### **METHODOLOGY**





# Associations between five indicators of epigenetic age acceleration and all-cause and cause-specific mortality among US adults aged 50 years and older

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### Abstract

**Background** Although DNA methylation age estimators (DNAmAges) are reliable tools for predicting aging, their effectiveness in predicting mortality risk has not been fully validated. This study compared the predictive utility of five different DNAmAges (HorvathAge, HannumAge, PhenoAgeAge, GrimAge and GrimAge2) for all-cause and cause-specific mortality among adults aged ≥ 50 years.

**Methods** We screened 1966 participants adults aged ≥ 50 from the National Health and Nutrition Examination Survey (1999–2002) and linked them to the National Death Index to obtain cause and status of death. We used weighted Cox proportional hazards models to examine the associations between epigenetic age acceleration (EAA) measured by different DNAmAges and all-cause and cause-specific mortality in the general population, adjusting for various covariates including age, smoking status and chronic diseases. We used restricted cubic splines to explore nonlinear associations. Finally, stratified analyses were performed to assess the relationship between DNA age estimators and stratification variables.

**Results** The multivariable adjustment model showed that EAA measured by HorvathAge (AAHorvathAge), HannumAge (AAHannumAge), PhenoAge (AAPhenoAge), GrimAge (AAGrimAge) and GrimAge2 (AAGrimAge) were significantly associated with the risk of death, among which AAGrimAge and AAGrimAge2 had stronger statistical correlation and the correlation pattern was positively correlated. Specifically, each 5-year increase in AAGrimAge was associated with a 44% increased risk of all-cause death, a 33% increased risk of cardiovascular death and a 54% increased risk of non-cardiovascular death. And each 5-year increase in AAGrimAge2 was associated with a 40% increased risk of all-cause death, a 33% increased risk of cardiovascular death and a 47% increased risk of noncardiovascular death. In contrast, AAHorvathAge, AAHannumAge and AAPhenoAge showed a J-shaped correlation with the risk of all-cause mortality and non-cardiovascular mortality, with the inflection points of all-cause mortality and non-cardiovascular mortality occurring at AAHorvathAge of 2.29 and 2.8, AAHannumAge of 3.07 and 2.97,

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and AAPhenoAge of – 7.65 and 7.04, respectively. No interaction was found between DNAmAges and stratification variables.

**Conclusions** AAGrimAge and AAGrimAge2 outperformed AAHorvathAge, AAHannumAge and AAPhenoAge in predicting mortality risk, and the association pattern was positive.

**Keywords** Biological age, DNA methylation age estimator, Epigenetic age acceleration, All-cause mortality, Cause-specific mortality

### Background

Population aging has accelerated globally in recent years, with the population over 60 years old accounting for 11%, and it is expected to reach 22% by 2050 [1]. However, the extension of life expectancy does not mean a simultaneous increase in healthy life expectancy. What increases is mainly the number of years of survival with illness. Especially after the COVID-19 pandemic, the global life expectancy has dropped by 1.6 years, and the main causes of death have also shifted to chronic non-communicable diseases, such as ischemic heart disease, stroke and chronic obstructive pulmonary disease (COPD) [2, 3]. Aging, as the greatest risk factor for most common chronic diseases and causes of death, refers to the process in which the body loses the structure and function of organs, tissues and cells with chronological age (CA), ultimately leading to disease, disability and death. Therefore, it is necessary to explore biomarkers of aging that can accurately and efficiently quantify the functional state of human beings or organs and their changes with CA to identify individuals at risk of premature aging, which is crucial for developing interventions to slow down, stop or even reverse aging.

It is worth noting that aging is a complex process, and epigenetic alterations have been directly demonstrated to be a major driver of aging and age-related diseases, and remodeling the integrity of the epigenome can reverse aging [4, 5]. Therefore, scholars have integrated epigenetic alterations, clinical parameters and many types of omics data through algorithms to construct epigenetic clocks to evaluate aging and health status instead of CA [6–10]. DNA methylation (DNAm) is the most widely studied epigenetic phenomenon, and the DNAm age estimator (DNAmAge) constructed based on it is currently the most promising biomarker for predicting BA and is a hot topic in the field of aging [11]. However, given that these various kinds of DNAmAges use different sets of CpGs from different tissues and age profiles, the accuracy of their predictions of the age of the DNA source (e.g., cell, tissue or organ) and of age-related diseases varies accordingly. For example, the "first-generation" epigenetic clocks (HorvathAge, HannumAge) constructed based on physiological tissues were trained only for CA and are highly correlated with CA [12], while the "second-generation" epigenetic clocks (PhenoAge, GrimAge, GrimAge2) were specifically designed to predict BA and risk of death by testing them against a combination of clinical biomarkers and are therefore far superior to the "first generation" in predicting mortality, healthy life span or cardiovascular diseases [10, 13, 14].

Epigenetic age acceleration (EAA, the difference between the predicted epigenetic clock and CA, described below as the prefix AA) measured based on different DNAm patterns is associated with a variety of age-related diseases, including cancer, cardiovascular disease, diabetes and mental disorders, and may be associated with poor prognosis of the disease [15-21]. For example, a meta-analysis found that for every 5-year increase in AAHannumAge and AAHorvathAge, the population mortality risk would increase by 21% and 11%, respectively [19, 22]. A case-control study based on the ESTHER cohort showed that for every 5-year increase in AAHorvathAge, the risk of all-cause mortality would increase by 23%, the risk of cardiovascular mortality would increase by 19%, and the risk of cancer mortality would increase by 22%, but the association calculated using AAHannumAge was no longer statistically significant [23]. After eliminating confounding factors such as genetics and lifestyle, a cohort study of twins found that the risk of all-cause mortality increased by 35% for every 5-year increase in AAHorvathAge [24]. However, in another twin cohort study, it was found that for every 1 SD increase in AAGrimAge, the risk of death increased by 31%, while AAHorvathAge was not significantly associated with mortality [25]. These conflicting results highlight the need to systematically compare these different DNAmAge predictors of allcause and cause-specific mortality in older adults and to evaluate their patterns of association to identify the best predictors of cause-specific mortality.

Previous studies on DNAmAges and all-cause mortality have been extensive [13, 20, 23], but there is a lack of research on the strength of the association between specific cause mortality. To fill this gap, this study aims to verify the association between the first- and secondgeneration epigenetic clocks and all-cause mortality in people aged  $\geq$  50 years in the same cohort using data from the National Health and Nutrition Examination Survey (NHANES) from 1999 to 2002, and further explore the association with cause-specific mortality (such as cardiovascular disease, cancer and metabolic disease), in order to find the best predictive indicators for early identification of individuals at higher risk of death due to age-related diseases.

#### Methods

#### Study population

NHANES is a cross-sectional study that assesses the health and nutritional status of adults and children in the USA. Information on basic demographic information, socioeconomics, diet and health, medical treatment and physiological measurements is collected through interviews and physical examinations, and all participants signed written informed consent. We downloaded the dataset from 1999 to 2002 from the NHANES website (http://www.cdc.gov/nchs/nhanes.htm). Among the 21,004 participants, 19,038 were excluded because they were younger than 50 years old (n = 16,021), missing DNAm data (n = 2,451) or covariate data (n = 566). Finally, we included a total of 1966 eligible subjects to study the association between DNAmAges and all-cause mortality, cardiovascular mortality and non-cardiovascular mortality. The detailed screening process of the study population is shown in Fig. 1.

## DNAm measurement and DNAm epigenetic biomarker prediction

HorvathAge, HannumAge, PhenoAge, GrimAge and GrimAge2 were all adopted from DNA methylation data of NHANES. Specifically, the Duke University Institute of Molecular Physiology collected biological samples



Fig. 1 Study population screening flow diagram

from some adult participants aged 50 and above from 1999 to 2002 to analyze DNA methylation patterns and predict DNAm-derived epigenetic biomarkers (https:// wwwn.cdc.gov/nchs/data/nhanes/dnam/NHANES% 20DNAm%20Epigenetic%20Biomarkers%20Data% 20Documentation.pdf). Based on this, we calculated the corresponding EAA of the subjects by the difference between the predicted epigenetic clock and CA. The preprocessing and generation of all DNAm epigenetic biomarkers were completed using R language [version 4.3.2].

#### **Clinical endpoints**

The survival outcomes were all-cause and detailed causespecific mortality. Mortality data were linked to the CDC National Death Index (NDI) database (https://www.cdc. gov/nchs/data-linkage/mortality-public.htm) using a probabilistic matching algorithm to obtain information on participants'survival status and deaths as of December 31, 2019. Specific cause-of-death classification criteria in the NDI were defined and coded according to the International Statistical Classification of Diseases and Related Health Problems, Tenth Revision (ICD-10). The definition of all-cause mortality includes all types of deaths. Specific causes of death are categorized into cardiovascular causes of death (heart disease and cerebrovascular disease) and non-cardiovascular causes of death (including cancer, diabetes mellitus, respiratory disease, renal disease, Alzheimer's disease and deaths related to other causes).

#### Covariates

Age, sex, race, marital status, education level, povertyto-income ratio (PIR), body mass index (BMI), smoking status, alcohol use, physical activity and history of comorbidities were obtained from demographic and health questionnaires of the NHANES survey. Total cholesterol, albumin and eGFR levels were obtained from laboratory test results. Educational attainment was categorized as below high school, high school and above high school. Smoking status was determined by participants' responses to having smoked at least 100 cigarettes in their lifetime and whether they were currently smoking, categorizing participants as never smoker, former smoker and current smoker. Alcohol intake was categorized as nondrinking (< 12 drinks per year) and drinking ( $\geq 12$  drinks) based on participants' 24-h dietary recall. Physical activity status was categorized into inactive group (defined as not reporting leisure-time physical activity), active group (defined as meeting recommended physical activity levels, i.e., self-reported moderate leisure-time activity [metabolic equivalents of 3 to 6] 5 or more times per

week or vigorous leisure-time activity [metabolic equivalents of >6] 3 or more times per week) and insufficiently active group (defined as neither being inactive nor meeting recommended physical activity level criteria) [26].

Comorbidities included diabetes, renal disease, atherosclerotic cardiovascular disease (ASCVD), chronic heart failure (CHF), COPD and cancer. Diabetes mellitus was defined as a self-reported history of hyperglycemia, glycosylated hemoglobin A1c  $\geq$  6.5%, fasting blood glucose  $\geq$  126 mg/dL or using glucoselowering medications [27]. eGFR was calculated according to the Chronic Kidney Disease Epidemiology Collaboration 2021 (CKD-EPI) formula (the Chronic Kidney Disease Epidemiology Collaboration equation), with renal disease defined as eGFR < 60 mL/min [28]. ASCVD includes coronary artery disease and stroke. CHF and COPD are determined by physician diagnosis and prescription medications.

#### Statistical analysis

Participants were categorized into two groups by survival status to characterize the study population. Continuous variables were reported as mean ± standard deviation or median (interquartile range), with t-tests or Kruskal–Wallis tests for hypothesis testing based on distribution. Categorical variables were presented as counts and percentages, with Chi-square tests for hypothesis testing. Linear regression and Pearson's correlation assessed relationships between EAA, CA and other variables.

Cox proportional hazards models estimated HRs and 95% CIs for AAHorvathAge, AAHannumAge, AAPhenoAge, AAGrimAge and AAGrimAge2 in relation to mortality, adjusting for covariates like age, sex, race, marital status, education level, PIR, BMI, smoking status, alcohol status, physical activity, comorbidities and laboratory parameters. P-values for trends were calculated using quartile levels as ordinal variables. EAAs were analyzed as continuous variables to determine the effect size per 5-year increase, with exposure variables normalized to z-scores for per SD increase.

To account for nonlinear associations, inflection points were identified, and piecewise models were constructed. We performed stratified analyses according to age (< 65 years or  $\geq$  65 years), sex, race, smoking history, alcohol drinking history, physical activity, ASCVD and diabetes mellitus. Stratified analyses assessed potential effect modifications using Wald tests, with consistent adjustment levels across HR and 95% CI estimations for EAA and specific mortality causes. Statistical analyses were conducted using R version 4.3.2, with p < 0.05 indicating statistical significance.

#### Results

#### General characteristics of participants

A total of 1966 NHANES participants were included in the BA analysis; during a median follow-up of 208 months, 1014 participants died. Details of demographics, medical history, baseline laboratory test results, univariate analysis results and detailed cause-specific numbers and proportions of deaths are shown in Tables 1 and 2. It is worth noting that the EAA of deceased participants during follow-up was significantly lower than that of survivors (p < 0.05), but given that the baseline CA of the deceased group was significantly higher (mean 60.42 years vs. 71.10 years, p < 0.001), the relative EAA value would be higher than that of the survivors. Figure 2 shows the difference between CA and DNAmAges between survivors (red dots) and deceased participants (blue dots). Compared with the survivors, the deceased participants had higher DNAmAges, male proportion, smoking proportion and prevalence of diabetes, ASCVD, cancer, CHF and chronic kidney disease, but lower BMI, albumin and eGFR levels. The magnitude of the Pearson intercorrelations between the various DNAmAges is as follows: HorvathAge correlated 0.87, 0.85, 0.76, 0.76 and 0.72 with HannumAge, PhenoAge, GrimAge and Grim-Age2, respectively. HannumAge correlated 0.85, 0.78 and 0.76 with PhenoAge, GrimAge and GrimAge2, respectively. PhenoAgeAA correlated 0.79 with both GrimAge and GrimAge2, and GrimAge correlated 0.99 with Grim-Age2 (Fig. S1).

## Associations of EAA measured by DNAmAges with all-cause and cause-specific mortality

As shown in Fig. 3, after adjustment for potential confounders, we found that EAA measured by HorvathAge (AAHorvathAge), HannumAge (AAHannumAge), PhenoAge (AAPhenoAge), GrimAge (AAGrimAge) and GrimAge2 (AAGrimAge) was significantly associated with mortality risk when treated as continuous variables. Specifically, every 5-year increase in AAHorvathAge was associated with a 10% increased risk of all-cause mortality (P = 0.013) and a 3% increased risk of non-cardiovascular mortality (P = 0.005). For every 5-year increase in AAHannumAge, the risk of all-cause mortality and non-cardiovascular mortality increased by 17% (P < 0.001 and P = 0.002), and the risk of cardiovascular mortality increased by 24% (P= 0.015). Each 5-year increase in AAPhenoAge increased the risk of all-cause death by 13% and the risk of non-cardiovascular death by 18% (P <0.001). Each 5-year increase in AAGrimAge was associated with a 44% increased risk of all-cause death (P <

 Table 1
 Baseline characteristics of participants stratified by survival status

Characteristic	Surviving participants (n=952)	Dead participants (n=1014)	<i>p</i> value	
Age, years Biological age	60.42 ± 7.23	71.10±9.16	< 0.001	
HorvathAge, years	62.33 ± 7.03	70.97 ± 8.96	< 0.001	
HannumAge, years	62.02 ± 7.51	$71.52 \pm 9.19$	< 0.001	
PhenoAge, years	50.12 ± 8.24	$60.62 \pm 10.35$	< 0.001	
GrimAge, years	60.97 + 6.53	70.91 + 7.77	< 0.001	
GrimAge2, years	66.83 + 6.61	76.62 + 7.58	< 0.001	
Epigenetic age acceleration				
AAHorvathAge	1.91 ± 5.00	$-0.13 \pm 6.65$	< 0.001	
AAHannumAge	1.60 ± 5.05	$0.42 \pm 6.36$	< 0.001	
AAPhenoAge	$-10.30 \pm 6.06$	$-10.48 \pm 7.85$	0.559	
AAGrimAge	0.55 ± 4.98	$-0.19 \pm 5.74$	0.003	
AAGrimAge2	6.41 ± 5.46	5.52 ± 6.31	< 0.001	
PIR	2.94 (1.38–5.00)	1.84 (1.11–3.32)	< 0.001	
BMI, kg/m <sup>2</sup>	29.02 ± 5.65	$28.44 \pm 5.95$	0.029	
Male, n (%)	457 (48.00)	552 (54.44)	0.004	
Race, n (%)			< 0.001	
Non-Hispanic White	368 (38.66)	472 (46.55)		
Non-Hispanic Black	168 (17 65)	225 (22 19)		
Hispanic	376 (39.50)	292 (28.80)		
Other	40 (4 20)	25 (2 47)		
Education n (%)	10 (1120)	20 (2.07)	< 0.001	
Below high school	350 (36.76)	501 (49.41)		
High school	185 (1943)	228 (22 49)		
Above high school	417 (43.80)	285 (28.11)		
Marital status n (%)	117 (19.00)	203 (20.11)	< 0.001	
Married/cobabiting	670 (70 38)	605 (59 66)	0.001	
Separated/divorced/ widowed	244 (25.63)	369 (36.39)		
Never married/unknown	38 (3.99)	40 (3.94)		
Smoking status, n (%)			0.008	
Never smoker	471 (49.47)	435 (42.90)		
Former smoker	361 (37.92)	418 (41.22)		
Current smoker	120 (12.61)	161 (15.88)		
Drinking status, n (%)			0.03	
Nondrinking	627 (65.86)	620 (61.14)		
Drinking	325 (34.14)	394 (38.86)		
Physical activity, n (%)			< 0.001	
Inactive	436 (45.80)	601 (59.27)		
Insufficient	203 (21.32)	154 (15.19)		
Active	313 (32.88)	259 (25.54)		
Total cholesterol, mmol/L	5.47 ±0.99	$5.40 \pm 1.08$	0.174	
Albumin, g/L	42.96 ± 2.89	42.40 ± 3.06	< 0.001	
eGFR, mL/min	91.66 ± 16.34	79.44 ± 22.02	< 0.001	
Diabetes mellitus. n (%)	165 (17.33)	293 (28.90)	< 0.001	
ASCVD, n (%)	64 (6.72)	174 (17.16)	< 0.001	
CHF, n (%)	15 (1.58)	90 (8.88)	< 0.001	
COPD, n (%)	139 (14.60)	168 (16.57)	0.23	

#### Table 1 (continued)

Characteristic	Surviving participants (n=952)	Dead participants (n=1014)	<i>p</i> value	
Cancer, n (%)	90 (9.45)	184 (18.15)	< 0.001	
Chronic renal disease (eGFR < 60 mL/min), n (%)	39 (4.10)	199 (19.63)	< 0.001	

The data was shown as mean (standard deviation) or median (interquartile range) for continuous variables, n (%) for categorical variables

N, number of subjects; %, weighted percentage

*PIR* poverty income ratio, *BMI* body mass index, *eGFR* estimated glomerular filtration rate, *ASCVD* atherosclerotic cardiovascular disease, *CHF* chronic heart failure, *COPD* chronic obstructive pulmonary disease

 Table 2
 Number and proportion of detailed cause-specific deaths among participants

Characteristic	Frequency (n)	Percentage (%)		
Cardiovascular				
Heart diseases	265	26.13		
Cerebrovascular diseases	51	5.03		
Non-cardiovascular				
Cancer	209	20.61		
Diabetes mellitus	51	5.03		
Respiratory disease	76	7.50		
Renal disease	21	2.07		
Alzheimer disease	48	4.73		
All other causes	293	28.9		

0.001), a 33% increased risk of cardiovascular death (P= 0.019) and a 54% increased risk of non-cardiovascular death (P< 0.001). Every 5-year increase in AAGrimAge2 was associated with a 40% increased risk of all-cause mortality (P< 0.001), a 33% increased risk of cardiovascular death (P= 0.003) and a 47% increased risk of non-cardiovascular death (P< 0.001). When used as categorical variables, EAA measured by DNAmAges also showed a stepwise increasing association with the risk of all-cause and non-cardiovascular mortality (P-value for trend < 0.05), but in terms of cardiovascular mortality risk, only AAGrimAge and AAGrimAge2 were found to show an increasing trend with the risk of cardiovascular mortality (P-value for trend = 0.026 and P-value for trend = 0.006).

#### The detection of nonlinear relationships

On continuous scales, AAHorvathAge, AAHannumAge and AAPhenoAge showed a J-shaped correlation with the risk of all-cause mortality and non-cardiovascular mortality, with the inflection points of all-cause and noncardiovascular mortality occurring at AAHorvathAge of 2.29 and 2.8, AAHannumAge of 3.07 and 2.97, and AAPhenoAge of -7.65 and 7.04, respectively. The risk of death remained almost constant before the inflection



Fig. 2 Scatterplots and linear regression lines of biological age according to chronological age. A AAHorvathAge, B AAHannumAge, C AAPhenoAge, D AAGrimAge, and E AAGrimAge2

point and then increased sharply. AAHannumAge was linearly associated with the risk of cardiovascular death (*P* for nonlinear =0.149). More importantly, we found that AAGrimAge and AAGrimAge2 were positively associated with all-cause mortality, cardiovascular mortality and non-cardiovascular mortality(*P* for nonlinear >0.05), which means that as AAGrimAge and AAGrim-Age2 increase, i.e., the positive EAA, the risks of allcause, cardiovascular and non-cardiovascular mortality also increase, whereas as AAGrimAge and AAGrim-Age2 decrease, i.e., negative EAA, the risk of all-cause, cardiovascular and non-cardiovascular mortality also decreases. The results of the restricted cubic spline (RCS) are presented in Fig. 4, respectively.

#### Stratified analyses

As shown in Fig. 5, to further evaluate the impact of EAA measured by DNAmAges on outcome indicators, subgroup analyses were performed. Stratified by age, sex, race, smoking history, alcohol drinking history, physical activity, ASCVD and diabetes, there were no significant interactions among subgroups. These subgroup analyzes highlight the consistency and generalizability of the association between these DNAmAges and mortality risk across different populations.

## Association between EAA measured by DNAmAges and cause-specific mortality subcategories

We then used a multivariate-adjusted Cox proportional hazards model to compare the predictive ability of different DNAmAges for cause-specific mortality (Fig. 6). The results showed that for every 5-year increase in AAGrim-Age and AAGrimAge2, the risk of death from heart disease increased by 44% and 43%, respectively. Every 5-year increase in AAHannumAge was associated with an 85% increase in the risk of death from cerebrovascular disease. Among non-cardiovascular causes of death, for every 5-year increase in AAHorvathAge, AAHannum-Age, AAPhenoAge, AAGrimAge and AAGrimAge2, the risk of death from respiratory disease increased by 37%, 76%, 74%, 137% and 132%, respectively. For every 5-year increase in AAHannumAge, AAPhenoAge, AAGrim-Age and AAGrimAge2, the risk of death from renal disease increased by 59%, 99%, 85% and 94%, respectively; for every 5-year increase in AAHannumAge and AAGrimAge, the risk of death from cancer increased by 23% and 31%, respectively. Additionally, AAPhenoAge, AAGrimAge and AAGrimAge2 were also associated with increased hazard ratios for death from all other residual causes.

А	All-cause	mortality		Cardiovascular mortality			Non-cardiovascular mortality				
AAHorvathAge	e HR (95% CI)		P value	AAHorvathAg	je HR (95% CI)		P value	AAHorvathAge	e HR (95% CI)		P value
Per 5 years	1.10(1.02 - 1.18)	HEH	0.013	Per 5 years	1.07(0.93 - 1.22)	H <b>I</b> II	0.3	Per 5 years	1.13(1.04 - 1.23)	HEH	0.005
Per SD	1.12(1.02 - 1.22)	<b></b>	0.013	Per SD	1.08(0.92 - 1.27)	⊢∎I	0.3	Per SD	1.16(1.04 - 1.28)	⊢∎⊣	0.005
Quartile 1	Reference			Quartile 1	Reference			Quartile 1	Reference		
Quartile 2	0.97(0.78 - 1.21)	-	0.033*	Quartile 2	0.86(0.62 - 1.19)	⊢∎∔□	0.4*	Quartile 2	1.01(0.75 - 1.36) +	- <b>+</b>	0.034*
Quartile 3	1.05(0.83 - 1.33)			Quartile 3	1.30(0.86 - 1.96)	· <b>-</b>		Quartile 3	0.97(0.76 - 1.23)	- <b>-</b>	
Quartile 4	1.27(0.99 - 1.64)		-	Quartile 4	1.10(0.65 - 1.88)	·	-	Quartile 4	1.36(1.03 - 1.82)	<b>⊢</b> ∎	
B	0.7	5 1.00 1.25 1.50				1.0 1.5	2.0		0.7	51.001.251.50	1.75
AAHannumAge	e HR (95% CI)		P value	AAHannumAge	e HR (95% CI)		P value	AAHannumAge	e HR (95% CI)		P value
Per 5 years	1.17(1.09 - 1.27)	H <b>H</b> H	<0.001	Per 5 years	1.24(1.04 - 1.48)	H	0.015	Per 5 years	1.17(1.06 - 1.30)	HEH	0.002
Per SD	1.20(1.10 - 1.31)	HEH	<0.001	Per SD	1.28(1.05 - 1.57)	H=H	0.015	Per SD	1.20(1.07 - 1.35)	HEH	0.002
Quartile 1	Reference			Quartile 1	Reference			Quartile 1	Reference		
Quartile 2	1.17(0.98 - 1.40)		<0.001*	Quartile 2	1.57(1.01 - 2.42)		0.084*	Quartile 2	0.94(0.73 - 1.21)	-∎	<0.001*
Quartile 3	1.24(1.01 - 1.51)			Quartile 3	1.07(0.62 - 1.84)			Quartile 3	1.28(0.99 - 1.64)	┝╼╌┥	
Quartile 4	1.82(1.49 - 2.24)	<b>—</b>	-	Quartile 4	1.99(1.13 - 3.50)	<b>-</b>		Quartile 4	1.78(1.36 - 2.34)	<b></b>	
~	•	1.2 1.6 2.0				1 2 3				1.0 1.5 2.0	D
C											
AAPhenoAge	HR (95% CI)		P value	AAPhenoAge	HR (95% CI)		P value	AAPhenoAge	HR (95% CI)		P value
Per 5 years	1.13(1.05 - 1.20)	H	<0.001	Per 5 years	1.03(0.94 - 1.12)	H	0.6	Per 5 years	1.18(1.08 - 1.28)	HEH	<0.001
Per SD	1.18(1.07 - 1.30)	HEH	<0.001	Per SD	1.04(0.91 - 1.18)	H <b>-</b> 4	0.6	Per SD	1.26(1.12 - 1.42)	HEH	<0.001
Quartile 1	Reference			Quartile 1	Reference			Quartile 1	Reference		
Quartile 2	1.00(0.85 - 1.18)	H H	0.012*	Quartile 2	0.93(0.63 - 1.37)		0.5*	Quartile 2	1.05(0.82 - 1.36)	· •	0.005*
Quartile 3	0.90(0.71 - 1.14) ⊢	•		Quartile 3	0.82(0.58 - 1.17)	⊢∎∔≉		Quartile 3	0.95(0.66 - 1.37)		
Quartile 4	1.56(1.20 - 2.02)		-	Quartile 4	1.26(0.79 - 2.01)			Quartile 4	1.72(1.26 - 2.36)	┥┝┯╋┯	
D		1.0 1.5 2	.0			1.0 1.5	2.0			1.0 1.5 2.0	)
AAGrimAge	HR (95% CI)		P value	AAGrimAge	e HR (95% CI)		P value	AAGrimAge	HR (95% CI)		P value
Per 5 years	1.44(1.29 - 1.60)	HEH	<0.001	Per 5 years	1.33(1.05 - 1.68)	H	0.019	Per 5 years	1.54(1.32 - 1.79)	HEH	<0.001
Per SD	1.48(1.31 - 1.66)	H	<0.001	Per SD	1.36(1.05 - 1.75)	⊨ <b>-</b>	0.019	Per SD	1.59(1.35 - 1.87)	нен	<0.001
Quartile 1	Reference			Quartile 1	Reference			Quartile 1	Reference		
Quartile 2	1.41(1.12 - 1.76)	H <b></b>	<0.001*	Quartile 2	1.21(0.80 - 1.83)	H=1	0.024*	Quartile 2	1.59(1.21 - 2.10)	⊢∎⊸i	<0.001*
Quartile 3	1.83(1.48 - 2.25)	⊢∎		Quartile 3	1.62(1.03 - 2.54)			Quartile 3	2.05(1.55 - 2.70)	⊢∎→	
Quartile 4	2.22(1.71 - 2.88)	<b>—</b>	-	Quartile 4	1.91(1.06 - 3.45)			Quartile 4	2.58(1.77 - 3.76)		
F	1.	.0 1.5 2.0 2.5				1.0 1.5 2.0 2.5 3.	0 3.5			1 2 3	
AAGrimAge2	HR (95% CI)		P value	AAGrimAge	2 HR (95% CI)		P value	AAGrimAge	2 HR (95% CI)		P value
Per 5 years	1 40(1 27 - 1 54)		<0.001	Per 5 years	1 33(1 10 - 1 60)	<b>H</b>	0.003	Per 5 years	1 47(1 28 - 1 69)	HEH	<0.001
Per SD	1 49(1 32 - 1 67)		<0.001	Per SD	1 40(1 12 - 1 75)	н	0.003	Per SD	1 58(1 34 - 1 86)		<0.001
Quartile 1	Reference		-0.001	Quartile 1	Reference		0.005	Quartile 1	Reference	. <b>.</b> .	-0.001
Quartile ?	1 27(0 99 - 1 64)		<0.001*	Quartile ?	1 03(0 68 - 1 57)		0.006*	Quartile ?	1 48(1 09 - 2 01)		<0.001*
Quartile 3	1.27(0.33 - 1.04)		-0.001	Quartilo 3	1.00(0.00 - 1.07) 1.22(0.77 - 1.95)	<u> </u>	0.000	Quartile 3	1.32(1.33 - 2.01)		-0.001
Quartile 4	251(1.87 - 3.39)		-	Quartile 4	2 46(1 43 - 4 21)		_	Quartile 4	2.72(1.33 - 2.30)		
	2.51(1.67 - 0.36)	.0 1.5 2.0 2.5 3.0	·		2.40(1.40 - 4.21)	1 2 3	4		2.72(1.72 - 4.30)		4

Fig. 3 Cox proportional hazards regression analyses for the association between EAA and all-cause and cause-specific mortality. **A** AAHorvathAge, **B** AAHannumAge, **C** AAPhenoAge, **D** AAGrimAge, and **E** AAGrimAge2. Adjusted for age, sex, race, marital status, education level, PIR, BMI, smoking status, alcohol status, physical activity, diabetes, renal disease, ASCVD, CHF, COPD, cancer, total cholesterol, albumin and eGFR. \**P* value for trend. Effect sizes for per 5-year and per SD increase in EAA were also shown separately. *EAA* epigenetic age acceleration, *PIR* poverty income ratio, *BMI* body mass index, *ASCVD* atherosclerotic cardiovascular disease, *CHF* chronic heart failure, *COPD* chronic obstructive pulmonary disease, *eGFR* estimated glomerular filtration rate

#### Sensitivity analyses

Sensitivity analyses showed that the results remained stable when individuals with less than 2 years of follow-up were excluded (Fig. S2). A weakening pattern of association between DNAmAges and mortality was observed in individuals younger than 65 years, but not in those older than 65 years (Figs. S3-S4). The association between DNAmAges and mortality tended to be stronger in never smokers, while it was attenuated in former smokers and even lost statistical significance in current smokers (Figs. S5-S6). The weakening pattern of association was also observed when analyzing men and women separately (Figs. S7-S9). In addition, the results remained stable when individuals with severe comorbidities, including ASCVD, cancer, CHF and chronic kidney disease, were excluded (Figure S10).

#### Discussion

This study investigated the association of different DNAmAges measures with all-cause mortality in community-dwelling patients aged  $\geq$  50 years in the USA. In this cohort study of 1966 subjects from the NHANES study, we found that those with higher EAA in the elderly population had an increased risk of mortality, as both continuous and categorical variables, by multivariate Cox regression and RCS analysis, which is generally consistent with previous findings. More importantly, we found that AAGrimAge and AAGrimAge2 outperformed AAHorvathAge, AAHannumAge and AAPhenoAge in predicting the risk of death. In our study, GrimAge and GrimAge2 were positively associated with allcause, cardiovascular and non-cardiovascular death, among which AAGrimAge2 had a stronger statistical correlation. Specifically, every 5-year increase in AAGrimAge2 was associated with a 40% increased risk of all-cause mortality, a 33% increased risk of cardiovascular mortality and a 47% increased risk of noncardiovascular mortality. In contrast, this study found that the association pattern between AAHorvathAge, AAHannumAge and AAPhenoAge and all-cause and non-cardiovascular death was J-shaped, suggesting that these DNAmAges have the ability to predict mortality risk only when they rise above a certain threshold. In terms of predicting the risk of cardiovascular mortality, AAHannumAge was positively correlated with it, but no significant correlation was found between AAHorvathAge and AAPhenoAge and the risk of cardiovascular death. This is partly different from the results of previous studies, which may due to the small sample size and population heterogeneity that make these results inconsistent. Therefore, more participants from different regions and ethnicities are needed to verify our findings.

The predictive properties of EAA for cancer, diabetes and metabolic syndrome, neurodegenerative diseases and even infections and psychiatric disorders have also been reported [9, 29-32]. Our analysis provides new evidence for EAA in predicting cause-specific mortality in the aging population. Further analysis of more detailed causes of death subcategories revealed that EAA was significantly associated with deaths from heart disease, cerebrovascular disease, cancer, respiratory disease, kidney disease, etc. This can be explained by the pathophysiological mechanisms of aging, where aging hallmarks such as genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, disabled macroautophagy, deregulated nutrient-sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, altered intercellular communication, chronic inflammation and dysbiosis interact with each other, leading to chronic inflammation of cells and tissues, thereby promoting pathological damage to organs and inducing cancer [4, 33-35]. However, the predictive ability of various EAA measurements for different causes of death varies. Specifically, in terms of cardiovascular disease, both AAGrimAge and AAGrimAge2 may significantly predict heart disease mortality, while AAHannumAge significantly predicts the risk of death due to cerebrovascular disease. For non-cardiovascular causes of death, AAGrimAge and AAGrimAge2 may be the best predictors of respiratory disease mortality, while AAPhenoAge, AAGrimAge and AAGrimAge2 may be the best predictors of renal disease mortality. In addition, there was a statistically significant association between AAHannumAge and AAGrimAge and cancer mortality. AAPhenoAge, AAGrimAge and AAGrimAge2 were also associated with an increased hazard ratio for death from all other residual causes. These differences in predictive ability between different causes of death may be explained by the different algorithms used to construct different DNAmAges. In conclusion, these results might help clinicians select DNAmAges assessment tools that have the potential to more accurately predict mortality risk based on known clinical risk factors.

(See figure on next page.)

**Fig. 4** Restricted cubic spline curve for the association between EAA and all-cause and cause-specific mortality. **A** AAHorvathAge, **B** AAHannumAge, **C** AAPhenoAge, **D** AAGrimAge, and **E** AAGrimAge2. Solid lines represent hazard ratios, and shadows represent corresponding 95% confidence intervals. Adjusted for age, sex, race, marital status, education level, PIR, BMI, smoking status, alcohol status, physical activity, diabetes, renal disease, ASCVD, CHF, COPD, cancer, total cholesterol, albumin and eGFR. The shaded areas in the background show the distribution of EAA measured by DNAmAges in the population. Two-piece Cox proportional hazards models were used to estimate the risk inflection point, and effect sizes for per 1-year increase in EAA before and after the inflection point were shown separately



Fig. 4 (See legend on previous page.)

#### A. AAHorvathAge

	All-cause	mortality		Cardiovascular mortality			Non-cardiovascular mortality				
Characteristic 	Herefore Control (1997)		P for interaction 0.284 0.383 0.583 0.362 0.568 0.271 0.415 0.165	Characteristic «Söysaras Mala Female Remain Non-Hispanic White Non-Hispanic Black Hispanic Hispanic Remer smoker Former smoker Romer smoker Romer smoker Nordinking Insufficient Active Insufficient Active Non-ASCO Non-ASCO Non-Astates melitus	H H (49%C) 47 (138 - 1.02) 47 (138 - 1.02) 47 (138 - 1.02) 47 (138 - 1.02) 48 (1.03 - 1.02) 49 (108 - 1.02) 49 (108 - 1.02) 49 (108 - 1.02) 49 (108 - 1.03) 49 (108 - 1.03) 40 (108 -		2 for interaction 0.822 0.656 0.773 0.463 0.463 0.18 0.846 0.846 0.469 0.469	Characteristic <pre>doi:10.10.10.10.10.10.10.10.10.10.10.10.10.1</pre>	$\begin{array}{c} H8(856C)\\ H8(1657-107)\\ L6(10, 357-107)\\ L6(10, 357-107)\\ L0(10, 357-103)\\ L1(10, 35$	+ + + + + + + + + + + + + + + + + + +	P for interaction 0.251 0.755 0.62 0.475 0.621 0.33 0.217 0.435
Characteristic <65 years	HR(95%CI) 1.04 (1.01 - 1.06)	<b></b> -	P for interaction 0.133	Characteristic <65 years	HR(95%CI) 1.03 (0.98 - 1.09)	-	o for interaction 0.903	Characteristic <65 years	HR(95%CI) 1.04 (1.00 - 1.07)	<b>H4</b> -1	P for interaction 0.125
265 years Male Fenale Non-Hispanic Black Hispanic Black Other race Never smoker Condriving Inactive Insufficient Active Non-ASCVD Non-ASCVD Non-ABCVD Non-ABCVD Non-ABCVD	$\begin{array}{c} 100 (0.99 + 1.02) \\ 1.01 (0.99 + 1.02) \\ 1.02 (0.99 + 1.04) \\ 1.02 (1.09 + 1.04) \\ 1.03 (1.00 - 1.03) \\ 1.00 (0.98 + 1.03) \\ 1.00 (0.98 + 1.03) \\ 1.01 (0.98 + 1.03) \\ 1.01 (0.98 + 1.03) \\ 1.01 (0.98 + 1.03) \\ 1.01 (0.98 + 1.04) \\ 1.01 (0.98 + 1.04) \\ 1.01 (0.98 + 1.04) \\ 1.01 (0.98 + 1.04) \\ 1.01 (0.98 + 1.04) \\ 1.01 (0.98 + 1.04) \\ 1.01 (0.98 + 1.04) \\ 1.01 (0.98 + 1.04) \\ 1.01 (0.98 + 1.04) \\ 1.01 (0.98 + 1.04) \\ 1.01 (0.98 + 1.04) \\ 1.01 (0.98 + 1.04) \\ 1.01 (0.98 + 1.04) \\ 1.01 (0.98 + 1.05) \\ 1.0$		0.689 0.672 0.824 0.492 0.462 0.717 0.386	265 years Male Fernale Non-Haparic Ulback Non-Kington Other race Never smoker Former smoker Commonities Nondrinking Inschee Insufficient Non-ASCVD Non-ASCVD Non-Ascevitius Dabates melitus	$\begin{array}{c} 102 (0.86 + 1.06) \\ 112 (0.86 + 1.06) \\ 122 (0.86 + 1.07) \\ 100 (0.85 + 1.07) \\ 100 (0.85 + 1.06) \\ 100 (0.85 + 1.06) \\ 100 (0.85 + 1.06) \\ 100 (0.85 + 1.06) \\ 100 (0.85 + 1.06) \\ 100 (0.85 + 1.06) \\ 100 (0.85 + 1.06) \\ 100 (0.86 + 1.06) \\ 100 (0.86 + 1.06) \\ 100 (0.86 + 1.06) \\ 100 (0.86 + 1.06) \\ 100 (0.86 + 1.06) \\ 100 (0.86 + 1.16) \\ 110 (0.86 + 1.16) \\ 100 (0.86 + 1.16) \\$		0.645 0.407 0.254 0.238 0.869 0.485 0.44	265 years Male Fernále Non-Hispanic What Non-Kispanic Other race Never smoker Former smoker Comercial Non-driking Inactive Inactive Inactive Non-ASCVD ASCVD Non-dabetes melitus Dabetes melitus	$\begin{array}{c} 100 \\ 1088 \\ -100 \\ 1088 \\ -100 \\ -10$		0.488 0.737 0.562 0.799 0.51 0.911 0.2
C. AAPheno	oAge	0.95 1.00 1.05 1.10		0111-1	0.4 0	16 0.8 1.0		Oterestedetie		10 11 12 13	D (as istantia
di years di years Male Non-Hispanic Black Non-Hispanic Black Hispanic Black Hispanic Black Hispanic Black Non-dispanic Non-dispanic Pomer smoker Current smoker Current smoker Current smoker Current smoker Non-disbetes mellitus Diabetes mellitus	1 00 (0.89 - 105) 1 00 (0.89 - 102) 1 01 (0.89 - 102) 1 01 (0.89 - 102) 1 01 (0.89 - 103) 1 01 (0.89 - 104) 1 00 (0.87 - 104) 1 01 (0.89 - 103) 1 01 (0.89 - 102) 1 00 (0.89 -	+	0.128 0.502 0.521 0.477 0.297 0.282 0.478 0.723	- d5 years 265 years Male Non-Hispanic Write Non-Hispanic Black Hispanic Black Hispanic Black Hispanic Black Horizer smoker Current smoker Current smoker Current smoker Current smoker Insufficient Active Insufficient Active Diabetes melitus	$\begin{array}{c} 1 & 02 & 037^{-1} & 108) \\ 0.89 & 0.97^{-1} & 108) \\ 0.89 & 0.97^{-1} & 1091 \\ 1.07 & 0.88 & 1.165 \\ 0.97 & 0.88 & 0.896 \\ 1.02 & 0.896 & 0.896 \\ 1.02 & 0.896 & 1.046 \\ 1.01 & 0.97 & 1.049 \\ 0.01 & 0.97 & 1.049 \\ 0.88 & 0.048 & 1.049 \\ 1.02 & 0.88 & 0.166 \\ 0.88 & 0.048 & 1.070 \\ 0.88 & 0.048 & 1.070 \\ 0.88 & 0.048 & 1.070 \\ 0.88 & 0.048 & 1.070 \\ 0.88 & 0.048 & 1.070 \\ 0.88 & 0.048 & 1.070 \\ 0.98 & 0.088 & 1.020 \\ 0.98 & 0.088 & 0.080 \\ 0.98 & 0.080 & 0.080 \\ 0.98 & 0.080 & 0.080 \\ 0.98 & 0.080 & 0.080 \\ 0.98 & 0.080 & 0.080 \\ 0.98 & 0.080 & 0.080 \\ 0.98 & 0.080 & 0.080 \\ 0.98 & 0.080 & 0.080 \\ 0.98 & 0.080 & 0.080 \\ 0.98 & 0.080 & 0.080 \\ 0.98 & 0.080 & 0.080 \\ 0.98 & 0.0$		0.191 0.139 0.367 0.406 0.253 0.331 0.878 0.981	- d5 years 265 years Mate Non-Hispanic Write Non-Hispanic Black Hispanic Stack Current smoker Current smoker Current smoker Current smoker Current smoker Current smoker Current School Insufficient Active Insufficient Active Diabetes melitus	$\begin{array}{c} 1.22 \left( 288 - 1.68 \right) \\ 1.02 \left( 1.00 - 1.64 \right) \\ 1.02 \left( 1.00 - 1.64 \right) \\ 1.02 \left( 1.00 - 1.65 \right) \\ 1.01 \left( 1.03 - 1.65 \right) \\ 1.01 \left( 1.03 - 1.65 \right) \\ 1.01 \left( 1.03 - 1.65 \right) \\ 1.02 \left( 1.03 - 1.65 \right) \\ 1.01 \left( 1.05 - $		0.413 0.779 0.402 0.989 0.081 0.744 0.686 0.767
Characteristic	HR(95%CI)	1	P for interaction	Characteristic	HR(95%CI)	-	o for interaction	Characteristic	HR(95%CI)		P for interaction
So years and the set of the se	$\begin{array}{c} 1 & 0.0 & (1.90) - 1 & 0.0 \\ 1 & 0.0 & (1.00) - 1 & 0.07 \\ 1 & 0.0 & (1.00) - 1 & 0.07 \\ 1 & 0.0 & (1.00) - 1 & 0.07 \\ 1 & 0.0 & (1.00) - 1 & 0.07 \\ 1 & 0.0 & (1.00) - 1 & 0.07 \\ 1 & 0.0 & (1.00) - 1 & 0.07 \\ 1 & 0.0 & (1.00) - 1 & 0.07 \\ 1 & 0.0 & (1.00) - 1 & 0.07 \\ 1 & 0.0 & (1.00) - 1 & 0.07 \\ 1 & 0.0 & (1.00) - 1 & 0.07 \\ 1 & 0.0 & (1.00) - 1 & 0.07 \\ 1 & 0.0 & (1.00) - 1 & 0.07 \\ 1 & 0.0 & (1.00) - 1 & 0.07 \\ 1 & 0.0 & (1.00) - 1 & 0.07 \\ 1 & 0.0 & (1.00) - 1 & 0.07 \\ 1 & 0.0 & (1.00) - 1 & 0.07 \\ 1 & 0.0 & (1.00) - 1 & 0.07 \\ 1 & 0.0 & (1.00) - 1 & 0.08 \\ 1 & 0.0 & (1.00) - 0.08 \\ 1 & 0.0 & (1.00) - 0.08 \\ 1 & 0.0 & (1.00) - 0.08 \\ 1 & 0.0 & (1.00) - 0.08 \\ 1 & 0.0 & (1.00) - 0.08 \\ 1 & 0.0 & (1.00) - 0.08 \\ 1 & 0.0 & (1.00) - 0.08 \\ 1 & 0.0 & (1.00) - 0.08 \\ 1 & 0.0 & (1.00) - 0.08 \\ 1 & 0.0 & (1.00) - 0.08 \\ 1 & 0.0 & (1.00) - 0.08 \\ 1 & 0.0 & (1.00) - 0.08 \\ 1 & 0.0 & (1.00) - 0.08 \\ 1 & 0.0 & (1.00) - 0.08 \\ 1 & 0.0 & (1.00) - 0.08 \\ 1 & 0.0 & (1.00) - 0.08 \\ 1 & 0.0 & (1.00) - 0.08 \\ 1 & 0.0 & (1.00) - 0.08 \\ 1 & 0.0 & (1.00) - 0.08 \\ 1 & 0.0 & (1.00)$		0.329 0.347 0.573 0.286 0.084 0.843 0.783 0.783 0.473	do years book of the second second second Female Non-Hispanic Black Helpsnic Black Helpsnic Black Non-King Tomer smoker Current smoker Current smoker Current smoker Current smoker Current smoker Current smoker Current smoker Non-diabetes melitus Diabates melitus	1 01 (0.85 - 1.07) 1 01 (0.85 - 1.07) 1 04 (0.85 - 1.06) 0.86 (0.82 - 1.05) 1 04 (0.85 - 1.06) 1 04 (0.85 - 1.07) 0 16 (0.15 - 0.23) 1 04 (0.15 - 0.23) 1 04 (0.15 - 0.23) 1 04 (0.15 - 0.23) 1 04 (0.15 - 1.17) 1 04 (		0.289 0.429 0.384 0.638 0.164 0.501 0.479 0.162	dob years book with the Female Non-Hispanic Black Hispanic Black Hispanic Black Non-King Diraking Diraking Institute the Non-disbetes melitus Diabates melitus	$\begin{array}{c} 1.06 \\ 1.00 \\ - 1.06 \\ 1.00 \\ - 1.06 \\ 1.00 \\ - 1.06 \\ 1.00 \\ - 1.06 \\ - 1.00$		0.67 0.4 0.609 0.581 0.158 0.619 0.384 0.06
E. AAGrimA			P for interaction	Charactorietic			9 for interaction	Characteristic	HP(05%(CI)		P for interaction
del yvaris     del yvaris     del yvaris     del yvaris     Male     Female     Female     Non-Hispanic White     Mispanic     Other nace     Never smoker     Comer smoker     Comer smoker     Insufficient     Active     Non-ASCVD     ASCVD     ASCVD	$\begin{array}{c} 1 & 0.10 \\ \hline 0$	╘┱╪┿┿ <sup>┷</sup> ┿╽╈ <sup>╋</sup> ╢┿┿ <sup>┿</sup> ┿┿	0.272 0.366 0.51 0.493 0.083 0.807 0.631 0.533	des years ads years Male Nor-Fernale White Nor-Fernale Black Herein Black Herein Black Nord State Current smoker Current smoker Nordistret	$\begin{array}{c} 1 & 22 & (216)^{-5} - 1.06) \\ 1 & 10 & (10.97^{-1} - 1.06) \\ 1 & 10 & (10.97^{-1} - 1.06) \\ 1 & 22 & (0.97^{-1} - 1.06) \\ 1 & 20 & (0.97^{-1} - 1.04) \\ 1 & 40 & (0.98^{-1} - 1.16) \\ 1 & 40 & (0.98^{-1} - 1.16) \\ 1 & 40 & (0.98^{-1} - 1.16) \\ 1 & 40 & (0.98^{-1} - 1.16) \\ 1 & 40 & (0.98^{-1} - 1.16) \\ 1 & 40 & (0.98^{-1} - 1.06)$		0.213 0.289 0.342 0.89 0.241 0.595 0.635 0.305	des varant zes yvers Male Nor-Fernále Witte Nor-Fispanic Black Hor-Fispanic Black Hor-Fispanic Black Other race Nord marker Current smoker Current smoker Nondrinking Drinking Inactive te Non-ScVD ASCVD ASCVD	$\begin{array}{c} 1.03 \pm 0.03 \pm 0.01 \ \text{M}{\odot} \\ 1.03 \pm 1.00 \pm 1.05 \ M$	╡┿┿┿╪ <sub>╋╏</sub> ╋╞┿┿┿╪ ┥	0.685 0.505 0.619 0.747 0.14 0.618 0.413 0.112
Diabetes mellitus	1.00 (0.97 - 1.04)	09 10 11		Diabetes mellitus	0.95 (0.88 - 1.02)	06 08 10		Diabetes mellitus	1.03 (1.00 - 1.07)	0.7 0.8 0.9 10 1.1	

Fig. 5 Stratified analyses of the associations between EAA and mortality. A AAHorvathAge, B AAHannumAge, C AAPhenoAge, D AAGrimAge, and E AAGrimAge2. Adjusted for age, sex, race, marital status, education level, PIR, BMI, smoking status, alcohol status, physical activity, diabetes, renal disease, ASCVD, CHF, COPD, cancer, total cholesterol, albumin and eGFR, except the subgroup factors themselves

To our knowledge, this is the first study to compare the association between EAA measured by different DNAmAges (HorvathAge, HannumAge, PhenoAge, GrimAge and GrimAge2) and all-cause and specific mortality in a large cohort, and the current findings may have important clinical implications. First, our study showed that the five most studied DNAmAges may predict all-cause mortality well, especially AAGrimAge and AAGrimAge2. Therefore, the detection of BA in the general population has the potential to identify high-risk individuals as early as possible and initiate therapeutic interventions, which is particularly important for global

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А	Characteristics	HR (95% CI)	P value	HR (95% CI)	P value		-
	Cardiovascular						-
	Heart diseases	1.08(0.95 - 1.23)	0.23	1.10(0.90 - 1.28)	0.24		
	Cerebrovascular diseases	1.00(0.75 - 1.34)	0.9	1.00(0.71 - 1.42)	0.99		
	Non cardiovascular						
	Cancer	1.16(0.96 - 1.39)	0.12	1.19(0.95 - 1.49)	0.12		Per 5 years Per SD
	Diabetes mellitus	1.09(0.73 - 1.63)	0.66	1.11(0.69 - 1.80)	0.66		
	Respiratory disease	1.37(1.01 - 1.85)	0.044	1.45(1.01 - 2.09)	0.044		
	Renal disease	1.03(0.83 - 1.29)	0.77	1.04(0.80 - 1.36)	0.77		
	Alzheimer disease	1.14(0.86 - 1.52)	0.35	1.17(0.84 - 1.65)	0.35		
	All other causes	1.22(0.93 - 1.59)	0.15	1.27(0.92 - 1.74)	0.15		-
В	Characteristics	HR (95% CI)	P value	HR (95% CI)	P value	1.0 1.5 2.0	_
	Cardiovascular	(		(			-
	Heart diseases	1.16(0.96 - 1.40)	0.12	1.19(0.96 - 1.48)	0.12	<b>.</b>	
	Cerebrovascular diseases	1.85(1.24 - 2.76)	0.003	2.04(1.28 - 3.24)	0.003		
	Non cardiovascular						
	Cancer	1.23(1.00 - 1.52)	0.047	1.28(1.00 - 1.62)	0.047	<b>.</b>	Per 5 years
	Diabetes mellitus	1.22(0.91 - 1.64)	0.18	1.27(0.90 - 1.78)	0.18		L Fei SD
	Respiratory disease	1.76(1.20 - 2.59)	0.004	1.93(1.24 - 3.01)	0.004		
	Renal disease	1.59(1.11 - 2.29)	0.012	1.71(1.13 - 2.61)	0.012		
	Alzheimer disease	1.26(0.92 - 1.73)	0.16	1.31(0.90 - 1.89)	0.16		
	All other causes	1.13(0.88 - 1.47)	0.32	1.16(0.86 - 1.56)	0.32		
C						1.0 1.5 2.0 2.5 3.0	-
Č	Characteristics	HR (95% CI)	P value	HR (95% CI)	P value		_
	Cardiovascular					1	
	Heart diseases	1.03(0.92 - 1.16)	0.57	1.05(0.89 - 1.23)	0.57	. <b>5</b> .	
	Cerebrovascular diseases	0.96(0.74 - 1.23)	0.74	0.94(0.67 - 1.34)	0.74	184	
	Non cardiovascular					1	Bor E veere
	Cancer	1.05(0.89 - 1.22)	0.58	1.06(0.85 - 1.33)	0.58	Ē.	Per SD
	Diabetes mellitus	1.20(0.84 - 1.70)	0.31	1.29(0.79 - 2.12)	0.31	Ĥ <u>₽</u>	
	Respiratory disease	1.74(1.28 - 2.37)	<0.001	2.19(1.42 - 3.36)	<0.001		
	Renal disease	1.99(1.61 - 2.46)	<0.001	2.63(1.95 - 3.55)	<0.001		1
	Alzheimer disease	1.16(0.88 - 1.52)	0.3	1.23(0.83 - 1.80)	0.3		
	All other causes	1.28(1.05 - 1.57)	0.015	1.42(1.07 - 1.89)	0.015		-
D	Characteristics	HR (95% CI)	P value	HR (95% CI)	P value	1 2 5	-
	Cardiovascular	(		(			-
	Heart diseases	1.44(1.14 - 1.83)	0.002	1.49(1.15 - 1.92)	0.002	1 <b>7</b> 1	
	Cerebrovascular diseases	0.68(0.39 - 1.18)	0.17	0.66(0.36 - 1.20)	0.17		
	Non cardiovascular						
	Cancer	1.31(1.01 - 1.70)	0.04	1.34(1.01 - 1.77)	0.04	<b>1</b>	Per 5 years
	Diabetes mellitus	1.38(0.91 - 2.07)	0.13	1.41(0.91 - 2.19)	0.13		
	Respiratory disease	2.37(1.42 - 3.97)	0.001	2.54(1.46 - 4.42)	0.001		
	Renal disease	1.85(1.45 - 2.37)	<0.001	1.94(1.49 - 2.54)	<0.001		
	Alzheimer disease	1.26(0.65 - 2.44)	0.48	1.29(0.63 - 2.62)	0.48		
	All other causes	1.64(1.16 - 2.33)	0.005	1.71(1.17 - 2.49)	0.005		
F						1 2 3 4	_
-	Characteristics	HR (95% CI)	P value	HR (95% CI)	P value		_
	Cardiovascular						
	Heart diseases	1.43(1.19 - 1.71)	<0.001	1.53(1.23 - 1.89)	<0.001		
	Cerebrovascular diseases	0.74(0.43 - 1.26)	0.27	0.70(0.37 - 1.32)	0.27	Ē	
	Non cardiovascular					L.	B Dor F
	Cancer	1.24(0.97 - 1.57)	0.081	1.29(0.97 - 1.71)	0.081	<u>F</u> .	Per 5 years Per SD
	Diabetes mellitus	1.42(0.89 - 2.28)	0.15	1.52(0.87 - 2.66)	0.15		
	Respiratory disease	2.32(1.32 - 4.09)	0.004	2.71(1.39 - 5.32)	0.004		ı
	Renal disease	1.94(1.50 - 2.50)	<0.001	2.19(1.62 - 2.96)	<0.001	176-1	
	Alzheimer disease	1.24(0.71 - 2.19)	0.45	1.30(0.66 - 2.53)	0.45		
	All other causes	1.51(1.12 - 2.04)	0.008	1.63(1.14 - 2.33)	0.008		-
						1 2 3 4 5	

Fig. 6 Cox proportional hazards regression analyses for the association between EAA and cause-specific mortality subcategories. Adjusted for age, sex, race, marital status, education level, PIR, BMI, smoking status, alcohol status, physical activity, diabetes, renal disease, ASCVD, CHF, COPD, cancer, total cholesterol, albumin and eGFR

healthy aging practice. Second, EAA plays an important role in predicting mortality risk, and the specific cause of mortality risk of the general population should be stratified by selecting DNAmAges that may more accurately assess the corresponding cause of mortality risk, so as to facilitate personalized monitoring and management. Finally, given the dynamic and changeable nature of epigenetics, monitoring the dynamic changes of EAA provides a promising approach for treating age-related decline and diseases. Therefore, these DNAmAges are worthy of promotion and application in clinical practice.

The strengths and limitations of this study include the following. First, this study included multiple biological aging indicators, and the blood samples for detecting these indicators were all tested using standardized protocols within the same period, which greatly reduced potential bias. Second, we used a large national database and a prospective cohort study with a long follow-up period, long-term follow-up with a low loss rate and sufficient endpoint events to help evaluate the relationship between different DNAmAges and all-cause, cardiovascular and non-cardiovascular mortality in the general population. However, our study is not without limitations. First, due to the limitations of a crosssectional study, DNAmAges data were only detected at baseline, and the dynamic changes in EAA during follow-up were not detected to evaluate the trajectory of biological aging, nor could its causal relationship with different causes of death be determined. Second, our study population was the general population of the USA aged 50 years or older, and our conclusions are limited in their generalizability to other populations. Future studies should expand the sample size and include groups of different regions, races and age groups to verify the differences in the prediction of mortality risk of different DNAmAges. Despite these limitations, our results are still clinically important because we have demonstrated the association of different DNAmAges with all-cause mortality and specific mortality risks.

#### Conclusions

This study compared the mortality prediction effect of EAA measured by different DNAmAges (HorvathAge, HannumAge, PhenoAge, GrimAge and GrimAge2) in NHANES participants. The results showed that AAGrimAge and AAGrimAge2 were positively correlated with all-cause mortality and cause-specific mortality in the general population. They are reliable screening tools for predicting mortality risk and identifying high-risk individuals, and are of great significance for early risk stratification and formulation of intervention strategies for the elderly population.

#### Abbreviations

Chronic obstructive pulmonary disease
Chronological age
DNA methylation
DNAm age estimator
Biological age
National Health and Nutrition Examination Survey
Epigenetic age acceleration
National Death Index
Poverty-to-income ratio
Body mass index
Chronic heart failure
Atherosclerotic cardiovascular disease
Estimated glomerular filtration rate
Chronic Kidney Disease Epidemiology Collaboration 2021
Restricted cubic spline
EAA measured by HorvathAge
EAA measured by HannumAge
EAA measured by PhenoAge
EAA measured by GrimAge
EAA measured by GrimAge2

#### Supplementary Information

The online version contains supplementary material available at https://doi. org/10.1186/s13148-025-01872-6.

Additional file1

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#### Author contributions

J.X. and X.T. proposed and designed the study. Y.Z. and J.H. extracted data and performed corresponding analyses. Y.Z. and J.H. participated in the writing of the manuscript.

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#### Availability of data and materials

The datasets analyzed in our study were downloaded from the NHANES database, and the datasets used during the current study are available in the NHANES repository (https://www.cdc.gov/nchs/nhanes/).

#### Declarations

#### Ethics approval and consent to participate

The National Center for Health Statistics and Ethics Review Board approved the protocol for NHANES, and all participants provided written informed consent.

#### **Consent for publication**

All authors have reviewed and approved the final version of the manuscript.

#### Competing interest

The authors declare no competing interests.

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