (Q1; Q3)	2,157	-3.63; 3.32
Body mass index (kg/m^2) (mean, SD)	2,157	30.2 (6.2)
Current smoking (N, %)	2,157	533 (24.7)
Diabetes (N, %)	2,157	535 (24.8)
Hypertension (N, %)	2,157	1,169 (54.2)
Hypercholesterolemia (N, %)	2,157	1,139 (52.8)
APOE ε4 (at least 1 allele) (N, %)	2,157	892 (41.4)
Cognitive Tests		
DWRT		
All (mean, SD)	2,155	6.1 (1.6)
Quartile 1 (mean, SD)	703	4.3 (1.0)
Quartile 2 (mean, SD)	523	6.0 (0.0)
Quartile 3 (mean, SD)	489	7.0 (0.0)
Quartile 4 (mean, SD)	440	8.3 (0.6)
DSST		
All (mean, SD)	2,135	31.3 (13.4)
Quartile 1 (mean, SD)	581	15.9 (4.8)
Quartile 2 (mean, SD)	519	26.6 (2.3)
Quartile 3 (mean, SD)	506	35.1 (2.8)
Quartile 4 (mean, SD)	529	49.3 (8.3)
WFT		
All (mean, SD)	2,149	28.0 (13.1)
Quartile 1 (mean, SD)	565	12.7 (5.0)
Quartile 2 (mean, SD)	545	23.5 (2.3)
Quartile 3 (mean, SD)	517	31.7 (2.6)
Quartile 4 (mean, SD)	522	45.8 (7.7)

Note: SD, standard deviation; N, number; Q1, 25th percentile; Q3, 75th percentile; DWRT, Delayed Word Recall Test; DSST, Digit Symbol Substitution Test; WFT, Word Fluency Test

Table 2. Methylation age acceleration and cognitive function – ARIC Study (1990 – 1992)

		Model 1			Model 2			Model 3		
	N	β	SE	p	β	SE	p	β	SE	p
Cognitive Test										
DWRT										
Horvath age acceleration	2,155	-0.003	0.006	0.654	-0.001	0.006	0.868	-0.003	0.006	0.672
Hannum age acceleration	2,155	-0.005	0.006	0.457	-0.002	0.006	0.705	-0.004	0.006	0.530
DSST										
Horvath age acceleration	2,135	0.043	0.042	0.306	0.068	0.042	0.106	0.061	0.042	0.145
Hannum age acceleration	2,135	-0.053	0.042	0.207	-0.024	0.042	0.569	-0.014	0.044	0.751
WFT										
Horvath age acceleration	2,149	-0.025	0.044	0.564	-0.007	0.044	0.875	-0.013	0.045	0.764
Hannum age acceleration	2,149	-0.140	0.044	0.001	-0.119	0.044	0.007	-0.104	0.046	0.023

Note: N, number; β, beta coefficient; SE, standard error; DWRT, Delayed Word Recall Test; DSST, Digit Symbol Substitution Test; WFT, Word Fluency Test; Model 1: adjusted for age, sex, education; Model 2: covariates in Model 1 + *APOE* ε4 (at least 1 ε4 allele), smoking, body mass index, diabetes, hypertension, hypercholesterolemia; Model 3: covariates in Model 2 + white blood cell types

Table 3. Methylation age acceleration and 6-year cognitive change– ARIC Study (1990 - 1992) – (1996-1998)

		Model 1			Model 2			Model 3		
	N	β	SE	p	β	SE	p	β	SE	p
Cognitive Test										
DWRT										
Horvath age acceleration	1,401	0.014	0.009	0.105	0.015	0.009	0.078	0.017	0.009	0.059
Hannum age acceleration	1,401	0.004	0.009	0.614	0.006	0.009	0.494	0.008	0.009	0.384
DSST										
Horvath age acceleration	1,384	0.030	0.045	0.509	0.030	0.045	0.500	0.032	0.045	0.477
Hannum age acceleration	1,384	0.032	0.045	0.482	0.029	0.046	0.529	0.019	0.048	0.696
WFT										
Horvath age acceleration	1,394	-0.022	0.044	0.606	-0.021	0.044	0.628	-0.030	0.044	0.490
Hannum age acceleration	1,394	0.041	0.044	0.353	0.044	0.044	0.325	0.042	0.046	0.369

Note: N, number; β, beta coefficient; SE, standard error; DWRT, Delayed Word Recall Test; DSST, Digit Symbol Substitution Test; WFT, Word Fluency Test; Model 1: adjusted for age, sex, education; Model 2: covariates in Model 1 + *APOE* ε4 (at least 1 ε4 allele), smoking, body mass index, diabetes, hypertension, hypercholesterolemia; Model 3: covariates in Model 2 + white blood cell types