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# DNA methylation-based telomere length is more strongly associated with cardiovascular disease and long-term mortality than quantitative polymerase chain reaction-based telomere length: evidence from the NHANES 1999–2002

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## Abstract

**Background** Telomere length (TL) serves as a pivotal gauge of cellular aging, with shorter TL linked to various age-related ailments. Recently, a DNA methylation-based TL estimator, known as DNAmTL, has emerged as a novel TL measurement tool. Our current investigation scrutinized the correlation between DNAmTL and the risks of cardiovascular disease (CVD) and enduring mortality among middle-aged and elderly individuals.

**Methods** We enrolled a nationwide, population-based cohort of subjects from the National Health and Nutrition Examination Survey spanning 1999 to 2002, possessing data on both DNAmTL and quantitative polymerase chain reaction-based TL (qPCRTL). Logistic regression models and Cox proportional hazards models were employed to evaluate the associations of DNAmTL with CVD risk and mortality, respectively.

**Results** The cohort comprised 2532 participants, with a weighted CVD prevalence of 19.06%. Notably, each one-kilobase increase in DNAmTL was linked to a 53% diminished CVD risk [odds ratio (OR): 0.47, 95% confidence interval (CI): 0.23–0.95, $P=0.035$]. Over a median follow-up period of 206 months, 1361 deaths were recorded (53.75%), with 590 (23.30%) ascribable to CVD. Individuals with the lengthiest DNAmTL exhibited a 36% lower risk of all-cause mortality (hazard ratio (HR): 0.64, 95% CI: 0.49–0.85, $P=0.002$) and a 35% decrease in CVD mortality (HR: 0.65, 95% CI: 0.43–0.98, $P=0.044$) compared to those with shortest DNAmTL. Notably, a stronger association with age was observed for DNAmTL compared to qPCRTL ($r=-0.58$ vs. $r=-0.25$). Analysis of receiver operating characteristic (ROC) curves suggested superior predictive performance of DNAmTL over qPCRTL for CVD (area under curve (AUC): 0.63 vs. 0.55, $P<0.001$), all-cause (AUC: 0.74 vs. 0.62, $P<0.001$), and CVD mortality (AUC: 0.75 vs. 0.64, $P<0.001$).

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**Conclusion** Longer DNAmTL was positively correlated with reduced CVD risk and long-term mortality in middle-aged and elderly cohorts. Notably, DNAmTL outperformed qPCRTL as an aging biomarker in the stratification of CVD risks and mortality.

**Keywords** DNA methylation, Telomere length, Cardiovascular disease, Mortality, NHANES, Aging

## Introduction

Life expectancy has significantly increased over recent decades, largely due to advancements in technology and global economic development. Projections suggest that individuals born since 2000 in countries with high life expectancies, such as France, Germany, Italy, the UK, the USA, Canada, and Japan, could potentially reach the age of 100 years, assuming that the historical rate of increase observed in developed nations over the past two centuries continues throughout the twenty-first century[1]. However, the increase in life expectancy is accompanied by a rise in age-related diseases, including cardiovascular disease (CVD) [2], tumors [3], and cognitive dysfunction [4], which impose a significant burden on public health system.

Aging is a multifaceted biological process that progressively diminishes the resilience and adaptive capabilities of cells, tissues, and organs [5, 6]. Biological age, which reflects an individual's physiological state, may provide a more accurate assessment of aging compared to chronological age, which only measures the passage of time and does not necessarily correspond with an individual's overall health condition [7, 8].

Telomeres are complex structures composed of tandem DNA nucleotide repeats (TTAGGG)n and associated proteins, located at the ends of the chromosome to maintain chromosomal stability [9]. With each cell division, telomeres shorten, eventually leading to replicative senescence and/or apoptosis [10]. As a result, telomere length (TL) is widely recognized as a valuable marker for assessing biological age. Evidence indicates that shorter TL is significantly associated with an increased risk of various age-related diseases and health outcomes, including CVD, metabolic syndrome, and cancers [11].

Several methods have been developed to measure TL, each with its own advantages and limitations. Southern blot Telomere Restriction Fragment (TRF) analysis and fluorescence in situ hybridization (flow FISH) are considered gold standard methods for accurately measuring TL. However, these techniques require large quantities of high-quality DNA, special expertise, or costly, which limits their use in clinical practice or epidemiologic studies [12, 13]. Quantitative polymerase chain reaction (qPCR), which measures the ratio between telomere copy number and a single-copy gene (T/S) in the same DNA sample (qPCRTL), is a widely applied method due to its high-throughput and small DNA requirements [14]. Despite its advantages, qPCR's reliability can be affected by pre-analytic factors, such as DNA extraction and storage conditions.

Methylation of cytosine residues in cytosine-phosphate-guanine dinucleotides (CpGs) is another DNA-based biomarker that changes with age. Recently, Lu et al. developed a novel method to predict TL based on the methylation profiles of 140 CpGs in leukocytes (DNAmTL). This method has been shown to outperform TRF-based LTL in predicting mortality and time-to-heart disease [15]. Additionally, a recently published cohort study found that individuals with HIV infection had significantly shorter DNAmTL compared to those without HIV, and shorter DNAmTL was associated with an increased risk of mortality [16]. In this study, we aim to investigate the association between DNAmTL and CVD, as well as long-term mortality among middle-aged and older adults in the United States.

## Methods

### Study design and population

This study is a cross-sectional analysis based on data from 2532 participants from the 1999–2000 and 2001–2001 cycles of the National Health and Nutrition Examination Survey (NHANES). The NHANES project aims to assess the heath and nutritional status of adults and children in the United States. Managed by the Centers for Disease Control and Prevention (CDC), the NHANES protocols have received approval from the Research Ethics Review Board of the National Center for Health Statistics (NCHS). The program ensures participants' rights are protected through informed written consent obtained from all individuals involved.

Participants were excluded from the study if they met any of the following criteria (as depicted in Fig. 1): (1) Lack of CVD information, (2) Absence of data on either DNAmTL or qPCRTL.

CVD diagnosis in this study was based on self-reported physician diagnosis collected through a standardized questionnaire [17, 18]. Participants were asked, "Has a doctor or other health expert ever informed you that you have congestive heart failure (CHF)/coronary heart disease (CHD) /angina pectoris/myocardial infarction (MI)/stroke?" Those who answered "yes" were considered as having CVD.

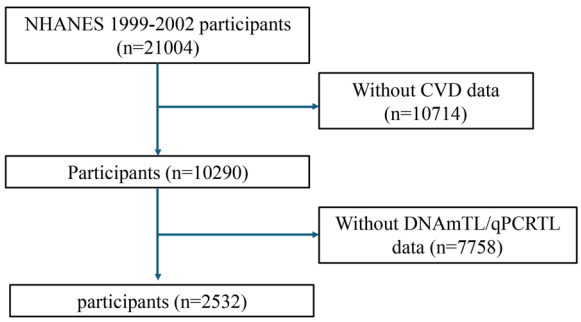


**Fig. 1** Study flowchart showing the inclusion and exclusion process

### DNA methylation measurement and DNAmTL calculation

Participants aged 50 years and over with blood samples for DNA purification were eligible for the DNAm measurement. DNA was extracted from whole blood samples and stored at −80 °C until analysis. The DNA methylation assay was conducted in Dr. Yongmei Liu's laboratory at Duke University. For bisulfite conversion, 500 ng of DNA was processed using the Zymo EZ DNA Methylation Kit (cat# D5001, Zymo Research, Irvine, CA, USA) following the manufacturer's specifications and applying PCR conditions optimized for Illumina's Infinium Methylation assay (95 °C for 30 s, 50 °C for 60 min over 16 cycles). Methylation data were obtained using the Illumina Infinium MethylationEPIC BeadChip v1.0 (cat# WG317-1001, Illumina, San Diego, CA, USA). A volume of 4 μL of bisulfite-converted DNA was hybridized to the BeadChip according to the manufacturer's protocols. Following hybridization, samples were denatured and amplified overnight for 20–24 h. Subsequent steps included fragmentation, precipitation, and resuspension of the samples, followed by an additional overnight incubation before hybridization to the EPIC BeadChip for 16–24 h. The BeadChip was then washed to remove any unhybridized DNA and labeled with nucleotides to extend the primers. Finally, imaging of the BeadChip was performed using the Illumina iScan system (Illumina, San Diego, CA, USA), in accordance with the Infinium HD Methylation protocol. DNAmTL was calculated using the elastic net regression model as described before based on DNA methylation levels [15].

### qPCRTL measurement

Blood samples were collected from participants and stored at -80 °C until DNA analysis. TL was measured using qPCR at the laboratory of Dr. Elizabeth Blackburn at the University of California, San Francisco, with TL quantified relative to a standard reference DNA (T/S ratio). Each sample underwent analysis in triplicate on separate days, yielding a total of six data points per sample. Assay plates were processed in triplicate batches, with no plate reused within the same batch. Each plate contained 96 control wells, incorporating one of eight distinct control DNA samples. Assay runs with eight or more invalid control wells were excluded, constituting less than 1% of total runs. Control DNA values were used to adjust for between run variability and runs with more than four control DNA values deviating beyond 2.5 standard deviations from the mean were excluded, accounting for less than 6% of runs. Outliers were identified and removed, affecting less than 2% of samples. The mean and standard deviation of the T/S ratio were computed, with an inter-assay coefficient of variation of 6.5%.

### Ascertainment of mortality

In the NHANES program, mortality data were linked to the National Death Index (NDI) by the NCHS through December 31, 2019. Disease-specific deaths were confirmed using the International Statistical Classification of Diseases, 10th Revision (ICD-10).

### Covariates

Demographic variables included age, sex, race/ethnicity (white, black, Mexican American, other races), education level (below high school, high school or equivalent, above high school), poverty income ratio (PIR), alcohol consumption (yes/no) and smoking status (never, former, current). Laboratory assessments measured total cholesterol (TC), triglycerides (TG), high-density lipoprotein cholesterol (HDL-C), and estimated glomerular filtration rate (eGFR). Physical exams recorded systolic blood pressure (SBP), diastolic blood pressure (DBP), and body mass index (BMI). Medication use, including antihypertensive drugs, hypoglycemic agents, and statins were also collected. Hypertension was defined as self-reported diagnosis, use of anti-hypertensive medications, SBP ≥ 140 mmHg, or DBP ≥ 90 mmHg [19]. Diabetes mellites (DM) was identified through self-reported diagnosis, use of insulin or oral hypoglycemic agents, fasting blood glucose ≥ 7.0 mmol/L, 2-h glucose ≥ 11.1 mmol/L post-oral glucose tolerance test, or HbA1c ≥ 6.5% [20].

### Statistical analysis

Due to the complex sampling design of NHANES, sample weights were incorporated into all analyses. Participants were categorized into three groups based on tertiles of DNAmTL levels (T1-T3). Continuous variables are reported as mean ± standard deviation (SD) or median (interquartile range) and analyzed using one-way ANOVA or Mann–Whitney tests, as appropriate. Categorical variables are presented as number (percent) and analyzed using the chi-square test. Pearson correlation

analysis was employed to assess the relationship between DNAmTL, qPCRTL, and age.

To investigate the association between DNAmTL and CVD, three logistic regression models were utilized. Model I adjusted for age; Model II further adjusted for gender, race/ethnicity, BMI, PIR, education level, alcohol consumption, and smoking status; Model III additionally accounted for hypertension, DM, TC, TG, HDL-C, SBP, DBP, eGFR, and medication use (antihypertensives, hypoglycemics, and statins). Cox proportional hazards models were employed to evaluate the relationship between DNAmTL and mortality, with three models constructed similarly: Models I and II adjusted for the same confounding factors as above, while Model III also adjusted for CVD. Restricted cubic splines were used to explore potential nonlinear dose–response relationships between DNAmTL, CVD risk, and mortality outcomes. Receiver operating characteristic (ROC) curves assessed the discriminative value of DNAmTL and qPCRTL for CVD risk and mortality, with the area under the curve (AUC) evaluated using the DeLong test. Subgroup analyses by age, gender, smoking, alcohol consumption, obesity, hypertension, DM, and CVD were conducted to assess the robustness of the DNAmTL-mortality association across different populations. Data analysis was performed using R software (version 4.0.3), with statistical significance set at a two-sided $p$ value < 0.05.

## Results

### Baseline characteristics of the study participants

In this study, 2,532 participants were enrolled with a mean age of 66.13 ± 10.08 years; 1,285 (50.75%) were male. Table 1 outlines the baseline characteristics of the participants. Those with the longest DNAmTL (T3) were younger and more likely to be female, non-smokers, Black, and had higher educational attainment compared to those with the shortest DNAmTL (T1). Additionally, participants in the T3 group exhibited lower SBP, DBP, and TC levels, alongside higher HDL-C and eGFR. DNAmTL showed a stronger negative correlation with age (r = −0.58, $P$ < 0.001) compared to qPCRTL (r = −0.25, $P$ < 0.001).

### Association between DNAmTL and CVD prevalence

Among the 2532 participants enrolled in this study, 501 had CVD, yielding a weighted prevalence of 19.06%. Table 2 details the cross-sectional associations between DNAmTL and CVD risk in middle-aged and older adults. The prevalence of CVD was significantly higher in the T3 group compared to the T1 group (weighted prevalence 31.01% vs. 9.80%, $P$ < 0.001). After adjusting for potential confounding factors, each 1 kilobases increment in DNAmTL was associated with a 53% reduced risk of CVD [odds ratio (OR): 0.47, 95% confidence interval (CI): 0.23–0.95, $P$ = 0.035]. Participants in the T3 group also exhibited a 50% reduced risk of CVD compared to those in the T1 group (OR: 0.50, 95%CI: 0.29–0.85, $P$ = 0.011). RCS analysis demonstrated a linear dose–response relationship between DNAmTL and CVD risk (P for nonlinearity: 0.546) (Fig. 2).

ROC analysis revealed that DNAmTL was more effective than qPCRTL in predicting CVD in middle-aged and older adults (AUC: 0.63 vs. 0.55, $P$ < 0.001) (Supplementary Fig. 1).

### Associations between DNAmTL and mortality

During a median follow-up period of 206 months, there were 1361 deaths (53.75%), including 590 (23.30%) attributable to CVD. Table 3 summarizes the impact of DNAmTL on all-cause and CVD mortality. After full adjustment, DNAmTL as a continuous variable was inversely associated with both all-cause and CVD mortality, with hazard ratios (HRs) and 95% CIs of 0.77 (0.70–0.85) and 0.45 (0.22–0.90), respectively. Relative to the T1 group, individuals in the T3 group had a 36% decreased risk of all-cause mortality (HR: 0.64, 95% CI: 0.49–0.85, $P$ = 0.002) and a 35% decreased risk of CVD mortality (HR: 0.65, 95% CI: 0.43–0.98, $P$ = 0.044). RCS analysis indicated that lower DNAmTL was linearly associated with an increased risk of all-cause and CVD mortality (Fig. 3).

Additionally, ROC analysis showed that DNAmTL was superior to qPCRTL for predicting long-term all-cause mortality (AUC: 0.74 vs. 0.62, $P$ < 0.001) and CVD mortality (AUC: 0.75 vs. 0.64, $P$ < 0.001) (Supplementary Fig. 2).

### Subgroup and sensitivity analysis

Subgroup analyses were conducted to assess the robustness of the association between DNAmTL and mortality across different populations. As illustrated in Supplementary Fig. 3A, CVD significantly modified the relationship between DNAmTL and all-cause mortality, with the association being stronger in individuals with CVD compared to those without. For CVD mortality, the effects of sex and DM were significant. Specifically, a longer DNAmTL was associated with a significantly reduced risk of CVD mortality only in women or individuals without DM (Supplementary Fig. 3B). Sensitivity analysis, excluding individuals who died within 2 years of follow-up, confirmed the consistency of these associations (Supplementary Table 1).

**Table 1** Baseline characteristics of participants according to the tertiles of DNAmTL

| Variables | Overall (n = 2532) | DNAmTL | | | *P* value |
|---|---|---|---|---|---|
| | | T1 (n = 849) | T2 (n = 839) | T3 (n = 844) | |
| Age, years | 66.13 ± 10.08 | 73.21 ± 8.69 | 65.79 ± 8.62 | 59.36 ± 7.65 | < 0.001 |
| BMI, Kg/m2 | 28.67 ± 5.82 | 27.68 ± 5.21 | 28.84 ± 5.74 | 29.47 ± 6.30 | < 0.001 |
| Male, n (%) | 1285 (50.75) | 520 (61.25) | 439 (52.32) | 326 (38.63) | < 0.001 |
| Smoking status, n (%) | | | | | < 0.001 |
| Never | 1174 (46.37) | 350 (41.22) | 379 (45.17) | 445 (52.73) | |
| Current | 388 (15.32) | 113 (13.31) | 149 (17.76) | 126 (14.93) | |
| Former | 970 (38.31) | 386 (45.47) | 311 (37.07) | 273 (32.35) | |
| Alcohol consumption, n (%) | 1506 (59.48) | 514 (60.54) | 499 (59.48) | 493 (58.41) | 0.672 |
| Race//Ethnicity, n (%) | | | | | < 0.001 |
| Mexican American | 721 (28.48) | 241 (28.39) | 293 (34.92) | 187 (22.16) | |
| Hispanic | 163 (6.44) | 36 (4.24) | 74 (8.82) | 53 (6.28) | |
| White | 1027 (40.56) | 455 (53.59) | 284 (33.85) | 288 (34.12) | |
| Black | 538 (21.25) | 94 (11.07) | 154 (18.36) | 290 (34.36) | |
| Others | 83 (3.28) | 23 (2.71) | 34 (4.05) | 26 (3.08) | |
| Education levels, n (%) | | | | | < 0.001 |
| Less than high school | 1167 (46.09) | 413 (48.65) | 418 (49.82) | 336 (39.81) | |
| High school or equivalent | 517 (20.42) | 183 (21.55) | 155 (18.47) | 179 (21.21) | |
| Above high school | 848 (33.49) | 253 (29.80) | 266 (31.70) | 329 (38.98) | |
| PIR | 2.20 (1.17, 4.19) | 1.93 (1.13, 3.40) | 2.08 (1.13, 3.92) | 2.67 (1.26, 4.92) | < 0.001 |
| CVD, n (%) | 501 (19.79) | 252 (29.68) | 145 (17.28) | 104 (12.32) | < 0.001 |
| Hypertension, n (%) | 1601 (63.23) | 594 (69.96) | 525 (62.57) | 482 (57.11) | < 0.001 |
| Diabetes, n (%) | 604 (23.85) | 211 (24.85) | 215 (25.63) | 178 (21.09) | 0.065 |
| Anti-hypertensive agents, n (%) | 1003 (39.61) | 360 (42.40) | 307 (36.59) | 336 (39.81) | 0.050 |
| Hypoglycemic agents, n (%) | 392 (15.48) | 140 (16.49) | 138 (16.45) | 114 (13.51) | 0.152 |
| Statins, n (%) | 444 (17.54) | 159 (18.73) | 152 (18.12) | 133 (15.76) | 0.238 |
| SBP, mmHg | 136.77 ± 22.19 | 140.84 ± 22.74 | 137.22 ± 21.56 | 132.15 ± 21.39 | < 0.001 |
| DBP, mmHg | 70.56 ± 16.29 | 66.24 ± 18.48 | 71.46 ± 15.13 | 74.08 ± 13.85 | < 0.001 |
| TC, mmol/L | 5.35 ± 1.05 | 5.25 ± 1.11 | 5.43 ± 1.04 | 5.36 ± 1.00 | 0.004 |
| HDL, mmol/L | 1.34 ± 0.42 | 1.31 ± 0.41 | 1.33 ± 0.40 | 1.38 ± 0.42 | < 0.001 |
| TG, mmol/L | 1.45 (1.03, 2.12) | 1.47 (1.03, 2.17) | 1.55 (1.10, 2.25) | 1.34 (0.98, 1.98) | < 0.001 |
| eGFR, ml/min/1.73m$^2$ | 85.38 ± 21.10 | 78.93 ± 21.34 | 86.28 ± 21.12 | 90.97 ± 19.02 | < 0.001 |

CVD: cardiovascular disease, BMI: body mass index, SBP: systolic blood pressure, DBP: diastolic blood pressure, TC: total cholesterol, HDL-c: high-density lipoprotein cholesterol, TG: triglyceride, UA: uric acid, eGFR: estimated glomerular filtration rate, PIR: poverty income ratio

**Table 2** Association between the risk of CVD and DNAmTL

| | Model I | Model II | Model III |
|---|---|---|---|
| Continuous | | | |
| Per 1 kilobases increment | 0.43 (0.26–0.72) < 0.001 | 0.52 (0.27–0.98) 0.044 | 0.47 (0.23–0.95) 0.036 |
| Categorical | | | |
| T1 | Reference | Reference | Reference |
| T2 | 0.70 (0.49–0.99) 0.042 | 0.74 (0.49–1.11) 0.145 | 0.74 (0.48–1.15) 0.186 |
| T3 | 0.43 (0.29–0.65) < 0.001 | 0.47 (0.28–0.77) 0.003 | 0.50 (0.29–0.85) 0.011 |
| *P* for trend | < 0.001 | 0.003 | 0.011 |

Data were shown as OR, 95% CI, and P value

Model I was adjusted for age, Model II was adjusted for gender, race/ethnicity, BMI, PIR, education levels, alcohol consumption, smoking status, Model III was further adjusted for hypertension, DM, TC, TG, HDL-C, SBP, DBP, eGFR, and medication use (anti-hypertensive agents, hypoglycemic agents, and statins)

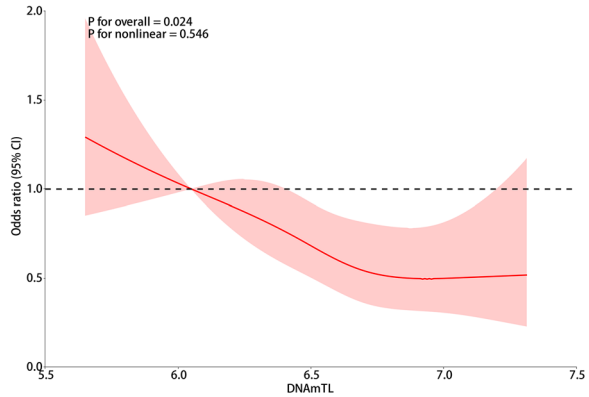


**Fig. 2** RCS analysis for the dose–response association between DNAmTL and risk of CVD

## Discussion

This study is the first to explore the relationship between DNAmTL and CVD risk as well as long-term mortality in middle-aged and older adults in the United States. Key findings include: (1) a negative association between DNAmTL and both CVD risk and mortality; (2) a linear dose–response relationship between DNAmTL and these outcomes; and (3) superior predictive capability of DNAmTL over quantitative qPCRTL for CVD and mortality.

Over recent decades, numerous biological aging biomarkers have been identified, including genomic instability, telomere attrition, and mitochondrial dysfunction [6]. TL is a well-established aging biomarker, with shortening observed across various species, including humans and mice, due to incomplete replication during cell division [21, 22]. Factors such as age, genetic variation, lifestyle, and social determinants significantly influence telomeric attrition rates, affecting cellular proliferation and lifespan predictions across species [23–25].

Despite TL's recognized role in linking aging to disease, its measurement remains challenging due to technical variations in DNA extraction, storage and analysis [26]. Quantitative PCR, due to its high-throughput capabilities and minimal DNA requirements, is commonly used to assess TL. However, DNAmLTL offers a simpler measurement process and may more reliably track clinically relevant traits, as methylation patterns reflect transcriptional changes and cumulative exposure effects over time [27, 28]. Lu et.al [15] found DNAmTL to be more closely associated with age and more predictive of time-to-death, CHD, and HF compared to qPCRTL. Hastings et.al [29] also reported a stronger association between DNAmTL and age compared to qPCRTL in a high-risk pediatric cohort ($r=-0.25$ vs. $r=-0.13$). A meta-analysis of over 100 studies showed a pooled age correlation of $r=-0.29$ for qPCR measures[30], while DNAmTL estimates often exceed $-0.60$ [31]. Our study similarly found DNAmTL to be more negatively correlated with age than qPCRTL ($r=-0.58$ vs. $r=-0.25$), aligning with prior findings.

**Table 3** Association between mortality and the DNAmTL

| | Model I | Model II | Model III |
|---|---|---|---|
| All-cause mortality | | | |
| Continuous | | | |
| Per 1 kilobases increment | 0.31 (0.23–0.40) < 0.001 | 0.78 (0.70–0.86) < 0.001 | 0.77 (0.70–0.85) < 0.001 |
| Categorical | | | |
| T1 | Reference | Reference | Reference |
| T2 | 0.71 (0.60–0.85) < 0.001 | 0.76 (0.62–0.93) 0.007 | 0.75 (0.61–0.93) 0.008 |
| T3 | 0.52 (0.41–0.66) < 0.001 | 0.66 (0.50–0.87) 0.003 | 0.64 (0.49–0.85) 0.002 |
| *P* for trend | < 0.001 | 0.001 | 0.001 |
| CVD mortality | | | |
| Continuous | | | |
| Per 1 kilobases increment | 0.26 (0.16–0.41) < 0.001 | 0.39 (0.20–0.74) 0.004 | 0.45 (0.22–0.90) 0.023 |
| Categorical | | | |
| T1 | Reference | Reference | Reference |
| T2 | 0.53 (0.39–0.71) < 0.001 | 0.56 (0.41–0.76) < 0.001 | 0.59 (0.41–0.83) 0.003 |
| T3 | 0.53 (0.37–0.76) 0.001 | 0.61 (0.40–0.92) 0.018 | 0.65 (0.43–0.98) 0.044 |
| *P* for trend | < 0.001 | 0.007 | 0.018 |

Data were shown as HR, 95% CI, and P value

Model I was adjusted for age, Model II was further adjusted for gender, race/ethnicity, BMI, PIR, education levels, alcohol consumption, smoking status, Model III was further adjusted for hypertension, DM, CVD, TC, TG, HDL-C, eGFR, SBP, DBP, and medication use (anti-hypertensive agents, hypoglycemic agents, and statins)

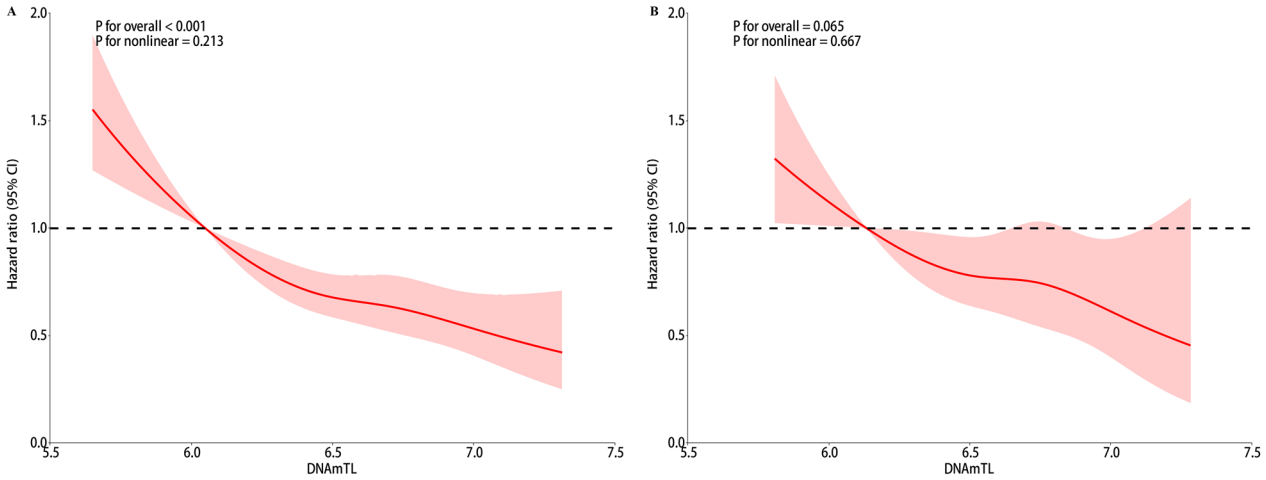


**Fig. 3** RCS analysis for the dose–response association between DNAmTL and risk of all-cause (**A**) and CVD (**B**) mortality

While evidence links shorter TL with increased risk of age-related diseases, research on DNAmTL's role in such diseases is limited. Liang et.al [16] reported associations between shorter DNAmTL and HIV infection, physiological frailty, and cancer, with each kilobase reduction in DNAmTL correlating with a 40% increase in mortality risk. Our findings indicate that each kilobase increase in DNAmTL is associated with a 53% reduced risk of CVD in middle-aged and older adults, and that longer DNAmLTL correlates with lower all-cause and CVD mortality risk. The precision in measuring methylated CpG sites underscores DNAmLTL's advantage, providing a nuanced perspective on TL dynamics in aging populations.

In this study, as mentioned above, we observed that DNAmTL was a superior predictor of CVD risk and long-term mortality compared to qPCRTL. These results underscore the value of DNAmTL as a biomarker in aging and disease stratification, particularly for CVD. The underlying molecular mechanisms linking DNAmTL to CVD risk and mortality may be multifaceted, involving complex epigenetic regulation pathways. One key aspect could be the role of DNA methylation in the modification of chromatin structure, thus influencing gene expression without altering DNA sequence [32]. The aging process itself is known to accelerate telomere shorting, which can be exacerbated by chronic low-grade inflammation and oxidative stress-conditions commonly associated with CVD [33, 34]. This is particularly relevant as methylation patterns are sensitive to external environmental factors [35–37]. For instance, the production of reactive oxygen species (ROS)/reactive nitrogen species (RNS), induced by $H_2O_2$ or nitric oxide, can alter the methylation state of cytosine bases, transforming 5-methylcytosine into 5-hydroxymethylcytosine [38]. This alteration potentially disrupts the normal binding of methylation-binding proteins and DNA methyltransferases, leading to changes in gene expression that may predispose to cardiovascular dysfunction [39, 40]. Inflammatory cytokines including IL-6, TNF, and CRP, which are elevated in CVD, can themselves be regulated by changes in DNA methylation. Studies have shown that such gene changes may affect their levels in plasma and thus modify the inflammatory status, contributing to cardiovascular pathology [41–44]. Considering these insights, the superior predictive value of DNAmTL may stem from its ability to capture these complex epigenetic modifications that are indicative of biological aging and the systemic conditions, which in turn affects CVD risk and mortality. More studies should further focus on elucidating the specific genes and methylation sites involved in these pathways to better understand the mechanistic links between DNAmTL, aging and cardiovascular health.

The strengths of this study include its national representativeness, extended follow-up period, large sample size, and rigorous statistical adjustments for potential confounders. Nevertheless, several limitations must be acknowledged. Firstly, the cross-sectional design precludes drawing definitive conclusions about causal relationships between DNAmTL and CVD risk. Secondly, the study population, consisting of individuals aged 50 and older, suggests that further research is needed in younger cohorts. Thirdly, the TLs in this study were measured by the DNA methylation method, which may not directly reflect the actual TL. However, the DNAmTL measure is indeed more reflective of epigenetic modifications associated with telomere biology and may capture broader aspects of telomere

maintenance mechanisms beyond mere length. Additionally, CVD diagnoses were based on self-reported data, which may overlook asymptomatic cases; however, self-reporting remains a common method in large-scale epidemiological research. Finally, the study's focus on U.S. participants indicates that further investigation is required in other populations, such as Asians or Europeans, to validate these findings.

## Conclusion

DNAmTL was inversely correlated with CVD risk and mortality among middle-aged and older adults. This biomarker demonstrated a stronger association with age and outperformed qPCRTL in predicting CVD and mortality outcomes. Consequently, DNAmTL holds promise as a valuable aging biomarker for risk stratification in cardiovascular disease and long-term mortality within this demographic.

### Abbreviations

| | |
|---|---|
| TL | Telomere length |
| DNAmTL | DNA methylation-based TL |
| CVD | Cardiovascular disease |
| qPCRTL | Quantitative polymerase chain reaction-based TL |
| TRF | Telomere restriction fragment |
| CHF | Congestive heart failure |
| CHD | Coronary heart disease |
| MI | Myocardial infarction |
| TC | Total Cholesterol |
| TG | Triglycerides |
| HDL-C | High-density lipoprotein cholesterol |
| eGFR | Estimated glomerular filtration rate |
| SBP | Systolic blood pressure |
| DBP | Diastolic blood pressure |
| BMI | Body mass index |
| OR | Odds ratio |
| HR | Hazards ratio |
| CI | Confidence interval |

## Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13148-024-01795-8.

Additional file1 (DOCX 628 KB)

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### Author contributions

WQ. designed the study and wrote the original manuscript, GY., DLR., and SJ performed the data analysis, DH. collected the data, YX., TB., and ZX. reviewed the manuscript.

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### Data availability

The data sets used and analyzed in this study are available from the corresponding author for reasonable use.

## Declarations

### Ethics approval and consent to participate

The study protocol of NHANES was approved by the National Center for Health Statistics of the Center for Disease Control and Prevention Institutional Review Board. Informed consent was obtained from all participants included in the study.

### Competing interests

The authors declare no competing interests.

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