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Research Article

Serum 25-Hydroxyvitamin D Levels as an Aging Marker: Strong Associations With Age and All-Cause Mortality Independent From Telomere Length, Epigenetic Age Acceleration, and 8-Isoprostane Levels

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Abstract

Background: A strong association of serum 25-hydroxyvitamin-D levels (25(OH)D) with all-cause mortality has been shown previously and 25(OH)D could be a useful aging marker.

Methods: The analysis was performed in a population-based, cohort study from Germany with 9,940 participants, aged 50–74 years at baseline. A general linear model was used to assess associations of 25(OH)D levels with chronological age and the aging markers leukocyte telomere length (LTL), epigenetic age acceleration, and 8-isoprostane levels. A multivariate Cox regression model was applied to explore the independent and combined associations of these biomarkers with all-cause mortality (2,204 deaths occurred during a median follow-up of 14.3 years).

Results: On average, study participants lost 2.9 nmol/L 25(OH)D each 10 years of age. Increasing 25(OH)D levels were significantly associated with decreasing levels of 8-isoprostane levels but neither with LTL nor epigenetic age acceleration. The association of 25(OH)D quartiles with mortality was almost unchanged after adjusting for all aging markers (1.6-fold increased mortality in bottom quartile compared with top quartile). All aging markers were independent mortality predictors and subjects with unfavorable values for 4, 3, 2, and 1 aging marker(s) had 4.3-, 2.9-, 2.2, and 1.4-fold increased mortality, respectively.

Conclusions: The 25(OH)D level can be regarded as an aging marker because it is linearly associated with age and an independent mortality predictor. Mechanisms linking vitamin D to healthy aging are unique and can neither be fully explained by aging of the epigenome, loss of telomeres, or antioxidative effects of vitamin D metabolites.

Keywords: Biomarkers, Epidemiology, Longevity, Successful aging

Previous cohort studies identified a strong association of vitamin D deficiency with all-cause mortality (1). Therefore, the biomarker of vitamin D status that can be best measured, the serum 25-hydroxyvitamin D (25(OH)D) level, could be a useful, novel aging marker. To serve as an aging marker, 25(OH)D levels should be associated with age and mortality and these associations should not be explained by other established aging markers.

To test the independence of 25(OH)D from other aging markers, we used leukocyte telomere length (LTL), epigenetic age acceleration and 8-isoprostane levels. Telomeres are a region of repetitive nucleotide sequences at each end of a chromosome, which protect the end of the chromosome from deterioration or from fusion with neighboring chromosomes (2). The telomere length theory of aging was recognized by the Nobel Prize in Medicine/Physiology in 2009.

The epigenetic clock can give an estimate of the tissue's biological age by measuring age-dependent DNA methylation (3). The difference between the epigenetic clock and the chronological age is being termed epigenetic age acceleration, which has been shown to be associated with mortality independently of the chronological age (4). Urinary 8-isoprostane levels are a global measure of lipid oxidation and the most reliably measurable biomarker of oxidative stress with evidence for an association with mortality (5). The Free Radical/oxidative stress theory of aging, postulating that reactive oxygen species (ROS) lead to cellular aging, is a widely acknowledged theory of aging (6).

The aims of this study are first to assess the cross-sectional associations of serum 25(OH)D levels with chronological age and the established markers of aging (LTL, epigenetic age acceleration and 8-isoprostane levels) and second to explore the potential independent and combined associations of these four biomarkers with all-cause mortality.

Methods

Study Design

This investigation is based on the ESTHER study (German: **E**pidemiologische **S**tudie zu Chancen der Verhütung, Früherkennung und optimierten **Th**erapie chronischer **Er**krankungen in der älteren Bevölkerung), an ongoing cohort study, details of which have been reported elsewhere (7). Briefly, the total number of participants amounts to 9,940 adults between 50 and 74 years of age, who were recruited by their respective general practitioners (GP) during a routine health check-up between 2000 and 2002 in the German federal state Saarland. Deaths until end of 2015 were obtained by inquiry at all residents' registration offices in Saarland and information about the vital status of almost all participants (99.7%) could be obtained.

Covariate Assessment

Information on age, sex, education, smoking behavior, physical activity, cardiovascular disease events, and cancer diagnoses were obtained by a comprehensive questionnaire from the study participants at baseline. Height, weight, and a history of coronary heart disease were assessed and documented on a standardized form by the general practitioners during the health check-up. Prevalent cardiovascular disease (CVD) was defined by physician-reported coronary heart disease (CHD), a self-reported history of myocardial infarction, stroke, pulmonary embolism, or revascularization of the coronary arteries (bypass or stent). Information on a life-time history of cancer (ICD-10-codes C00-D48) was obtained by record linkage with the Saarland Cancer Registry and supplemented by self-reported cancer diagnoses.

Laboratory Methods

Blood and urine samples were taken during the health check-up, centrifuged, sent to the study center, and stored at −80°C until biomarker analysis. The aging markers of interest were all measured from stored baseline blood sample. Measurements of 25(OH)D levels in 9,572 serum samples of women and men of the ESTHER study with enzyme-linked immunosorbent assays (ELISAs) and their standardization with isotope-dilution liquid chromatography tandem-mass spectrometry (LC-MS/MS) have been described previously (7,8). In brief, the automated Diasorin-Liaison analyzer (Diasorin Inc., Stillwater) was employed to measure 25(OH)D levels in women in an earlier study that had been restricted to females, and the automated IDS-iSYS (Immunodiagnostic Systems GmbH, Frankfurt Main, Germany) analyzer was later used for measurements in men. For each of the two assays employed, random baseline serum samples of 100 study participants were drawn and remeasured with isotope-dilution LC-MS/MS in the Department of Clinical Chemistry, Canisius Wilhelma Hospital, Nijmegen, The Netherlands (9). Spearman rank correlation between measurements with Diasorin-Liaison and LC-MS/MS and between IDS-iSYS and LC-MS/MS was high (r = .83 and r = .86, respectively) and ordinary least squares linear regression equations were fitted and results were employed for standardization of 25(OH)D levels.

The 8-isoprostane concentration was measured in urine samples, which were obtained at baseline for most of the study participants (n = 9,638). The 8iso ELISA kit (Detroit R&D Inc., Detroit) was used with a two- or fourfold dilution as described previously (10). The measurements were adjusted to renal function by dividing the 8-isoprostane levels by urinary creatinine levels.

Two different epigenetic clocks were calculated. Horvath's epigenetic clock was calculated from DNA methylation information of 353 cytosine-phosphate-guanine sites (CpGs) with the published R tutorial (3). Hannum's epigenetic clock was calculated as the sum of DNA methylation values at 71 CpGs multiplied by the respective regression coefficients published by Hannum et al. (11). The epigenetic age acceleration was obtained by subtracting the chronological age from the respective epigenetic clocks. Epigenome-wide DNA methylation measurements to calculate the two epigenetic clocks were conducted in 1,547 randomly selected samples, which were measured at two time-points. First, 999 blood samples of study participants enrolled within the first 6 months of study recruitment were measured. Second, 548 randomly selected samples of the remaining cohort participants were measured. For all 1,547 samples, DNA was extracted and quantified by the PicoGreen© (Invitrogen, Darmstadt, Germany). Methylation status was measured by using the Infinium HumanMethylation450K BeadChip Assay (Illumina.Inc, San Diego) and data were normalized with GenomeStudio.

LTL was determined in a representative sample of n = 3,564 blood samples, whose extracted DNA became available first. As described in detail elsewhere (12), relative LTL was obtained by quantitative polymerase chain reaction, which was done on a LightCycler 480 (Roche Diagnostics GmbH, Mannheim, Germany). The ratio of telomere repeat copy number to the number of single-copy gene was assessed with 36B4 as the single-copy gene. Additionally, terminal restriction fragment analysis was performed to gain data for absolute LTL in a subset of 20 samples. A linear regression equation of relative and absolute LTL in the subset of 20 samples was applied to the total sample to convert relative LTL to absolute LTL.

Statistical Analyses

Multiple imputation was employed to impute missing values in all 9,940 study participants of the cohort for all variables except epigenetic age acceleration and LTL, which were not measured for the total cohort. As shown in Supplementary Table 1 (Supplementary Material), the proportion of missing values was not higher than 3.7% for all variables and to the best of our knowledge, data were missing at random. Separately by sex, 20 complete data sets were imputed with the SAS 9.3 procedure "PROC MI", using the Markov chain Monte Carlo method. The imputation model contained the variables age, education, body mass index (BMI), smoking, physical activity, history of cancer, history of CVD, 25(OH)D levels, season, 8-isoprostane levels, and 14-year mortality status. All multivariable

analyses were performed in the 20 imputed data sets and results of the individual data sets were combined by the SAS 9.3 procedure "PROC MIANALYZE". In a sensitivity analysis, analyses with complete data were compared with the results obtained with imputed data.

For the cross-sectional analyses, generalized linear models were used to investigate associations of chronological age, Horvath's and Hannum's estimators of epigenetic age acceleration, 25(OH)D levels, LTL and 8-isoprostane levels with each other while adjusting for chronological age, sex, season (in models incl. 25(OH)D), batch (in models incl. epigenetic age or LTL), and leukocyte subtype distribution (in models incl. epigenetic age). Longitudinal analyses with the endpoint all-cause mortality were conducted with Cox proportional hazards regression models adjusting for the same covariates as for the preceding cross-sectional analysis and additionally for BMI, education, smoking behavior, physical activity, history of cancer, and history of CVD. Each aging marker was assessed individually first and afterwards in a model combined with all other aging markers. To make obtained effect estimates of the aging markers comparable and to allow for nonlinear associations, all markers were modeled in quartiles. In addition, dose–response curves were plotted with restricted cubic splines for each aging marker to graphically illustrate their association with mortality (13).

All statistical tests were two-sided using an alpha level of 0.05 and all analyses were conducted with the software package SAS, version 9.3 (Cary, NC).

Results

Main characteristics of the study population are shown in Supplementary Table 1 (Supplementary Material). The mean age (±*SD*) was 62.1 ± 6.6 years and 45.1% were male. The mean ± *SD* of Horvath's and Hannum's estimators of epigenetic age acceleration, 8-isoprostane levels, LTL, and 25(OH)D level were 0.16 ± 5.16 years, 6.17 ± 5.51 years, 0.24 ± 0.28 nmol/mmol creatinine, 6.3 ± 0.62 kilobase pairs (kBP), and 51.1 ± 24.6 nmol/L, respectively. Histograms showing the distributions of the aging markers are shown in Supplementary Figures 1 and 2 (Supplementary Material).

All aging markers were statistically significantly associated with chronological age (Table 1). The association of age and 25(OH)D levels was linear and could be modelled with a linear regression model, which revealed a mean loss of 2.9 nmol/L 25(OH)D per 10 years increase in age (Figure 1). The aging markers were not associated with each other except the two estimators of epigenetic age acceleration with each other as well as 8-isoprostane with 25(OH)D levels (Table 1). Additional adjustment for BMI, education, smoking behavior, physical activity, history of cancer, and history of CVD did not substantially change the results shown in Table 1 (data not shown).

During a mean ± *SD* follow-up time of 13.2 years ± 3.1 years (Median: 14.3 years), 2,204 (22.2%) study participants died. Table 2 shows the hazard ratios (HR) and 95% confidence intervals (95% CI) for the associations of the aging markers with all-cause mortality. If modeled alone, Horvath's estimator of epigenetic age acceleration, 8-isoprostane levels, LTL, and 25(OH)D levels were associated with mortality and results were not relevantly altered when all aging markers were included in the same model. The corresponding dose–response curves were not linear. Instead, S-shaped curves for Horvath's estimator of epigenetic age acceleration and 8-isoprostane levels (Figure 2) and L-shaped curves for LTL and 25(OH)D levels were observed (Figure 3). The dose–response curve for Hannum's estimator of epigenetic age acceleration was flat at low values and showed increasing mortality at values of epigenetic age acceleration of 9 years and more (statistically significant for 14 years and more). When Hannum's and Horvath's estimators of epigenetic age acceleration were included in the same model of mortality prediction, the HRs for Hannum's estimator of epigenetic age acceleration were attenuated to null effect values (Table 2). Therefore, only Horvath's estimator of epigenetic age acceleration was used in a joint analysis of the aging markers.

To conduct the joint analysis of the remaining four aging markers, the results with aging marker tertiles (Table 2) and the dose–response analyses were used to define quartiles of the aging markers with an increased mortality (Table 3). Compared to subjects with Horvath's estimator of epigenetic age acceleration in the bottom quartile, LTL in the top two quartiles, 8-isoprostane levels in the bottom three quartiles and 25(OH)D levels in the top three quartiles, subjects at risk for mortality due to 1, 2, 3, and 4 aging marker(s) had a 1.35-, 2-, 19-, 2.86-, and 4.34-fold increased mortality, respectively. Complete case data analysis without imputed missing values did not lead to substantially different results (data not shown).

Discussion

In this large population-based cohort study, 25(OH)D levels were associated with chronological age and 8-isoprostane levels but not with LTL and the epigenetic age acceleration. Statistically significant associations of 25(OH)D levels, Horvath's estimator of epigenetic age acceleration, LTL, and 8-isoprostane levels with all-cause mortality persisted when all aging markers were put in the same model, which speaks for independent mechanisms of these four aging markers.

To the best of our knowledge, this is the first study analyzing the joint association of 25(OH)D levels with multiple aging markers in the same population. Only a previous work from our group already showed that 25(OH)D levels and an epigenetic mortality risk score were independently associated with mortality (14). The current analyses added that 25(OH)D levels were also neither associated with Horvath's nor Hannum's estimators of epigenetic age acceleration. The independency of 25(OH)D levels and methylomic survival predictors is not surprising when keeping in mind that 25(OH)D levels were not associated with DNA methylation at any CpG site in a large epigenome-wide screening study (15).

Furthermore, 25(OH)D levels were also not associated with LTL in our study. This is in agreement with the literature. Although two older studies observed an association of 25(OH)D levels and LTL (16,17), this was not confirmed in newer studies when analyses were comprehensively adjusted and conducted in the total population (which excludes results from subgroup analyses, which can be random findings) (18–22).

The only aging marker that showed an association with 25(OH)D was the 8-isoprostane level. This is the first cohort study conducted in the general older population to establish this association. To the best of our knowledge, only a small intervention study with seven obese children previously reported that normalizing vitamin D deficiency/insufficiency also led to significantly decreased values of this biomarker of oxidative stress at a 3-month follow-up visit (23). Urinary hydrogen peroxide also decreased but not statistically significantly. However, it needs to be noted that the study did not have a control group. Furthermore, a study with 66 obese school children observed an inverse association of 25(OH)D levels with 3-nitrotyrosine, a marker of nitrosative stress (24). With respect to

Table 1. Cross-sectional Analysis: Associations of Aging Markers With Each Other

			Dependent Variables					
			Chronological Age	Horvath's Epigenetic Age Acceleration	Hannum's Epigenetic Age Acceleration	8-isoprostane Levels	LTL	25(OH)D
Independent variables	Chronological age	β (per 1 *SD*)[a]	–	**−1.524 ± 0.122**	**−1.492 ± 0.100**	**−0.008 ± 0.003**	**−0.066 ± 0.008**	**−1.710 ± 0.237**
		p		**<.0001**	**<.0001**	**.006**	**<.0001**	**<.0001**
		N		1,547	1,547	9,940	3,546	9,940
	Horvath's epigenetic age acceleration	β (per 1 *SD*)[a]	–	–	**2.306 ± 0.092**	−0.005 ± 0.010	−0.005 ± 0.013	0.113 ± 0.863
		p			**<.0001**	.574	.724	.896
		N			1,547	1,547	1,516	1,547
	Hannum's epigenetic age acceleration	β (per 1 *SD*)[a]	–	–	–	0.007 ± 0.010	−0.017 ± 0.014	−1.083 ± 0.912
		p				.484	.218	.235
		N				1,547	1,516	1,547
	8-isoprostane levels	β (per 1 *SD*)[a]	–	–	–	–	−0.016 ± 0.009	**−1.245 ± 0.234**
		p					.086	**<.0001**
		N					3,564	9,940
	LTL	β (per 1 *SD*)[a]	–	–	–	–	–	0.564 ± 0.635
		p						.382
		N						3,564

Note: 25(OH)D = 25-hydroxyvitamin D; LTL = Leucocyte telomere length; *N* = Sample size.

Bold print: Statistically significant association ($p < .05$).

[a]The table shows β coefficients ± standard error by 1 *SD* for generalized linear models adjusted for chronological age and sex. Furthermore, models including 25-hydroxyvitamin D were additionally adjusted for season, models incl. epigenetic age acceleration or LTL were additionally adjusted for batch, and models incl. epigenetic age acceleration were additionally adjusted for leukocyte subtype distribution. 1 *SD* (age) = 6.63 years; 1 *SD* (Horvath's epigenetic age acceleration) = 5.16 years; 1 *SD* (Hannum's epigenetic age acceleration) = 4.51 years; 1 *SD* (8-isoprostane levels) = 0.278 nmol/mmol creatinine; 1 *SD* (LTL) = 0.624 kilobase pairs.

the oxidative stress marker advanced oxidation protein products (AOPPs), results of the studies were mixed. Whereas an observational study with 357 healthy adults did not find an association (25),

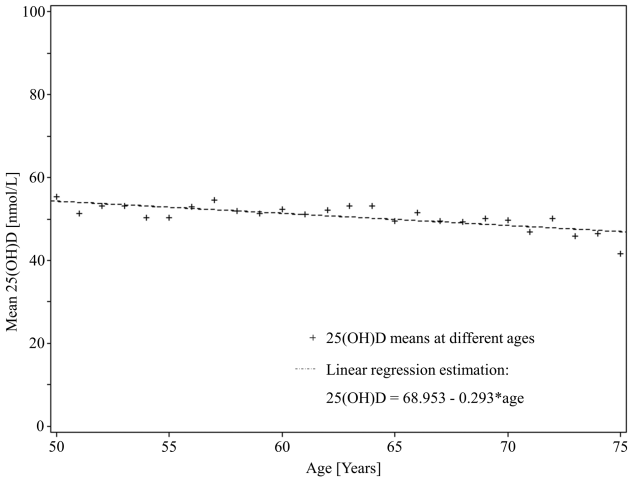

Figure 1. Trend of mean 25-hydroxyvitamin D (25(OH)D) levels with increasing age

a study with 65 patients with diabetes mellitus or impaired fasting glucose observed a statistically significant inverse association (26). The latter study also showed an inverse association of 25(OH)D levels with oxidized LDL (oxLDL) and nitric oxide metabolic pathway products (NOx) but the latter was not statistically significant (26). Moreover, a randomized controlled trial (RCT) with diabetes patients, which administered a vitamin D_3 fortified Persian yogurt drink to the verum group and a nonfortified yogurt drink to the placebo group twice a day for 12 weeks, observed significantly increased serum 25(OH)D, glutathione and total antioxidant capacity levels and significantly decreased malondialdehyde levels in the verum compared to the placebo group (27).

The antioxidative mechanism of 25(OH)D was nicely shown in an experiment with human umbilical vein endothelial cells (28). These cells were exposed to H_2O_2 and pretreatment with vitamin D was able to reduce superoxide anion production. These antioxidative capacities of vitamin D can explain the attenuation of the association of 8-isoprostane levels and mortality after 25(OH)D levels were added to the model (The effect estimate for comparison of quartile 3 and 1 lost statistical significance [Table 2]). However, the attenuation of the effect estimate for comparison of 8-isoprostane level quartile 4 and 1 was weak and the association of 25(OH)D levels and mortality even got stronger. Therefore, further mechanisms in addition to

Table 2. Associations of Aging Markers Alone and Combined in One Model With All-Cause Mortality

Aging Marker	Each Marker Alone (N between 1,547 and 9,940)[a]		All markers in one model (n = 1,516)[b]	
	N_{deaths}[c] (%)	HR (95% CI)[d]	N_{deaths}[c] (%)	HR (95% CI)[d]
Horvath's epigenetic age acceleration (years)				
Q1 (−21.118-<−3.364)	96 (24.8)	Ref	95 (25.2)	Ref
Q2 (−3.364-<−0.009)	97 (25.1)	1.13 (0.84; 1.52)	95 (25.5)	1.16 (0.84; 1.58)
Q3 (−0.009-<3.369)	102 (26.3)	**1.41 (1.04; 1.90)**	102 (26.6)	**1.47 (1.04; 2.06)**
Q4 (3.369–28.853)	92 (23.8)	1.22 (0.87; 1.70)	90 (23.6)	1.17 (0.80; 1.71)
Hannum's epigenetic age acceleration (years)				
Q1 −4.954-<3.056)	90 (23.3)	Ref	89 (23.6)	Ref
Q2 (3.056-<6.041)	90 (23.4)	0.95 (0.69; 1.29)	89 (23.7)	0.95 (0.68; 1.32)
Q3 (6.041-<9.924)	98 (25.2)	1.02 (0.74; 1.41)	98 (25.6)	0.93 (0.65; 1.33)
Q4 (9.024–23.493)	109 (28.2)	1.21 (0.87; 1.70)	106 (27.8)	1.05 (0.71; 1.53)
8-isoprostane (nmol/mmol crea)				
Q1 (0.01-<0.16)	470 (18.9)	Ref	109 (23.3)	Ref
Q2 (0.16-<0.21)	487 (19.6)	1.05 (0.92; 1.20)	82 (22.0)	0.89 (0.64; 1.23)
Q3 (0.21-<0.28)	554 (22.3)	**1.19 (1.05; 1.36)**	83 (23.9)	1.02 (0.74; 1.41)
Q4 (0.28–8.08)	700 (28.1)	**1.46 (1.29; 1.66)**	108 (33.0)	**1.39 (1.01; 1.91)**
LTL (kilobase pairs)				
Q1 (4.7-<5.8)	211 (23.7)	**1.44 (1.11; 1.87)**	57 (23.4)	**1.61 (1.02; 2.56)**
Q2 (5.8-<6.2)	245 (27.3)	**1.37 (1.10; 1.70)**	108 (30.1)	**1.42 (1.02; 1.99)**
Q3 (6.2-<6.7)	212 (24.7)	1.17 (0.95; 1.44)	111 (26.4)	1.02 (0.75; 1.39)
Q4 (6.7–9.4)	191 (20.9)	Ref	106 (21.5)	Ref
25(OH)D (nmol/L)				
Q1 (7.0-<34.3)	687 (27.4)	**1.47 (1.29; 1.67)**	126 (31.0)	**1.59 (1.14; 2.22)**
Q2 (34.3-<45.7)	532 (21.8)	**1.21 (1.06; 1.38)**	80 (23.1)	1.28 (0.91; 1.79)
Q3 (45.7-<61.6)	504 (20.2)	1.06 (0.93; 1.21)	82 (22.6)	0.97 (0.70; 1.35)
Q4 (61.6–330.7)	488 (19.6)	Ref	94 (23.5)	Ref

Note: CI = Confidence interval; crea = Creatinine; HR = Hazard ratio; LTL = Leucocyte telomere length; N_{deaths} = Number of deaths; Q = Quartile; Ref = Reference; 25(OH)D = 25-hydroxyvitamin D.

Bold print: Statistically significant association ($p < .05$).

[a]Total study populations varied for the analyses depending on available measurements of the respective aging marker. Analyses on epigenetic age acceleration, 8-isoprostane levels, LTL and 25(OH)D levels were conducted with 1,547, 9,940, 3,546 and 9,940 study participants respectively. [b]Total study population for this analysis is n = 1,516 because subjects with missing epigenetic age acceleration or LTL measurement were excluded. Measurement for 8-isoprostane and 25(OH)D levels were complete. [c]Exemplarily number of deaths in first imputed data set is shown. [d]Cox proportional hazards regression model adjusted for chronological age, sex, body mass index, education, smoking behavior, physical activity, history of cancer, history of CVD, season (in models incl. 25(OH)D), batch (in models incl. epigenetic age acceleration or LTL), and leukocyte subtype distribution (in models incl. epigenetic age acceleration).

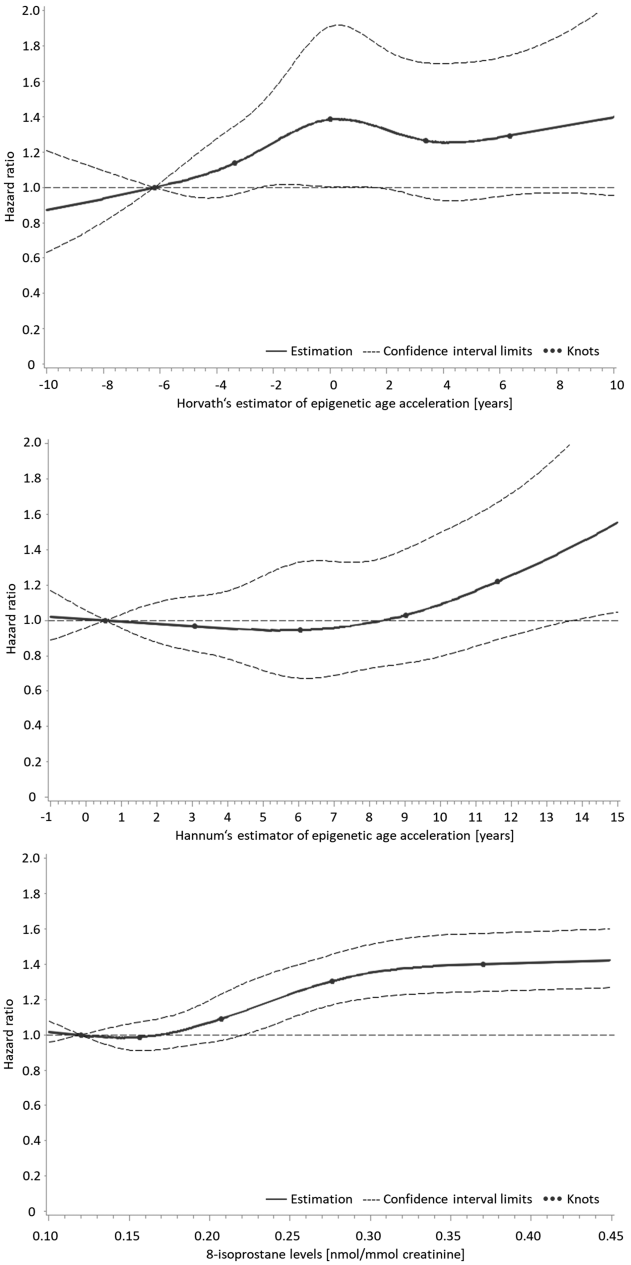

Figure 2. Dose–response curve of the positive associations of Horvath's and Hannum's estimators of epigenetic age acceleration and 8-isoprostane levels with all-cause mortality. *Note*: The curves were estimated with a Cox proportional hazards regression model adjusted for chronological age, sex, body mass index, education, smoking behavior, physical activity, history of cancer and history of CVD (and additionally for epigenetic age acceleration: batch and leukocyte subtype distribution). Knots were placed on the 10th, 25th, 50th, 75th, and 90th percentile of the biomarkers and the 10th percentile was used as the reference.

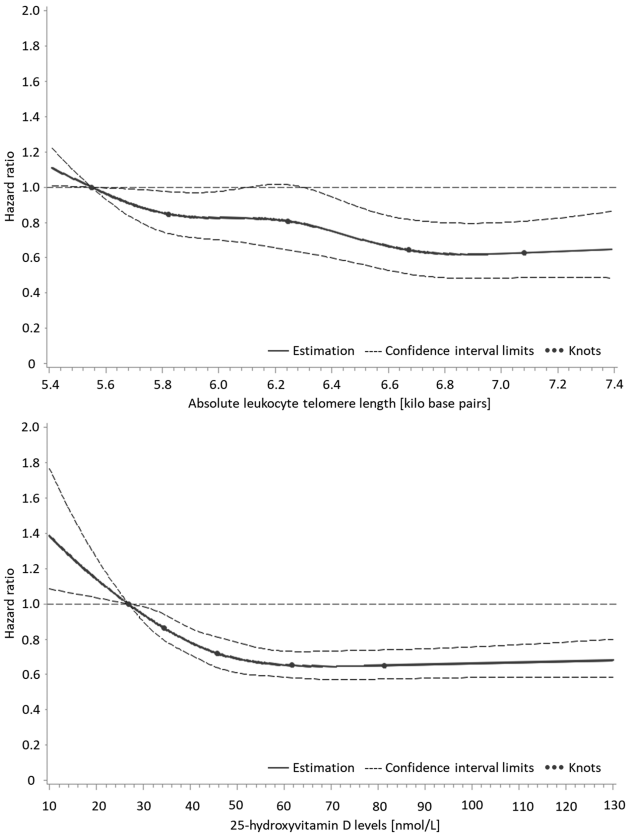

Figure 3. Dose–response curve of the inverse associations of leukocyte telomere length and 25-hydroxyvitamin D levels with all-cause mortality. *Note*: The curves were estimated with a Cox proportional hazards regression model adjusted for chronological age, sex, body mass index, education, smoking behavior, physical activity, history of cancer and history of CVD (and additionally for leukocyte telomere length: batch). Knots were placed on the 10th, 25th, 50th, 75th, and 90th percentile of the biomarkers and the 10th percentile was used as the reference.

its antioxidative capacities need to exist to explain the association of 25(OH)D and mortality and several are currently being discussed, which include effects on cell proliferation, differentiation and apoptosis, signaling pathways, mitochondrial function, and a modulation of the immune system (29–31).

The active hormone 1,25-hydroxyvitamin D3 ($1,25(OH)_2D$) can bind to the vitamin D receptor (VDR), which is present on virtually all cell types (29). Once dimerized, the $1,25(OH)_2D$-VDR complex can induce expression of genes associated with cellular proliferation, differentiation and apoptosis and thus contribute to cellular aging via cell-cycle regulation (31).

Another important function of $1,25(OH)_2D$ in cellular aging is mediated by the renal hormone α-klotho. Together, $1,25(OH)_2D$ and α-klotho maintain important signaling pathways, including the insulin-like growth factor 1 (IGF-1) (aging suppression), Wnt (stem cell proliferation) and Nrf2 (protection of cells against oxidative stress) pathway, which all have been shown to have an impact on the life span in animal models (30–32).

Furthermore, vitamin D deficiency may also be involved in mitochondrial dysfunction, a key process in aging leading to increased ROS production (33). By regulating the level of Ca^{2+} in mitochondria through its ability to promote the expression of Ca^{2+} pumps and Ca^{2+} buffers, $1,25(OH)_2D$ may promote autophagy, a process of removing damaged proteins and malfunctioning organelles from cells (28, 30, 34). These are the most plausible pathways but vitamin D deficiency could contribute to mitochondrial dysfunction also via other mechanisms and these are being discussed in detail elsewhere (30).

In addition, $1,25(OH)_2D$ has been ascribed immunosuppressive effects with reduction of lymphocyte proliferation and inhibition of the production of proinflammatory cytokines (35). We previously postulated the "resilience factor hypothesis" stating that sufficient

Table 3. Joint Association of Aging Markers (Horvath's estimator of epigenetic age acceleration, leukocyte telomere length, 8-isoprostane levels, and 25(OH)D concentrations) With All-Cause Mortality

No. of Aging Markers With Values in an Unfavorable Range[a]	N_{total}[b]	N_{deaths} (%)	HR (95% CI)[c]
0	140	28 (20.0)	Ref
1	581	124 (21.3)	**1.35 (0.83; 2.22)**
2	527	143 (27.1)	**2.19 (1.25; 3.83)**
3	231	72 (31.2)	**2.86 (1.50; 5.47)**
4	37	15 (40.5)	**4.34 (1.83; 10.30)**

Note: CI = Confidence interval; HR = Hazard ratio; N_{deaths} = Number of deaths; N_{total} = Total sample size; Ref = Reference; 25(OH)D = 25-hydroxyvitamin D.

Bold print: Statistically significant association ($p < .05$).

[a]Based on the preceding results in Table 2, the unfavorable ranges were defined as top three quarter of Horvath's estimator of epigenetic age acceleration distribution, bottom half of LTL distribution, top quartile of 8-isoprostane distribution and bottom quartile of 25(OH)D level distribution. [b]Exemplarily number of deaths in first imputed data set is shown. Total study population for this analysis is n = 1,516 because subjects with missing epigenetic age acceleration or LTL measurement were excluded. [c] Cox proportional hazards regression model adjusted for chronological age, sex, body mass index, education, smoking behavior, physical activity, history of cancer, history of CVD, season, and LTL measurement batch.

levels of $1,25(OH)_2D$ may be needed as a regulator of the response of the immune system when it is challenged by severe diseases to prevent a fatal course of the disease (eg, by down-regulation of the production of proinflammatory cytokines and ROS production) (36).

This is just a selection of the most promising mechanisms that could explain potential effects of $1,25(OH)_2D$ on the life span. The final proof that vitamin D deficiency is a cause for premature death can only origin from RCTs. Two systematic reviews on RCTs, evaluating the efficacy of vitamin D supplementation on mortality, already confirmed that vitamin D supply has an effect on mortality but the effects were weaker than suggested by observational studies and vitamin D supplementation seemed to be only effective for the administration of vitamin D_3 in subjects with low 25(OH)D levels at baseline (37). Our group recently estimated how strongly an untargeted approach reduces the statistical power compared to a trial that focuses on subjects with low 25(OH)D levels (38). If future RCTs focus on subjects with vitamin D deficiency, the gap between results from observational studies and RCTs may not be as large as it currently seems. Further evidence for a causal association of vitamin D deficiency and mortality is available from a Mendelian randomization study (39).

Some limitations have to be kept in mind when interpreting our study results. The main limitation of our prospective cohort study is its observational nature and residual confounding cannot be completely excluded. A further limitation is the unavailability of repeated measurements of the biomarkers of aging, which could have led to an underestimation of effect estimates. However, with respect to 25(OH)D, we previously reported that this biomarker has a relatively low intraindividual variability and repeated measurements analysis did not lead to other results for the endpoint mortality than the conventional approach of using only one-time measurements from the baseline examination (7). Strengths of our study are the large size of the cohort, a large number of events identified via an almost complete nation-wide registry-based follow-up and availability of the multiple established aging markers.

We conclude that the 25(OH)D level can be regarded as an aging marker because it was linearly associated with age and its association with mortality was independent of other established aging markers. Low 25(OH)D predicted increased mortality beyond mortality increases associated with low LTL, high epigenetic age acceleration, and high 8-isoprostane levels. The mechanisms linking vitamin D to healthy aging are unique and can neither be fully explained by aging of the epigenome, the telomere length theory, nor the oxidative stress theory of aging.

Supplementary Material

Supplementary data is available at *The Journals of Gerontology, Series A: Biological Sciences and Medical Sciences* online.

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Conflict of Interests

None reported.

Authors' Contributions

B.S. and L.H. wrote research proposal and conducted data analyses. B.S. wrote first draft of the manuscript. Y.Z. computed Horvath's epigenetic clock and leukocyte composition. X.G. conducted 8-isoprostane measurements. B.H. collected cancer data. H.B. contributed to data collection as principal investigator of the ESTHER study. All authors contributed intellectual input to parts of the final version of the manuscript.

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