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Comparative validation of an epigenetic mortality risk score with three aging biomarkers for predicting mortality risks among older adult males

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Abstract

Background: A 'mortality risk score' (MS) based on ten prominent mortality-related cytosine-phosphate-guanine (CpG) sites was previously associated with all-cause mortality, but has not been verified externally. We aimed to validate the association of MS with mortality and to compare MS with three aging biomarkers: telomere length (TL), DNA methylation age (DNAmAge) and phenotypic age (DNAmPhenoAge) to explore whether MS can serve as a reliable measure of biological aging and mortality.

Methods: Among 534 males aged 55–85 years from the US Normative Aging Study, the MS, DNAmAge and DNAmPhenoAge were derived from blood DNA methylation profiles from the Illumina HumanMethylation450 BeadChip, and TL was measured by quantitative real-time polymerase chain reaction (qRT-PCR).

Results: A total of 147 participants died during a median follow-up of 9.4 years. The MS showed strong associations with all-cause, cardiovascular disease (CVD) and cancer mortality. After controlling for all potential covariates, participants with high MS (>5 CpG sites with aberrant methylation) had almost 4-fold all-cause mortality (hazard ratio: 3.84, 95% confidence interval: 1.92–7.67) compared with participants with a low MS (0–1 CpG

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site with aberrant methylation). Similar patterns were observed with respect to CVD and cancer mortality. MS was associated with TL and DNAmPhenoAge acceleration but not with DNAmAge acceleration. Although the MS and DNAmPhenoAge acceleration were independently associated with all-cause mortality, the former exhibited a higher predictive accuracy of mortality than the latter.

Conclusions: MS has the potential to be a prominent predictor of mortality that could enhance survival prediction in clinical settings.

Key words: Mortality risk score, DNA methylation, biological aging, mortality, epigenetic epidemiology

Key Messages

- An epigenetic mortality risk score (MS) was previously associated with mortality; our study verified and compared it with three aging biomarkers in an independent cohort for the first time.
- MS was highly associated with all-cause, cardiovascular disease and cancer mortality during a median follow-up of 9.4 years among 534 males.
- MS was associated with telomere length and the acceleration of DNA methylation phenotypic age (DNAmPhenoAge), but not with the acceleration of DNA methylation age.
- MS and DNAmPhenoAge acceleration were independently associated with all-cause mortality, and the former exhibited a higher predictive accuracy of mortality than the latter.
- MS has the potential to be an informative biomarker for survival prediction.

Introduction

As the global population ages and has increasing life expectancy, healthy aging is becoming an increasingly relevant public health challenge. Identifying indicators that measure aging and predict age-related mortality is a major emerging topic of biomedical research. Biologically, aging is associated with the gradual accumulation of a wide variety of molecular and cellular changes over time. Telomere length (TL), which varies among individuals and gradually shortens by 50–100 base pairs (bp) with each cell division,[1] is associated with a number of aging-related diseases, including cardiovascular disease (CVD), diabetes, Alzheimer's disease, cancers and disease-specific mortality,[2–5] and therefore has been increasingly recognized as one of the most prominent biomarkers of biological aging.

Another popular indicator of biological aging is DNA methylation age (DNAmAge), proposed by Horvath in 2013.[6] DNAmAge was highly correlated with chronological age and may estimate the biological age of a tissue, cell or organ based on the methylation profiles of multiple cytosine-phosphate-guanine (CpG) sites across the genome. DNAmAge acceleration, i.e. the discrepancy between DNAmAge and chronological age, is heritable and has been brought forward as a new index of accelerated biological aging. DNAmAge acceleration has been linked to lifestyle factors, environmental hazards and stressful life events, as well as mortality.[7–14] Most recently, Horvath and his colleagues also proposed another biomarker named 'phenotypic age (DNAmPhenoAge)' based on 513 CpG sites that show associations with age-related phenotypes. The discrepancy between DNAmPhenoAge and chronological age is defined as the DNAmPhenoAge acceleration, and is a marker of risks for an array of diverse outcomes across multiple tissues and cells. Indeed, it may provide novel insights into important pathways of biological aging.[15,16]

DNA methylation of CpG sites other than age-related loci has also been found to be strongly associated with mortality.[17] In a recent epigenome-wide association study (EWAS), based on approximately 1900 older adults followed for 14 years of the ESTHER study and a validation study among 1727 participants of the KORA study, Zhang *et al.* found a total of 58 CpG sites within 19 chromosomes that were associated with all-cause mortality.[18] The author further constructed a 'mortality risk score' (MS) based on the ten most robustly mortality-related loci, which was a strong and informative predictor of all-cause, CVD and cancer mortality. While the identified CpG sites were mapped in genes known to be related to various diseases, MS linked the DNA methylation profile seen in common disease-related genes with mortality. Even though three recent studies showed strong independent associations

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between MS and mortality, along with frailty, telomere length and vitamin D deficiency,[19–21] potential overestimation of the predictive power of this MS may exist since the findings were all based on the studies (ESTHER and KORA) in the German population.

MS has yet to be validated as a predictive measure of mortality in an independent cohort. To fill this gap, we sought to validate the association between the 58 previously identified mortality-related CpG sites, MS and mortality. We then assessed and compared the individual and joint associations between MS, TL, DNAmAge acceleration, DNAmPhenoAge acceleration and mortality over the course of a median follow-up of 9.4 years. This investigation was carried out through the Normative Aging Study (NAS), which is an all-male cohort of older veterans living in the Greater Boston area.

Methods

Study design and population

The NAS is an ongoing longitudinal study on aging established by the US Department of Veterans Affairs in 1963. Details of the study have been published previously.[22] Briefly, the NAS is a closed cohort of 2280 male veterans from the Greater Boston area. They were enrolled after an initial health screening to determine that they were free of known chronic medical conditions. DNA from blood samples was collected from 657 participants, most of whom were examined up to four times between 1999 and 2013. Participants have been re-evaluated every 3–5 years on a continuous rolling basis using detailed on-site physical examinations and questionnaires. We restricted the current analysis to data from the first visit of 534 Caucasian participants (aged 55–85 years) with available DNA methylation profiles to control for the heterogeneity of race. The NAS was approved by the Department of Veterans Affairs Boston Healthcare System and written informed consent was obtained from each subject before participation.

Data collection

As previously described,[23] participants were asked to provide detailed information about their lifestyles, dietary habits, activity levels and demographic factors. Height and weight were used to calculate body mass index (BMI, in kg/m^2). Blood samples were collected to assess blood-based biomarkers. Major diseases were assessed based on participants' medical history and prior diagnoses.[24] Regular mailings to study participants have been used to acquire vital-status information and official death certificates were obtained from the appropriate state health department to be reviewed by a physician; cause of death was coded by an experienced research nurse using ICD-9. Participant deaths and causes of death are routinely updated by the research team and the last available update was on 31 December 2013.

DNA methylation data

As previously described,[25,26] we used the QIAamp DNA Blood Kit (Qiagen, CA, USA) to extract DNA from buffy coat and performed bisulfite conversion with the EZ-96 DNA Methylation Kit (Zymo Research, CA, USA). To minimize batch effects, we randomized chips across plates and randomized samples based on a two-stage age-stratified algorithm so that age distributed similarly across chips and plates. We measured DNA methylation of CpG probes using the Illumina HumanMethylation450 BeadChip. After quality control, the remaining samples were preprocessed using the Illumina-type background correction, dye-bias adjustment and BMIQ normalization[27] to generate methylation status. The methylation status of a specific CpG site was quantified as a β value ranging from 0 (no methylation) to 1 (full methylation).

Estimations of mortality risk score, DNA methylation age and phenotypic age

We retrieved a total of 58 mortality-related CpG sites reported by Zhang *et al.* from whole epigenome data.[18] As described by the author, three forms of MS (ordinal, risk level and continuous) were further built based on ten CpG sites (cg01612140, cg05575921, cg06126421, cg08362785, cg10321156, cg14975410, cg19572487, cg23665802, cg24704287 and cg25983901). Values within the 4th quartile of cg08362785 (positively correlated with mortality) and within the 1st quartile of the other nine loci (negatively correlated with mortality) were defined as 'aberrant' methylation for each CpG site.[18] The ordinal MS was determined as the cumulative number of aberrantly methylated CpG sites (0–10) and the participants were further classified into three risk levels: low, MS = 0–1; moderate, MS = 2–5; and high, MS >5. Continuous MS was constructed as the sum of the methylation β values multiplied by the regression coefficients of each of the ten CpG sites for all-cause mortality derived from the least absolute shrinkage and selection operator (LASSO) regression in Zhang *et al.*'s study.[18] Ordinal MS ranged from 0 to 10, and continuous MS ranged from –4 to 0 (Supplementary Figure S1, available as Supplementary data at *IJE* online).

A total of 353 CpG sites were retrieved from the methylation profiles for the estimation of DNAmAge for each participant based on the algorithm proposed by Horvath.[6] This algorithm was derived from a range of tissues and cell

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types using 353 probes targeted in the Illumina 27 K and 450 K methylation arrays. In this study, we performed the estimation using an online calculator (http://labs.genetics.ucla.edu/horvath/dnamage/),[6] and used the basic Horvath DNAmAge estimated by the 353 loci in our study. DNAmAge acceleration was determined as discrepancies between DNAmAge and chronological age in the form of residuals, which had a mean of 0 and represented positive and negative deviations from chronological age in years. The residuals were calculated by a linear regression procedure in which methylation age was the outcome and chronological age was the independent variable.

Another batch of 513 CpG sites was retrieved for the estimation of DNAmPhenoAge for each participant based on the algorithm proposed by Levine *et al.*[15] With the coefficient and intercept values provided by the authors, we estimated the DNAmPhenoAge as:

$$\text{DNAmPhenoAge} = CpG1_1 \times \beta_1 + CpG_2 \times \beta_2 + \cdots CpG_{513} \times \beta_{513} + \text{intercept}$$

The difference between phenotypic and chronological age (DNAmPhenoAge – chronological age) was defined as the DNAmPhenoAge acceleration.

Telomere length

As previously described,[28] TL was measured by quantitative real-time polymerase chain reaction (qRT-PCR). Relative TL was measured by determining the ratio of the telomere repeat (T) copy number to a single-copy gene (S) copy number (T/S) in a given sample. Human beta-globin was used as the single copy gene. To control for batch effects of plate, TL was calculated as relative units, which was the ratio between TL in the test DNA and TL in a DNA pool used to generate a standard curve in each PCR. The standard pool consisted of DNA from participants randomly selected from the NAS (50 ng per sample) and was used in each run to create a standard curve, which ranged from 20 to 0.25 ng/μL of pooled DNA. An eight-point standard curve ranging from 30 to 0.234 ng/μL and derived from serially diluted pooled DNA was included in each PCR plate so that relative quantities of T and S could be determined. We ran all samples in triplicate and the average of three T measurements was divided by the average of three S measurements to calculate the average T/S.

Statistical analysis

Descriptive statistics were used to summarize sociodemographics, lifestyle factors and distributions of TL, DNAmAge and DNAmPhenoAge at baseline of all 534 participants and subsets based on the risk levels of MS.

We then evaluated the associations of 58 loci identified by Zhang *et al.* with all-cause mortality in NAS during a median follow-up of 9.4 years. Three multivariate Cox regression models were used to test their associations by increasingly controlling for potential confounding factors and treating the batch of DNA methylation measurement as the random effect. Model 1 adjusted for age (years) and the leukocyte distribution estimated by the Houseman algorithm.[29] Model 2 additionally adjusted for smoking status (current/former/never smoker), alcohol consumption (abstainer/low/intermediate/high), BMI [underweight or normal weight (<25.0)/overweight (≥25.0 to <30.0)/obese (≥30.0)], physical activity [metabolic equivalent of task (MET), low (≤12 kcal/kg hours/week), median (12–30 kcal/kg hours/week), high (≥30 kcal/kg hours/week)] and education (≤12 years, 13–16 years, >16 years). Model 3 additionally adjusted for total cholesterol (mg/dL), high-density lipoprotein (HDL, mg/dL), triglycerides (mg/dL), systolic blood pressure (mm Hg), hypertension, stroke, coronary heart disease (CHD), diabetes and cancer (yes/no). After the correction of multiple testing by false discovery rate [(FDR), Benjamini-Hochberg method[30]] we identified CpG sites with a FDR <0.05 as mortality-related CpG sites. The same three Cox models were used to evaluate the association between the MS (ordinal/risk level/continuous) and all-cause, CVD and cancer mortality.

We further employed three linear mixed models to test the associations between MS, TL, DNAmAge acceleration and DNAmPhenoAge acceleration, in which the batch of DNA methylation measurement was treated as the random effect. Model 1 adjusted for age and leukocyte distribution. Model 2 additionally adjusted for smoking status, alcohol consumption, BMI, physical activity and education. Model 3 additionally adjusted for total cholesterol, HDL, triglycerides, systolic blood pressure, hypertension, stroke, CHD, diabetes and cancer.

Finally, we examined the associations of TL, DNAmAge acceleration and DNAmPhenoAge acceleration with all-cause, CVD and cancer mortality using multivariate Cox regression models that adjusted for all the potential covariates as described in the previous survival analyses for MS. The leukocyte distribution was adjusted for in models with epigenetic-based indicators. In addition to models including a single biomarker as the predictor, we also evaluated their independent associations with mortality in models that mutually controlled for the ordinal MS and one of the aging biomarkers. The dose–response curves of identified mortality-related biomarkers with all-cause mortality were assessed by restricted cubic spline regression using the SAS macro from Desquilbet and Mariotti.[31] All models for dose–response analyses were adjusted for the covariates described above. The 25th, 50th and 75th

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percentiles were selected as knots for the continuous markers and MS = 2, 5 and 8 were selected as the knots for the ordinal MS. Harrell's and Uno's C statistics were employed to evaluate the predictive abilities of the identified mortality-related indicators and their combinations[32,33] and potential overestimation by fitting to our data was corrected using bootstrap analysis with 1000 replications to quantify the degradation in model predictive accuracy. Final estimates were the averages and standard deviations (SDs) of the results from each bootstrap sample. The corresponding receiver operating characteristic curves (ROC) were derived by logistic regression and showed areas under the curve (AUC) that were very similar to Harrell's and Uno's C statistics for the Cox model.

All analyses were performed by SAS version 9.4 (SAS Institute Inc., Cary, NC, USA), and all statistical tests were two-sided with *P*-values of <0.05.

Results

Participant characteristics

Table 1 shows the baseline characteristics and distributions of three aging biomarkers, TL, DNAmAge and DNAmPhenoAge, among the total population of 534 participants and the subsets based on the risk levels of MS. Overall, the average age at the baseline was about 72 years and the high MS group had a higher average age than the other two subsets. Of the participants, >60% were former smokers and <5% were current smokers; the high MS group (MS > 5) had the lowest proportion of never smokers. The majority of participants were overweight or obese, consumed no or low amounts of alcohol, reported a low level of physical activity and had <16 years of education. The high MS group had the highest proportion of people with hypertension, CVD and cancer at baseline. Figure 1 shows the distributions of the three aging biomarkers. Men with a high MS had shorter TL, higher DNAmAge and DNAmPhenoAge accelerations (Table 1). During a follow-up of 9.4 years, 147 (27.5%), 85 (15.9%) and 47 (8.8%) of participants died from any cause, CVD and cancer, respectively.

Validations of 58 mortality-related CpG sites and the mortality risk score

We first tested the associations of the 58 CpG sites previously identified by Zhang *et al.* with all-cause mortality (Table 2 and Supplementary Table S1, available as Supplementary data at *IJE* online). Fourteen of the 58 loci showed strong associations with all-cause mortality after controlling for all potential covariates and multiple testing. Hazard ratios (HRs) of the 58 CpG sites for a SD decrease in methylation levels in our study were highly correlated with those described by Zhang *et al.* (Pearson correlation coefficient =0.68, *P*-value <0.0001). Among the 14 loci (Table 2), the demethylation of cg08362785 (*MKL1*) and cg23842572 (*MPRIP*) showed negative correlations with mortality. HRs and 95% confidence intervals (CIs) for a decrease in methylation levels by one SD were 0.62 (0.46–0.83) and 0.65 (0.50–0.86), respectively. Demethylation of the remaining 12 loci showed positive associations with mortality, with HRs (95% CIs) ranging from 1.28 (1.06–1.54) to 1.73 (1.14–2.63) per SD decrease in methylation levels. Five of the ten loci used to construct the MS were also verified in NAS participants (cg01612140, cg05575921, cg06126421, cg08362785 and cg23665802). The five loci along with three other CpG sites (cg14975410, cg19572487 and cg24704287) were smoking-related loci reported by previous studies.[34,35]

Associations of the MS with all-cause and disease-specific mortality are demonstrated in Table 3. After controlling for age and technical covariates (Model 1), the MS showed strong associations with deaths by any cause, CVD and cancer. Additional adjustment for other potential covariates did not alter the pattern in any relevant manner. In the fully-adjusted model (Model 3), a unit increase of ordinal and continuous MS was associated with a 1.25-fold (95% CI: 1.13–1.38) and 2.53-fold (95% CI: 1.43–4.47) increase in all-cause mortality, respectively. In the categorical analysis of the MS risk level, HRs (95% CIs) for moderate and high-risk groups were 1.38 (0.86–2.19) and 3.84 (1.92–7.67), respectively, compared with the low-risk group (MS = 0–1). Similar patterns were observed with respect to CVD and cancer mortality.

Subgroup analyses were additionally performed by smoking status (Supplementary Table S2, available as Supplementary data at *IJE* online). As the self-reported smoking status is the subject of underreporting or other information bias, and cg05575921 can objectively distinguish cigarette consumption,[36] we performed the analysis in four subgroups, group 1: ever smokers (current or former smokers, $n = 361$), group 2: never smokers ($n = 173$), group 3: never smokers who had a cg05575921 level >1st quartile of never smokers ($n = 119$) and group 4: never smokers who had a cg05575921 level ≥0.75 ($n = 156$). The distribution of cg05575921 based on smoking status is provided in Supplementary Figure S2, available as Supplementary data at *IJE* online. All forms of MS mostly demonstrated increasing patterns with all-cause, CVD and cancer mortality as identified from the total population, albeit with larger CIs due to the limited number of deaths. In particular, the ordinal MS still had robust associations

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Table 1. Characteristics of participants from the Normative Aging Study at baseline[a]

Characteristics	$n_{Total} = 534$	Subsets based on risk levels of mortality risk score			
		0–1 / Low ($n = 238$)	2–5 / Moderate ($n = 251$)	>5 / High ($n = 45$)	*P*-value
Age (years)	71.6 (6.5)	70.5 (6.3)	72.2 (6.6)	73.2 (6.1)	0.003
Total cholesterol (mg/dL)	198.9 (36.9)	202.7 (38.4)	197.2 (35.6)	188.9 (34.3)	0.04
Serum triglyceride (mg/dL)	138.9 (85.6)	139.6 (96.3)	137.8 (75.6)	141.8 (78.8)	0.95
HDL cholesterol (mg/dL)	49.8 (13.5)	49.7 (13.0)	49.8 (13.8)	50.0 (14.9)	0.99
Systolic blood pressure (mm Hg)	131.4 (17.3)	132.4 (16.2)	131.3 (17.5)	127.2 (21.3)	0.18
Smoking status					0.0004
Current smoker	19 (3.6%)	0 (0%)	16 (6.4%)	3 (6.7%)	
Former smoker	342 (64.0%)	149 (62.6%)	160 (63.7%)	33 (73.3%)	
Never smoker	173 (32.4%)	89 (37.4)	75 (29.9%)	9 (20.0%)	
Body mass index (kg/m^2)					0.61
Underweight or normal weight (<25.0)	102 (19.1%)	51 (21.4%)	45 (17.9%)	6 (13.3%)	
Overweight (≥25.0 to <30.0)	292 (54.7%)	124 (52.1%)	143 (57.0%)	25 (55.6%)	
Obese (≥30.0)	140 (26.2%)	63 (26.5%)	63 (25.1%)	14 (31.1%)	
Alcohol consumption[b]					0.39
Abstainer	112 (22.5%)	54 (24.4%)	50 (21.5%)	8 (18.6%)	
Low (0 to <40 g/d)	349 (70.2%)	157 (71.0%)	161 (69.1%)	31 (72.1%)	
Intermediate (40 to <60 g/d)	24 (4.8%)	8 (3.6%)	13 (5.5%)	3 (7.0%)	
High (≥60 g/d)	12 (2.4%)	2 (1.0%)	9 (3.9%)	1 (2.3%)	
Physical activity (MET-hours/week)[c]					0.02
Low (≤12 kcal/kg hours/week)	312 (61.1%)	122 (54.2%)	156 (64.5%)	34 (77.3%)	
Median (12–30 kcal/kg hours/week)	128 (25.0%)	65 (28.9%)	54 (22.3%)	9 (20.5%)	
High (≥30 kcal/kg hours/week)	71 (13.9%)	38 (16.9%)	32 (13.2%)	1 (2.2%)	
Years of education[d]					0.86
≤12 years	227 (44.3%)	95 (42.2%)	109 (45.1%)	23 (51.1%)	
13–16 years	217 (42.4%)	99 (44.0%)	101 (41.7%)	17 (37.8%)	
>16 years	68 (13.3%)	31 (13.8%)	32 (13.2%)	5 (11.1%)	
Major diseases					
Hypertension	365 (68.4%)	151 (63.5%)	180 (71.7%)	34 (75.6%)	0.08
Stroke	32 (6.0%)	11 (4.6%)	15 (6.0%)	6 (13.3%)	0.08
Coronary heart disease (CHD)	141 (26.4%)	51 (21.4%)	73 (29.1%)	17 (37.8%)	0.03
Diabetes	66 (12.4%)	29 (12.2%)	31 (12.4%)	6 (13.3%)	0.98
Cancer	267 (50.0%)	117 (49.2%)	120 (47.8%)	30 (66.7%)	0.06
Telomere length (T/S)	1.28 (0.48)	1.31 (0.51)	1.28 (0.47)	1.16 (0.38)	0.15
DNAmAge (Horvath, years)	72.6 (6.7)	71.9 (6.6)	72.9 (6.5)	74.8 (7.4)	0.02
DNAmAge acceleration (Horvath, years)	0.21 (5.2)	0.14 (4.9)	0.06 (5.2)	1.39 (6.0)	0.27
DNAmPhenoAge (years)	66.5 (8.1)	64.3 (8.0)	67.5 (7.8)	71.7 (6.8)	<0.0001
DNAmPhenoAge acceleration (years)	–5.0 (6.2)	–5.9 (5.9)	–4.8 (6.1)	–1.6 (6.8)	0.0002

[a]Mean values (standard deviation) for continuous variables and *n* (%) for categorical variables; differences among risk levels of mortality risk score were tested for statistical significance by Kruskal-Wallis test (continuous variables) and Chi-square test (categorical variables).

[b]Data missing for 37 participants.

[c]Data missing for 23 participants.

[d]Data missing for 22 participants.

with all-cause and disease-specific mortality, but its HRs attenuated in groups 2–4.

Figure 2a and b further depicts the dose–response relationships of the ordinal and continuous MS with all-cause mortality after controlling for all potential covariates in the total population. All-cause mortality robustly increased for ordinal MS >2 (Figure 2a) and one SD increase of continuous MS was roughly associated with a 2-fold increase in the risk of all-cause mortality (Figure 2b).

Additionally, two CpG sites, cg06126421 and cg23665802, are not covered by the Illumina EPIC methylation array. We tested the associations between a revised MS [ordinal (0–8) and continuous] without the two loci and all-cause, CVD and cancer mortality as a sensitivity analysis (Supplementary

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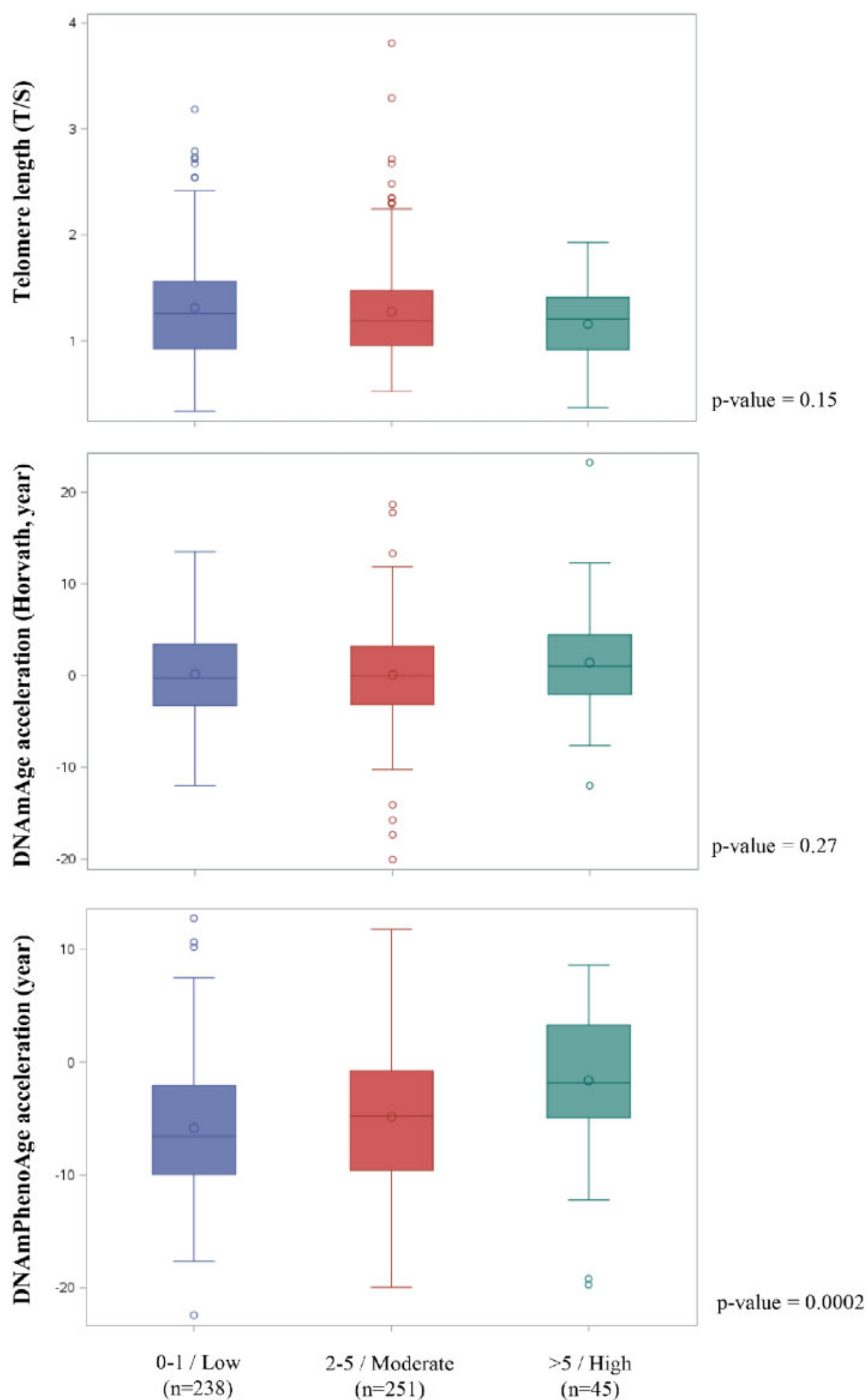

Figure 1. Distributions of telomere length, DNAmAge acceleration and DNAmPhenoAge acceleration based on the mortality risk score.

Table S3, available as Supplementary data at *IJE* online). This revised indicator still showed positive associations with the mortality after fully controlling for potential covariates, but the corresponding HRs of both forms of this revised MS were attenuated and the CIs were wider than the original.

Associations of mortality risk score with telomere length, DNAmAge and DNAmPhenoAge

Next, we evaluated the associations of the MS with three aging biomarkers: TL, DNAmAge acceleration and DNAmPhenoAge acceleration (Table 4, Supplementary

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Table 2. Fourteen previously reported CpG sites that were associated with all-cause mortality in NAS

CpG Sites[a]	Genes	Chromosome[b]	Dead ($n=147$) Mean (SD)[c]	Alive ($n=387$) Mean (SD)	HR per SD decrease (95% CI)[d]	*P*-value	FDR[e]
cg01612140*	unassigned	6q14.1	0.24 (0.060)	0.27 (0.063)	1.47 (1.11–1.95)	0.0070	0.0370
cg03636183*	F2RL3	19p13.11	0.67 (0.077)	0.70 (0.067)	1.28 (1.06–1.54)	0.0096	0.0427
cg03725309*	SARS	1p13.3	0.07 (0.024)	0.08 (0.025)	1.43 (1.10–1.85)	0.0083	0.0403
cg05575921*	AHRR	5p15.33	0.80 (0.086)	0.83 (0.078)	1.36 (1.09–1.70)	0.0059	0.0370
cg06126421*	unassigned	6p21.33	0.63 (0.104)	0.67 (0.089)	1.33 (1.09–1.64)	0.0064	0.0370
cg08362785*	MKL1	22q13.1	0.70 (0.044)	0.69 (0.039)	0.62 (0.46–0.83)	0.0013	0.0255
cg11341610	CALR	19q13.2	0.08 (0.031)	0.09 (0.031)	1.62 (1.22–2.16)	0.0010	0.0255
cg15342087*	unassigned	6p21.33	0.86 (0.040)	0.88 (0.042)	1.34 (1.10–1.65)	0.0043	0.0370
cg18181703*	SOCS3	17q25.3	0.42 (0.065)	0.45 (0.057)	1.37 (1.12–1.68)	0.0028	0.0322
cg20732076*	TRERF1	6p21.1	0.07 (0.031)	0.08 (0.030)	1.44 (1.11–1.87)	0.0060	0.0370
cg23665802*	MIR19A	13q31.3	0.20 (0.064)	0.23 (0.052)	1.63 (1.22–2.18)	0.0009	0.0255
cg23842572*	MPRIP	17p11.2	0.79 (0.031)	0.77 (0.033)	0.65 (0.50–0.86)	0.0021	0.0307
cg25763716	VCAM1	1p21.2	0.11 (0.042)	0.13 (0.041)	1.58 (1.15–2.17)	0.0047	0.0370
cg26709988	CRISPLD2	16q24.1	0.31 (0.081)	0.33 (0.078)	1.73 (1.14–2.63)	0.0107	0.0442

[a]CpG sites with *smoking-related loci reported by previous studies[34,35] and in bold loci selected to construct the mortality risk score[17].

[b]According to GRCh37/hg19.

[c]SD, standard deviation.

[d]Model adjusted for age (years), leukocyte distribution (Houseman algorithm) and random batch effect of methylation measurement, smoking status (current/former/never), alcohol consumption (abstainer/low/intermediate/high), body mass index (BMI, underweight or normal weight/overweight/obese), physical activity [metabolic equivalent of task (MET), low (≤12 kcal/kg hours/week), median (12–30 kcal/kg hours/week), high (≥30 kcal/kg hours/week)], education (≤12 years, 13–16 years, >16 years), total cholesterol (mg/dL), HDL (mg/dL), triglycerides (mg/dL), systolic blood pressure (mm Hg), hypertension, stroke, coronary heart disease, diabetes and cancer (yes/no); HR, hazard ratio; CI, confidence interval.

[e]FDR = false discovery rate.

Table 3. Associations between mortality risk score and all-cause, cardiovascular disease and cancer mortality in NAS

Mortality	Indicator		n / n_{death}	Model 1[a]		Model 2[b]		Model 3[c]	
				HR (95% CI)	*P*-value	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value
All-cause	Ordinal MS (per one unit)		534/147	1.27 (1.16–1.38)	<0.0001	1.24 (1.12–1.37)	<0.0001	1.25 (1.13–1.38)	<0.0001
	MS risk level	Low	238/47	Ref		Ref		Ref	
		Moderate	251/76	1.45 (0.95–2.19)	0.084	1.26 (0.81–1.96)	0.298	1.38 (0.86–2.19)	0.180
		High	45/24	4.10 (2.26–7.42)	<0.0001	3.26 (1.72–6.20)	0.0003	3.84 (1.92–7.67)	0.0001
	Continuous MS (per one unit)		534/147	2.53 (1.59–4.01)	<0.0001	2.19 (1.30–3.69)	0.0032	2.53 (1.43–4.47)	0.0014
Cardiovascular disease	Ordinal MS (per one unit)		534/85	1.30 (1.16–1.45)	<0.0001	1.24 (1.09–1.41)	0.0014	1.29 (1.12–1.47)	0.0003
	MS risk level	Low	238/26	Ref		Ref		Ref	
		Moderate	251/42	1.61 (0.90–2.89)	0.112	1.33 (0.71–2.47)	0.373	1.42 (0.74–2.72)	0.377
		High	45/17	5.62 (2.59–12.18)	<0.0001	4.26 (1.84–9.88)	0.0007	5.20 (2.11–12.83)	0.0004
	Continuous MS (per one unit)		534/85	3.21 (1.74–5.92)	0.0002	2.60 (1.30–5.18)	0.007	3.09 (1.42–6.70)	0.004
Cancer	Ordinal MS (per one unit)		534/47	1.31 (1.13–1.52)	0.0003	1.34 (1.13–1.58)	0.0006	1.29 (1.09–1.53)	0.004
	MS risk level	Low	238/14	Ref		Ref		Ref	
		Moderate	251/26	1.64 (0.81–3.30)	0.169	1.58 (0.76–3.26)	0.220	2.50 (1.11–5.60)	0.026
		High	45/7	5.40 (1.85–15.75)	0.002	5.09 (1.66–15.67)	0.005	6.56 (1.87–23.06)	0.003
	Continuous MS (per one unit)		534/47	3.57 (1.67–7.64)	0.001	3.34 (1.39–8.03)	0.007	3.90 (1.47–10.35)	0.006

[a]Model 1: adjusted for age (years), leukocyte distribution (Houseman algorithm) and random batch effect of methylation measurement; HR. hazard ratio; CI, confidence interval; MS, mortality risk score.

[b]Model 2: additionally adjusted for smoking status, alcohol consumption (abstainer/low/intermediate/high), body mass index (BMI, underweight or normal weight/overweight/obese), physical activity [metabolic equivalent of task (MET), low (≤12 kcal/kg hours/week), median (12–30 kcal/kg hours/week), high (≥30 kcal/kg hours/week)] and education (≤12 years, 13–16 years, >16 years).

[c]Model 3: additionally adjusted for total cholesterol (mg/dL), HDL (mg/dL), triglycerides (mg/dL), systolic blood pressure (mm Hg), hypertension, stroke, coronary heart disease, diabetes and cancer (yes/no).

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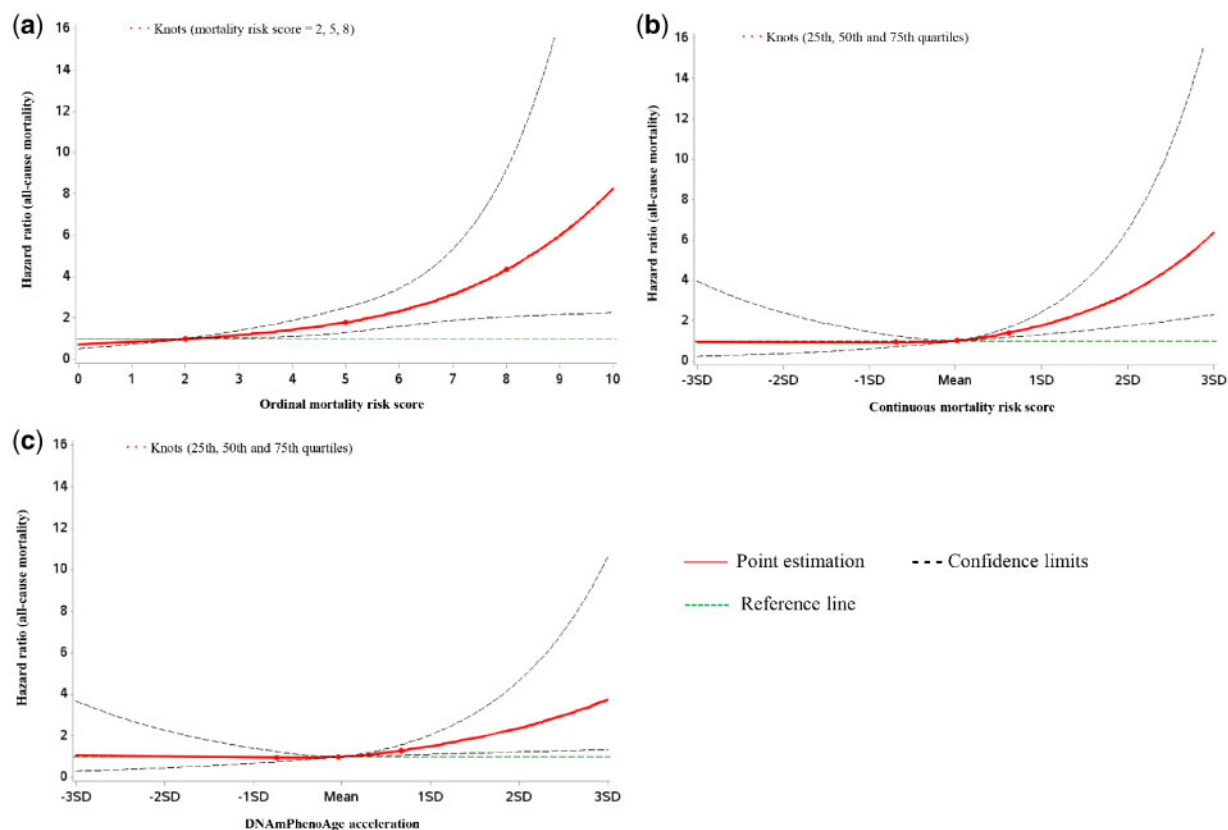

Figure 2. Graphs of the best-fitting models for relationships of mortality risk score (a and b) and DNAmPhenoAge acceleration (c) with all-cause mortality.

Table 4. Associations between mortality risk score and telomere length, DNAmAge acceleration and DNAmPhenoAge acceleration

Biomarkers	Forms of MS		Model 1[a]		Model 2[b]		Model 3[c]	
			Estimate (SE)	*P*-value	Estimate (SE)	*P*-value	Estimate (SE)	*P*-value
Telomere length (T/S ratio)	Ordinal MS (per one unit)		–0.027 (0.011)	0.018	–0.020 (0.013)	0.122	–0.019 (0.013)	0.149
	MS risk level	Low	Ref		Ref		Ref	
		Moderate	–0.074 (0.046)	0.109	–0.047 (0.050)	0.356	–0.039 (0.052)	0.451
		High	–0.193 (0.087)	0.027	–0.138 (0.093)	0.136	–0.142 (0.096)	0.139
	Continuous MS (per one unit)		–0.200 (0.056)	0.0004	–0.183 (0.064)	0.005	–0.194 (0.068)	0.004
DNAmAge acceleration (Horvath, years)	Ordinal MS (per one unit)		0.167 (0.123)	0.174	0.164 (0.135)	0.223	0.122 (0.138)	0.378
	MS risk level	Low	Ref		Ref		Ref	
		Moderate	0.065 (0.496)	0.896	0.083 (0.532)	0.876	–0.069 (0.538)	0.898
		High	1.924 (0.928)	0.039	1.430 (0.975)	0.143	0.830 (0.991)	0.403
	Continuous MS (per one unit)		1.405 (0.601)	0.020	1.417 (0.674)	0.036	0.824 (0.701)	0.241
DNAmPhenoAge acceleration (years)	Ordinal MS (per one unit)		0.564 (0.139)	<0.0001	0.480 (0.150)	0.002	0.392 (0.152)	0.010
	MS risk level	Low	Ref		Ref		Ref	
		Moderate	1.388 (0.561)	0.014	1.176 (0.592)	0.048	0.953 (0.591)	0.108
		High	4.367 (1.050)	<0.0001	3.403 (1.087)	0.002	2.347 (1.090)	0.032
	Continuous MS (per one unit)		2.512 (0.684)	0.0003	2.032 (0.756)	0.008	1.537 (0.773)	0.047

[a]Model 1: adjusted for age (years), leukocyte distribution (Houseman algorithm) and random batch effect of methylation measurement; SE, standard error; MS, mortality risk score.

[b]Model 2: additionally adjusted for smoking status, alcohol consumption (abstainer/low/intermediate/high), body mass index (BMI, underweight or normal weight/overweight/obese), physical activity [metabolic equivalent of task (MET), low (≤12 kcal/kg hours/week), median (12-30 kcal/kg hours/week), high (≥30 kcal/kg hours/week)] and education (≤12 years, 13–16 years, >16 years).

[c]Model 3: additionally adjusted for total cholesterol (mg/dL), HDL (mg/dL), triglycerides (mg/dL), systolic blood pressure (mm Hg), hypertension, stroke, coronary heart disease, diabetes and cancer (yes/no).

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Figure S3, available as Supplementary data at *IJE* online). No overlap was found between the CpG sites used by the MS and those used by the DNAmAge or DNAmPhenoAge. All types of MS were negatively associated with TL. One unit increase of the continuous MS was associated with about 0.19 unit decrease in the T/S ratio of TL. DNAmAge acceleration was not associated with any forms of MS. In contrast, DNAmPhenoAge acceleration showed a robust positive association with MS. After controlling for potential covariates, one unit increase of the ordinal and continuous MS were associated with about 0.4 and 1.5 years increase in DNAmPhenoAge acceleration, respectively. Compared with the low MS risk group, the moderate and high risk groups were associated with about 0.9 and 2.3 years increase in the acceleration of DNAmPhenoAge, respectively.

We further assessed the associations between the three aging biomarkers and mortality without and with mutual adjustment for ordinal MS (Table 5). After the mutual adjustments, the HRs of ordinal MS, DNAmAge acceleration and DNAmPhenoAge acceleration were essentially unchanged, whereas the HRs of TL changed considerably. The ordinal MS still showed the strongest associations with mortality compared with other markers even after the mutual adjustments. DNAmPhenoAge acceleration was the only aging biomarker that was highly associated with all-cause and CVD mortality after the mutual adjustment. A 1 year increase in DNAmPhenoAge acceleration was associated with a 1.05-fold (95% CI: 1.01–1.08) and 1.07-fold (1.02–1.12) increase in all-cause and CVD mortality, respectively. One SD increase in DNAmPhenoAge acceleration predicted a 1.5-fold increase in the risk of all-cause mortality (Figure 2c). Nevertheless, none of the aging biomarkers was associated with cancer mortality.

As the MS and DNAmPhenoAge acceleration were the only two indicators associated with all-cause mortality in the present study, we additionally estimated the performances of both in the prediction of all-cause mortality. Summary indicators are presented in Table 6 and Figure 3. The ordinal MS outperformed DNAmPhenoAge acceleration in predicting all-cause mortality. Harrell's C statistics for the ordinal MS was 0.627 (95% CI: 0.587–0.666) and 0.527 (95% CI: 0.490–0.564) for the DNAmPhenoAge acceleration (*P*-value for their difference <0.0001). Continuous MS was better than DNAmPhenoAge acceleration in mortality prediction with a Harrell's C of 0.585 (95% CI: 0.543–0.627), but not as good as the ordinal MS. The combination of ordinal MS and DNAmPhenoAge acceleration slightly increased the C statistics of the prediction model (*P*-value = 0.09). A very similar pattern was seen for Uno's C statistics.

Discussion

In this study of 534 older male adults from the NAS with a median follow-up of 9.4 years, we validated 58 previously identified mortality-related CpG sites and the newly constructed MS in relation to mortality. We further assessed associations between MS and three popular aging biomarkers and evaluated their predictive performances of mortality. Fourteen of the 58 loci were found to be associated with all-cause mortality and the MS demonstrated strong associations with all-cause, CVD and cancer mortality. Furthermore, the MS was associated with TL and DNAmPhenoAge acceleration, but not with DNAmAge acceleration. DNAmPhenoAge acceleration was the only aging biomarker that showed independent associations with all-cause and CVD mortality along with MS. Compared with the DNAmPhenoAge acceleration, MS was more predictive of all-cause mortality. This comparative validation of MS with other aging surrogates provides evidence in favour of the potential use of MS in the assessment of mortality risk in clinical settings.

Not only is our study the first external validation of MS, we also conducted for the first time a comparison of MS with three popular aging markers in the prediction of mortality using an independent cohort. The strong predictive ability of MS could be partly explained by tobacco smoking, which not only strongly correlates with mortality,[37,38] but also plays an important role in the modification of MS. As noted, 11 out of the 14 validated mortality-related CpG sites and eight out of the ten loci for the construction of MS were smoking-related as reported by previous studies.[34,35] The discrepancies between MS and DNAmAge in relation to smoking exposure might also partly explain their null association. In contrast to its strong linkage with MS, a previous study has reported that smoking status was not related to DNAmAge in older adults.[39] Furthermore, MS could have the potential to reflect more than the mortality risks triggered by smoking exposure. In the subgroup analyses by smoking status, we observed consistent patterns of the ordinal MS with all-cause and disease-specific mortality among never and ever smokers, albeit the CIs were much wider due to a limited number of cases. This suggests that the MS could be associated with mortality risks via pathways other than smoking-related pathological mechanisms Altogether, the full landscape of underlying biological mechanisms and functional connections of the mortality-related CpG sites/MS with the development of individual mortality risks still need to be further elucidated by multidisciplinary studies.

A key question with critical importance for gerontology is whether and to what extent MS reflects the biological aging and mortality risk separately. We found that MS was not associated with DNAmAge acceleration but with TL,

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Table 5. Associations of telomere length, DNAmAge acceleration and DNAmPhenoAge acceleration with all-cause, cardiovascular disease and cancer mortality with and without mutual adjustment for mortality risk score

Mortality (n/n_{death})	Biomarker	Model with one biomarker only[a]		Model with one of the aging biomarkers and the ordinal MS[b]			
				Biomarker		Ordinal MS	
		HR (95% CI)	*P*-value	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value
All-cause (534/147)	Telomere length (T/S ratio)	0.78 (0.48–1.27)	0.31	0.72 (0.44-1.17)	0.19	1.27 (1.15-1.41)	<0.0001
	DNAmAge acceleration (Horvath, years)	1.01 (0.97–1.05)	0.64	1.00 (0.96–1.04)	0.99	1.26 (1.13–1.39)	<0.0001
	DNAmPhenoAge acceleration (years)	1.05 (1.01–1.08)	0.008	1.04 (1.00–1.08)	0.041	1.26 (1.13–1.40)	<0.0001
	Ordinal MS (per one unit)	1.25 (1.13–1.38)	<0.0001				
Cardiovascular disease (534/85)	Telomere length (T/S ratio)	0.92 (0.49–1.74)	0.81	0.83 (0.44–1.58)	0.57	1.32 (1.14–1.53)	0.0002
	DNAmAge acceleration (Horvath, years)	1.00 (0.95–1.06)	0.93	0.99 (0.94–1.05)	0.78	1.30 (1.13–1.50)	0.0003
	DNAmPhenoAge acceleration (years)	1.07 (1.02–1.12)	0.003	1.05 (1.00–1.11)	0.04	1.31 (1.13–1.53)	0.0003
	Ordinal MS (per one unit)	1.29 (1.12–1.47)	0.0003				
Cancer (534/47)	Telomere length (T/S ratio)	0.83 (0.28–1.59)	0.36	0.66 (0.27–1.59)	0.35	1.28 (1.08–1.53)	0.006
	DNAmAge acceleration (Horvath, years)	1.00 (0.94–1.07)	0.96	0.98 (0.92–1.05)	0.57	1.30 (1.09–1.55)	0.003
	DNAmPhenoAge acceleration (years)	1.02 (0.96–1.08)	0.52	1.01 (0.95–1.07)	0.70	1.31 (1.15–1.64)	0.004
	Ordinal MS (per one unit)	1.29 (1.09–1.53)	0.004				

[a]Adjusted for age (years), leukocyte distribution (Houseman algorithm, for models with epigenetic-based indicators), smoking status, alcohol consumption (abstainer/low/intermediate/high), body mass index (BMI, underweight or normal weight/overweight/obese), physical activity [metabolic equivalent of task (MET), low (≤12 kcal/kg hours/week), median (12–30 kcal/kg hours/week), high (≥30 kcal/kg hours/week)], education (≤12 years, 13–16 years, >16 years), total cholesterol (mg/dL), HDL (mg/dL), triglycerides (mg/dL), systolic blood pressure (mm Hg), hypertension, stroke, coronary heart disease, diabetes and cancer (yes/no), and random batch effect of methylation measurement; HR, hazard ratio; CI, confidence interval.

[b]Additionally adjusted for one of the aging biomarkers and the ordinal MS simultaneously with other covariates controlled for in the model above.

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Table 6. Overall Harrell's and Uno's C statistics of the mortality risk score (ordinal and continuous) and DNAmPhenoAge acceleration in prediction of all-cause mortality

Characteristic	Harrell's C statistics	95% CI	Uno's C statistics	95% CI
Ordinal MS	0.627	0.587–0.666	0.612	0.569–0.654
Continuous MS	0.585	0.543–0.627	0.561	0.515–0.606
DNAmPhenoAge acceleration	0.527	0.490–0.564	0.500	0.449–0.553
Ordinal MS + DNAmPhenoAge acceleration	0.653	0.612–0.694	0.635	0.586–0.684
Continuous MS + DNAmPhenoAge acceleration	0.602	0.558–0.646	0.582	0.530–0.635

CI, confidence interval.

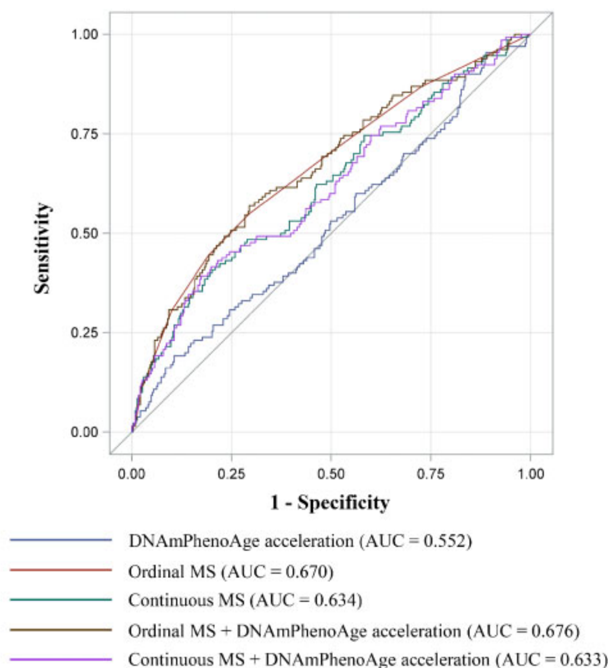

Figure 3. ROC curves for DNAmPhenoAge acceleration, mortality risk score (MS) and their combination for all-cause mortality.

two predictors that were considered to reflect biological aging. The MS–TL relationship is in line with the finding of a recent study based on the ESTHER cohort.[21] However, Zhang *et al.*'s study found that MS was associated with frailty, another well-known aging indicator.[19] Altogether, our data suggest that MS might reflect biological aging only to a limited extent without being able to perform as a reasonably accurate biomarker of accelerated aging. Conversely, MS had a robust association with DNAmPhenoAge acceleration, an 'updated' DNA methylation age built to be more closely related to a cadre of mortality predictors,[15] and even outperformed DNAmPhenoAge in predicting all-cause mortality. Since the DNAmPhenoAge primarily represents the impact of biological aging on mortality, rather than biological aging itself, we suggest that MS might additionally predict the impacts on the risks of death from factors other than simply biological aging. Independent associations of MS and DNAmPhenoAge with mortality further indicate that they may reflect different pathological mechanisms in the development of mortality risks. Together with similar independent associations between vitamin D and MS in relation to mortality that were observed,[20] we believe that the MS could be another important biomarker for predicting mortality risk along with other established mortality-related factors.

Given the different relationships with biological aging and mortality demonstrated in this study and previous epigenetic epidemiological investigations, we suggest that the DNA methylation-based age estimators (e.g. DNAmAge and DNAmPhenoAge) and mortality indicators (e.g. MS) have varied but equally important implications for clinical research and diagnosis. Age estimators could be utilized to describe the individual pathological changes and morbidities that are caused by or mostly sensitive to accelerated aging, such as physical functioning,[40] psychosocial disorders[8,41,42] and aging-related diseases. In contrast, the MS and other mortality-related epigenetic patterns that are under development are initially created for mortality prediction and could therefore be more specialized for reflecting the integrated mortality risks led by biological aging and other external risk factors, such as being overweight, tobacco smoking and other unhealthy lifestyles. Although DNA methylation-based age estimators showed robust association with mortality within larger populations ($n > 1000$)[11,40,43] and meta-analysis,[44] due to the potentially limited weight of aging in the contribution to mortality risks, their power in predicting mortality was much less than mortality-specific indicators for individuals or relatively small populations. This limitation restricts the applications of current available epigenetic aging estimators for mortality risk stratification in clinical settings and makes the indicators like MS good candidates. Nevertheless, we are still far from establishing reliable epigenetic indicators for the whole population. Current epigenetic indicators are required to be updated and optimized for compatibility with the advent of epigenetic measurement methods. For instance, 2/10 and 17/353 CpG sites for the construction of the MS and Horvath's DNAmAge were no longer available after the introduction of Illumina EPIC methylation array. As shown in Supplementary Table S3, available as

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Supplementary data at *IJE* online, the lack of the two loci for MS attenuated its prediction of mortality to a certain extent. Population heterogeneity is another obstacle that could hinder the promotion of such epigenetic indicators that are sensitive to racial difference.[10,45] More efforts are warranted to unify the currently available epigenetic candidates into universally applicable epigenetic indicators for aging and mortality and to translate them into reliable clinical tools.

Major strengths of the present study include detailed information on a broad range of covariates and estimates of different aging and mortality biomarkers. Several limitations should also be noted in the interpretation of results. First, shifts of leukocyte distribution might affect DNA methylation changes in whole blood samples.[46] Hence, we adjusted for leukocyte distribution by the Houseman algorithm to restrict confounding from differential blood counts to the greatest possible extent.[29] Second, the selected study participants were Caucasians and all older males, which limits the generalizability of our results to other racial/ethnic groups and women. Finally, although our overall sample size was relatively large, some of the results with weak associations in the subgroup analyses may have been due to the lack of statistical power with the relatively limited number of deaths.

In summary, this comparative validation showed strong associations between MS based on ten mortality-related CpG sites and all-cause, CVD and cancer mortality, and further demonstrated that it outperformed TL, DNAmAge acceleration and DNAmPhenoAge acceleration in predicting mortality as a more relevant mortality indicator. This study sheds some light on the utilization of epigenetic markers as an informative approach for predicting mortality and mortality-related health risks at the population level. Our findings need to be further assessed in larger cohorts to evaluate the clinical applications of the epigenetic-based MS in routine medical practice aimed at assessing mortality risks along with other robust survival predictors.

Supplementary Data

Supplementary data are available at *IJE* online.

Acknowledgements

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Conflict of interest: None declared.

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