

Effects of Vitamin D₃ supplementation on epigenetic aging in overweight and obese African Americans with suboptimal vitamin D status: a randomized clinical trial

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Abstract

Background: We have previously shown that vitamin D supplementation increases telomerase activity, suggesting an anti-aging effect. In this study, we aim to test the hypothesis that vitamin D supplementation would slow down epigenetic aging, a new marker of biological aging.

Methods: A randomized clinical trial was previously conducted among 70 overweight/obese African-Americans with serum 25-hydroxyvitamin D [25(OH)D] < 50 nmol/L, who were randomly assigned into four groups of 600 IU/day, 2000 IU/day, 4000 IU/day of vitamin D₃ supplements or placebo followed by 16-weeks interventions. Whole genome-wide DNA methylation analysis was conducted in 51 participants. DNA methylation ages were calculated according to the Horvath and the Hannum methods. Methylation-based age acceleration index (Δ Age) is defined as the difference between DNA methylation age and chronological age in years. Mixed-effects models were used to evaluate the treatment effects.

Results: Fifty-one participants (aged 26.1 ± 9.3 years, 16% are male) were included in the study. After the adjustment of multi-covariates, vitamin D₃ supplementation of 4000 IU/day was associated with 1.85 years decrease in Horvath epigenetic aging compared with placebo (p-value=0.046), and 2000 IU/day was associated with 1.90 years decrease in Hannum epigenetic aging (p-value=0.044). Serum 25(OH)D concentrations were significantly associated with decreased Horvath Δ Age only (p-values = 0.002), regardless of treatments.

Conclusions: Our results suggest that vitamin D supplementation may slow down Horvath epigenetic aging. But the effect on Hannum epigenetic aging is not

conclusive. Large-scale and longer duration clinical trials are needed to replicate the findings.

Keywords: 25-hydroxyvitamin D, DNA methylation age, vitamin D insufficiency.

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Introduction

In recent years, the concept of epigenetic aging has emerged. In 2013, Horvath's epigenetic clock was developed by Horvath *et al.* which predicts DNA methylation (DNAm) age based on 353 CpG sites¹. In the same year, Hannum *et al.* also formulated a quantitative model of aging based on the methylation levels of 71 CpG sites². Since then, epigenetic aging has been associated with several aging diseases, such as obesity^{3,4}, Down syndrome⁵, Huntington's disease⁶, Werner syndrome⁷ and schizophrenia in observational studies⁸. Whether epigenetic clock is sensitive to changes in response to a geroprotective intervention remains unknown.

DNAm age measures the cumulative effect of the epigenetic maintenance system. The difference between DNAm age and chronological age has been defined as an index of disproportionate aging. Although it is not yet fully understood what exactly is measured by DNAm age, its relation to individual health and longevity has been observed in several studies. DNAm age acceleration is found to be related to increased incidence of frailty, dementia, cancer and mortality⁹⁻¹³.

Some factors are suggested to influence epigenetic aging. Epigenetic age accelerators were found in observational studies, which include obesity^{4,14}, virus infection¹⁵, stress^{16,17} and exposure to violence¹⁸. Fish intake¹⁴ and exercise¹⁴ may slow down epigenetic aging. Beneficial effects of vitamin D have been widely studied for decades, including improving bone¹⁹ and cardiovascular health²⁰⁻²², reducing the risk of diabetes^{23,24}, autoimmune disease²⁵⁻²⁷, cancer²⁸, and even overall mortality²⁹. Recent evidence suggests that epigenetic regulation may be one of the underlying mechanisms. We have previously conducted a series of epigenome-wide association studies in youth and young adults, and have shown that vitamin D deficiency is

associated with global DNA hypomethylation³⁰ and locus-specific leukocyte DNA methylation changes³¹. In addition, we found that 16 weeks of vitamin D₃ supplementation increased global methylation level in a dose-dependent manner³⁰. Furthermore, we demonstrated that 16 weeks of vitamin D₃ supplementation increases telomerase activity, suggesting an anti-aging property³².

Therefore, in this study we tested the hypothesis that vitamin D supplementation would slow down epigenetic aging by taking advantage of a previously completed randomized clinical trial (RCT) among overweight/obese African-Americans with suboptimal vitamin D status [25(OH)D < 50 nmol/L].

Materials and Methods

Participants

As previously described³³, 70 overweight/obese African-Americans residing in Augusta, Georgia and surrounding areas were randomized into a double-blinded and placebo-controlled clinical trial (NCT01583621) during December 2011 and November 2012. Inclusion criteria were self-reported African-American race, aged between 13-45 years, overweight or obese (BMI ≥ 25 kg/m² for adults and $\geq 85^{\text{th}}$ percentile for age and sex otherwise), no pregnancy, no known acute or chronic illnesses, no use of any prescription medications, birth control pills, herbal, multi-vitamin or mineral supplementations, and suboptimal vitamin D status [serum 25(OH)D concentrations of ≤ 50 nmol/L]. A total of six participants were lost during the follow-up. The genome-wide DNA methylation was performed in 64 subjects with DNA available samples. Fifty-one participants (aged 26.1 ± 9.3 years, 16% are male) with 99 observations were finally included in this study with completed data of both DNA methylation data and laboratory measurements, among which 48 participants

had both baseline and posttest data and were included in the pre-post paired tests, while another 3 participants only had baseline data or posttest data. In accordance with the intention to treat principle, all 99 observations were included in the mixed-effects models³⁴. Informed consents were obtained from the adults and the guardians of adolescents. The study was approved by the institutional review board (IRB) at the Augusta University.

Randomization and treatments

The participants were randomly assigned to any one of the four groups 18,000 IU/month (~600 IU/day), 60,000 IU/month (~2000 IU/day), 120,000 IU/month (~4000 IU/day) of vitamin D₃ or placebo, and the interventional capsules were provided to the participants by supervised dosing for 16-weeks to maximize compliance. The vitamin D₃ and placebo capsules were provided by the Bio-Tech Pharmacal, Fayetteville, AR, and the AU clinical research pharmacy generated the randomization codes and dispensed the study capsules. The AU clinical pharmacy maintained the randomization codes until the end of the study and did not have any direct role in the data collection³³.

Measurements and laboratory assessments

Height and weight were obtained according to standard procedures and BMI was calculated as weight (kg) divided by height (m²). Fasting blood samples were obtained at baseline and 16-week, which were frozen and stored at -80 °C until assayed. Serum 25(OH)D concentrations were measured using enzyme immunoassay (Immunodiagnostic Systems, Fountain Hills, AZ). The intra- and inter-assay coefficients of variation (CV) were 5.6 and 6.6 %, respectively. Our laboratory is certified by the vitamin D₃ external quality assessment scheme (DEQAS), an

international program monitoring accuracy of 25(OH)D measurements. Peripheral blood was collected and sent to the clinical pathology core lab at the Medical College of Georgia within 2 hours for the complete blood count with differential, which included the total leukocyte count and percentages of peripheral blood cell types including neutrophils, lymphocytes, monocytes, eosinophils and basophils. Immune phenotype (CD3+, CD4+ and CD8+) was performed using flow cytometry.

Genome-wide DNA methylation and DNAm age calculation

DNA was extracted from stored buffy coat samples using the QIAamp DNA Mini Kit (QIAGEN, Valencia, CA). Genome-wide DNA methylation levels were analyzed by the Illumina Infinium Human Methylation 850K Beadchip (Illumina Inc., Denver, CO). In the quality control stage, DNA methylation data was processed using the Minfi package³⁵. Detectable probes were defined as the probes with detection p-value < 1E-16 in more than 95% samples; detectable samples were those with detection p-value < 1E-16 in more than 95% CpG sites.

Horvath DNAm age is defined as a prediction of age based on the DNA methylation levels of 353 CpG sites using the statistical pipeline developed by Horvath where background-corrected β values were pre-processed using the calculator's internal normalization method¹. Hannum DNAm age is calculated based on 71 CpG sites using the prediction function developed by Hannum and colleagues². Beta values were normalized via the beta-mixture quantile normalization method³⁶.

Methylation-based age acceleration index (Δ Age) is defined as the difference between DNA methylation age and chronological age in years (Δ Age = DNAm age – chronological age). Therefore, a negative Δ Age suggests that an individual is biologically younger than the chronological age, while a positive Δ Age means

accelerated aging. DNA methylation data processing and DNAm age calculation were accomplished using R version 3.4.0 (R Foundation for Statistical Computing Vienna, Austria).

Statistical analysis

The general characteristics of the subjects are presented as mean $\pm$ standard deviation (SD) for continuous variables and N (%) for categorical variables. Normality of each continuous variable was tested based on a combination test statistics of skewness and kurtosis. Baseline group differences were determined by analysis-of-variance (ANOVA) for normally distributed variables or by Kruskal-Wallis test, otherwise. Group differences in proportions at baseline were tested by Fisher's exact test. To test for changes of continues measurements from baseline to posttest, two-tailed paired t-test was conducted for variables with normal distribution. Wilcoxon matched-pairs signed-ranks test was used instead to test for non-normal distributed variables.

Mixed-effects regression models for repeated measures were used in an intention-to-treat analysis using all available data. Models for epigenetic aging included the fixed effects of intervention groups (placebo, 600 IU/day, 2000 IU/day, or 4000 IU/day), measurement time (baseline or posttest) and their interaction. Participant nested within group was considered a random effect. Previous studies have shown that male³⁷, obesity³ and immune function³⁸ may be associated with the aging process. In addition, a strong negative association between CD4 cells and Hannum aging predictor has been observed in previous studies⁹. Therefore, the models were also adjusted for gender, BMI and CD4 percent. As shown in this study as well as other studies³⁹, since vitamin D may also affect the immune function, so to estimate the total effect of vitamin D₃ supplementation on epigenetic aging, only the

baseline level of CD4 percent was included. Month of visit was also included in the model to account for the seasonal effect. Baseline 25(OH)D concentration was also included in the regression model. Khoury *et al.* observed that a decrease in slope of the predicted epigenetic age by Horvath clock took place at approximately 60 years, indicating that some loci in the model may change differently with age and that age acceleration measures will themselves be age-dependent. Thus age was further included in the regression model ⁴⁰.

We also tested the relationship between serum 25(OH)D and DNAm age. Mixed-effects models were carried out using natural log transformed 25(OH)D concentration as the independent variable, and adjusted for sex, BMI and CD4 percent.

As we assume that the causal effects of the vitamin D₃ supplementations are mediated by increasing the serum concentrations of 25(OH)D, we further tested the effects using the contamination adjusted intention to treat (CA ITT) technique ⁴¹. Instrumental variable (IV) regression analysis was carried out, which took serum concentration of 25(OH)D as endogenous variable, and intervention groups as IVs estimated by limited-information maximum likelihood (LIML). All statistical analysis was performed using Stata version 12.0 (College Station, Texas 77845 USA).

Results

General characteristics and DNA methylation age

Demographics of the participants are shown in Table 1. Fifty-one individuals were included (aged 26.1 ± 9.3 years, 16% are male), 48 of whom had both baseline data and posttest data. Overall DNAm ages were significantly correlated with

chronological age (Horvath DNAm age $r=0.90$, p -value < 0.001 , Hannum DNAm age $r=0.82$, p -value < 0.001) as shown in Figure 1 and Figure 2. The correlations were higher in the baseline (Horvath $r=0.92$, p -value < 0.001 , Hannum DNAm age $r=0.82$, p -value < 0.001) than those at posttest (Horvath $r=0.88$, p -value < 0.001 , Hannum DNAm age $r=0.81$, p -value < 0.001) in both DNAm ages. There was no difference in age, DNAm ages, Horvath Δ Age, gender, BMI, 25(OH)D, CD4 percent and CD4 count among the four groups at baseline (p -values > 0.05). However, Hannum Δ Age was significantly different among the four groups ranging from -0.7 years in placebo group to 5.6 years in 2000 IU/day group (p -value=0.012).

Changes in epigenetic aging and serum 25(OH)D from pre to post vitamin D₃ supplementation

In Table 2, we compared the changes in Δ Age and 25(OH)D concentration before and after treatment. Average serum 25(OH)D concentration was significantly raised by 22.10, 47.01 and 47.20 nmol/L in 600 IU/day, 2000 IU/day and 4000 IU/day groups (p -values < 0.001) as we previously reported⁴². Horvath Δ Age was decreased by 1.83 years (p -value = 0.002) and 1.62 years (p -value = 0.031) in 2000 IU/day and 4000 IU/day groups after the treatment, respectively. Changes in Hannum Δ Age were not significant (p -values >0.05).

Effects of vitamin D₃ supplementation on epigenetic aging

In Table 3, model 1 reassigned participants into two groups (assignment 1), which were group 1 including placebo and 600 IU/day, and group 2 including 2000 IU/day and 4000 IU/day. Assignment 2 in model 2 was the original assignment of this study, which split the participants into four groups. Effect 1 derived from model 1 was the interaction term of wave and assignment 1, the coefficient of which represented the

treatment effect of group 2 compared with group 1. Effect 2 derived from model 2 was the interaction of wave and assignment 2, which reflected dose-response of vitamin D₃ supplementation on epigenetic aging. Model 1 and model 2 were both adjusted for sex, BMI, group assignment, measure time, month of visit, baseline 25(OH)D concentration and CD4 percent. In model 1, treatment was associated with 1.49 years (p-value = 0.023) decrease in Horvath Δ Age, not with Hannum Δ Age. In model 2, vitamin D₃ supplementation was also associated with decreased Δ Age in both epigenetic clocks, but only significant with Horvath Δ Age in 4000 IU/day group (p-value = 0.046), and Hannum Δ Age in 2000 IU/day group (p-value = 0.044). We further adjusted for chronological age in the models, and the effects of vitamin D₃ supplementation on epigenetic aging remained unchanged. A set of regressions were also performed to investigate the interaction of age by including the interaction terms of chronological age and treatment, which were not significant.

Association between serum 25(OH)D concentration and epigenetic aging

In Table 4, regardless of treatment, the increase in serum 25(OH)D concentrations were only significantly associated with decreased Horvath Δ Age (p-values = 0.002), and the association remained significant after the adjustment of gender, BMI and CD4 percent (p-values = 0.002). There was no association between Hannum clock and changes in serum 25(OH)D concentrations.

Dose-effect of vitamin D₃ supplementation on the Horvath epigenetic aging

mediated by the changes of serum 25(OH)D concentration

To test whether serum 25(OH)D mediated the dose-response of vitamin D₃ supplementation on epigenetic aging, we conducted the IV regression analysis using

the Horvath clock since that only the Horvath clock was associated with serum 25(OH)D as mentioned above. The dose responsive increases in serum 25(OH)D concentration induced by vitamin D₃ supplementations were significantly associated with the decreased Horvath epigenetic aging in both IV regression models, which used assignment 1 as IV in model 1 (p-value = 0.019), and assignment 2 as IV in model 2 (p-value = 0.038).

Discussion

In this study, we found that the DNAm age estimated by either Horvath clock or Hannum clock was highly correlated with chronological age in African Americans with suboptimal vitamin D status at the baseline. Vitamin D₃ supplementation decreased DNA methylation aging. More specifically, the epigenetic aging was significantly decreased by 4000 IU/day vitamin D₃ supplementation according to the Horvath clock, and 2000 IU/day was more effective according to the Hannum clock. Moreover, serum 25 (OH)D levels were only negatively associated with DNA methylation aging measured by the Horvath clock.

Studies in mice suggest that caloric restriction slows epigenetic aging and DNA methylation drift^{43,44}. However, whether epigenetic clock changes in response to a geroprotective intervention in humans remains unknown. To the best of our knowledge, this is the first study to evaluate the effect of vitamin D₃ supplementation on epigenetic aging in humans using a RCT design.

DNAm age is a prediction of age based on individual DNA methylation levels with high accuracy¹, and has been employed by a number of studies and validated with a high correlation between DNAm age and chronological age^{8,45}. In the present

study, we observed that DNA methylation age calculated by either the Horvath method or the Hannum method was both highly correlated with chronological age in African Americans. The unadjusted correlation analysis showed that this correlation was higher at the baseline than posttest, suggesting that the vitamin D₃ supplementation may have driven the DNAm age to deviate from the chronological age.

The present study explored the treatment effect of vitamin D₃ supplementation on three doses (600 IU/day, 2000 IU/day and 4000 IU/day). Compared with placebo, both 2000 IU and 4000 IU group decreased epigenetic aging by either clock. However, the associations between vitamin D₃ supplementations and epigenetic aging were different between the Horvath and Hannum clocks. The epigenetic aging was significantly decreased by 4000 IU/day vitamin D₃ supplementation according to the Horvath clock, and 2000 IU/day was more effective according to the Hannum clock. This discrepancy may be due to the fact that only 6 CpG sites overlap between these two epigenetic clocks⁹. In addition, they were built to represent different tissues. Horvath is a multi-tissue clock built to capture more variation while Hannum only applies to blood leukocytes⁴⁵. It may also due to the small sample size. In addition, the increase in serum 25(OH)D concentration by vitamin D₃ supplementations was associated with the decrease in Horvath epigenetic aging regardless of groups. Future large-scale studies are needed to confirm our findings.

Accelerated epigenetic aging was considered a predictor of mortality⁴⁶, and was associated with higher all-cause mortality, cancer mortality and cardiovascular mortality¹⁰. Meta-analyses have shown that vitamin D supplementation was associated with decreased overall mortality^{47,48}. We have also shown that vitamin D

supplementation increases telomerase activity, which may help slow down the cellular aging process in the African American population³². The present study suggests that vitamin D supplementation may also slow down the epigenetic aging process.

Most of previous studies on epigenetic aging are cross-sectional, which cannot establish causal relationships between risk factors, such as nutrients, physical measurements and environments, and epigenetic aging, and unable to observe the changes of DNAm age overtime^{14,16}. Some studies have tracked down the longitudinal changes of epigenetic aging and transcriptional response, however, RCTs can establish causal relationships^{4,18,49,50}. In this study, an RCT was carried out and measurements were taken both before and after the intervention, which allowed us to observe the longitudinal changes of the epigenetic aging, and infer the causal relationship between vitamin D supplementation and epigenetic aging.

We tested the test-retest reliability of these two epigenetic clocks in our study (Supplementary Table S1). The coefficients of variation (CV) between the baseline and posttest measurements were 8.36% for the Horvath clock and 8.74% for the Hannum clock suggesting that the measurements of epigenetic clock between the two visits were highly correlated and reproducible. The correlation between the baseline and posttest of the Horvath clock was the highest in the placebo group, possibly due to that fact that vitamin D supplementation may have driven the DNAm age to deviate from the chronological age.

There are several strengths in our study. First, to the best of our knowledge, this is the first study to evaluate the effect of a nutritional intervention on epigenetic aging utilizing a randomized controlled clinical trial design, which could help to establish a causal relationship. Second, our study focused on the African American

population with suboptimal vitamin D status and who are prone to higher risk for age-related diseases⁵¹. However, our power for repeated measurements was estimated to be modest with 16% to detect the difference in Δ Age changes between placebo and the 4000IU/day group, and 70% to detect the difference in Δ Age changes between two groups (placebo/600 IU/day vs. 2000/4000IU/day). Of note, this is an ancillary study, which was not originally designed to test this hypothesis. Therefore, there is a considerable likelihood of both false-positive and false-negative results. Further large-scale studies are needed to validate our results and address dose-response effects of vitamin D supplement on epigenetic aging. We observed a decreased epigenetic aging by 16-week vitamin D supplementation in overweight African American population. Whether this geroprotective effect of vitamin D supplementation can be sustained over longer period is unknown. Also unknown is whether this anti-biological aging effect exists in other populations. Future longer term RCTs in other populations are warranted.

Biological aging measures have been proposed as proxies for extension of healthy lifespan. Belsky *et al.* found that biological aging measured by two other clinical algorithms are sensitive enough to detect effects of geroprotective therapy in a small randomized trial⁵². We were also able to detect significant changes in DNA methylation aging induced by vitamin D₃ supplementation in a relatively small RCT, which suggest that markers of biological aging such as epigenetic aging can be used as endpoints in RCTs that aim to extend healthy lifespan.

Conclusion

For the first time, our results suggest that vitamin D₃ supplementation may slow down the Horvath epigenetic aging process in overweight/obese African

Americans with suboptimal vitamin D status. But the effect on the Hannum epigenetic aging is not conclusive. Future longer term and large scale RCTs in other populations are warranted to validate our findings.

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Declarations of interest

None

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Figure legend

Figure 1. Correlation of Horvath DNA methylation age and chronological age.

Y-axis is Horvath DNAm age, and X-axis is chronological age. Round symbols represent baseline observations, while the triangle symbols represent posttest observations. Lines linking round symbols to triangle ones represent changes of each participant from baseline to posttest. Overall pairwise correlation coefficient of Horvath DNAm age and chronological age is 0.90 (p-value < 0.001). The correlation was higher in the baseline ($r=0.92$, p-value < 0.001) than posttest ($r=0.88$, p-value < 0.001).

Figure 2. Correlation of Hannum DNA methylation age and chronological age.

Y-axis is Hannum DNAm age, and X-axis is chronological age. Round symbols represent baseline observations, while the triangle symbols represent posttest observations. Lines linking round symbols to triangle ones represent changes of each participant from baseline to posttest. Overall pairwise correlation coefficient of Hannum DNAm age and chronological age is 0.82 (p-value < 0.001).

Tables

Table 1. Baseline characteristics among different groups

Characteristics	Total	Groups				p-values
		Placebo (N=11)	600 IU/day (N=12)	2000 IU/day (N=15)	4000 IU/day (N=12)	
Age (year)	26.1±9.3	30.9±10.5	26.9±10.2	24.1±8.7	23.6±7.1	0.347
Horvath DNAm age (year)	27.3±9.9	31.2±11.3	28.3±10.8	26.6±9.9	23.4±6.6	0.353
Horvath age acceleration index (year)	1.1±3.8	0.2±4.5	1.5±3.8	2.5±3.4	-0.2±3.2	0.676
Hannum DNAm age (year)	28.4±8.2	30.3±10.4	27.2±8.5	29.7±7.3	26.2±6.7	0.559
Hannum age acceleration index (year)	2.6±5.3	-0.7±4.1	0.3±4.9	5.6±5.4	2.6±4.8	0.012
Male (N)	8 (16)	3 (27)	2 (17)	2 (13)	1 (8)	0.719
BMI (kg/m ²)	35.2±6.9	36.2±7.7	34.2±6.1	35.6±6.6	34.9±7.9	0.784
25(OH)D	34.4±9.6	34.4±8.1	33.5±6.8	38.8±11.0	30.1±10.0	0.129

(nmol/L)						
CD4 percent						
(%)	46.6±6.3	46.4±4.2	47.4±7.5	45.4±6.2	47.5±7.3	0.705
CD4 counts						
(cell/mm ³)	861±293	960±324	758±244	824±289	920±307	0.333

Note: Statistics display as mean±SD for continuous variables, and N(%) for categorical variables. Baseline group differences of continuous variables were determined by ANOVA for normally distributed variables or by Kruskal-Wallis test, otherwise. Fisher's exact test was carried out on categorical variables.

Table 2. Changes of age acceleration index and serum 25(OH)D concentration between pre and post intervention among different treatment groups

Characteristics	Placebo (N=11)	600 IU/day (N=10)	2000 IU/day (N=15)	4000 IU/day (N=12)
Horvath age acceleration index (years)				
Pre	0.22±4.52	0.89±3.87	2.52±3.38	-0.17±3.19
Post	-0.42±5.63	1.52±4.14	0.69±3.40	-1.80±4.11
Difference	-0.64±2.46	0.63±2.68	-1.83±1.86	-1.62±2.27
p-values	0.410	0.477	0.002	0.031
Hannum age acceleration index				

(years)				
Pre	-0.69±4.06	0.62±5.02	5.65±5.52	2.56±4.75
Post	-0.28±4.19	1.19±4.04	4.45±5.31	2.30±5.01
Difference	0.40±2.65	0.58±1.97	-1.20±3.38	-0.26±5.54
p-values	0.623	0.375	0.192	0.728
25(OH)D (nmol/L)				
Pre	34.36±8.14	33.27±7.28	38.76±10.98	30.13±10.02
Post	41.27±13.67	55.37±11.48	85.77±31.54	77.33±14.79
Difference	6.91±10.79	22.10±12.86	47.01±27.03	47.20±16.64
p-values	0.060	<0.001	<0.001	<0.001

Note: Pre-post changes of continuous variables were determined by t-test for normally distributed variables or by Wilcoxon matched-pairs signed-ranks test, otherwise. Fisher's exact test was carried out on categorical variables.

Table 3. Effects of vitamin D₃ supplementation on Horvath and Hannum epigenetic aging based on mixed-effects models

		Horvath epigenetic aging				Hannum epigenetic aging			
		Model 1		Model 2		Model 1		Model 2	
		β (95% CI)	p	β (95% CI)	p	β (95% CI)	p	β (95% CI)	p
Effect 1									
Group 1									
(placebo, 600 IU/day)	Reference group			--	--	Reference group		--	--
Group 2									
(2000, 4000 IU/day)	-1.49 (-2.78, -0.21)	0.023	--			-1.08 (-2.46, 0.30)	0.124	--	--
Effect 2									
Placebo	--	--		Reference group		--	--	Reference group	
600 IU/day	--	--		0.14 (-1.86, 2.13)	0.892	--	--	-0.81 (-2.94, 1.33)	0.940

2000 IU/day	--	--	-1.07 (-2.81, 0.67)	0.228	--	--	-1.90 (-3.75, -0.05)	0.044
4000 IU/day	--	--	-1.85 (-3.66, -0.03)	0.046	--	--	-0.78 (-2.72, 1.16)	0.344
Female	-1.95 (-4.88, 0.98)	0.193	-2.23 (-5.04, 0.58)	0.120	0.34 (-3.67, 4.36)	0.868	0.40 (-3.52, 4.33)	0.840
BMI	0.14 (-0.00, 0.11)	0.050	0.13 (-0.01, 0.27)	0.067	0.06 (-0.13, 0.25)	0.532	0.03 (-0.16, 0.22)	0.723
Baseline 25(OH)D	0.003 (-0.10, 0.11)	0.950	-0.03 (-0.15, 0.08)	0.561	-0.09 (-0.23, 0.06)	0.248	-0.13 (-0.28, 0.03)	0.101
CD4 percent	-0.12 (-0.28, 0.03)	0.125	-0.12 (-0.27, 0.03)	0.117	-0.27 (-0.48, -0.06)	0.012	-0.25 (-0.46, -0.04)	0.017

Note: Totally 99 observations of 51 participants. Models were adjusted for sex, BMI, group assignment, measure time, month of visit, baseline 25(OH)D concentration and CD4 percent. Model 1 used assignment 1 that reassigned participants into two groups, which were group 1 including placebo and 600 IU/day, and group 2 including 2000 IU/day and 4000 IU/day. Effect 1 was the interaction term of

wave and assignment 1. Model 2 used assignment 2, which was the original design of this study. Effect 2 was the interaction of wave and assignment 2.

Table 4. Association between of changes in serum 25(OH)D concentration and Horvath and Hannum epigenetic aging based on mixed-effects models

	Horvath epigenetic aging				Hannum epigenetic aging			
	Model 1		Model 2		Model 1		Model 2	
	β (95% CI)	p	β (95% CI)	p	β (95% CI)	p	β (95% CI)	p
25(OH)D	-1.35 (-2.21, -0.48)	.002	-1.42 (-2.29, -0.54)	0.002	-0.27 (-1.28, 0.74)	0.598	-0.33 (-1.34e0.67)	0.514
Female	--	--	-1.12 (-4.28, 2.05)	0.489	--	--	0.81 (-3.20, 4.82)	0.693
BMI	--	--	0.09 (-0.05, 0.24)	0.220	--	--	0.09 (-0.09, 0.27)	0.320
CD4 percent	--	--	-0.15 (-0.32, 0.02)	0.089	--	--	-0.27 (-0.49, -0.05)	0.016

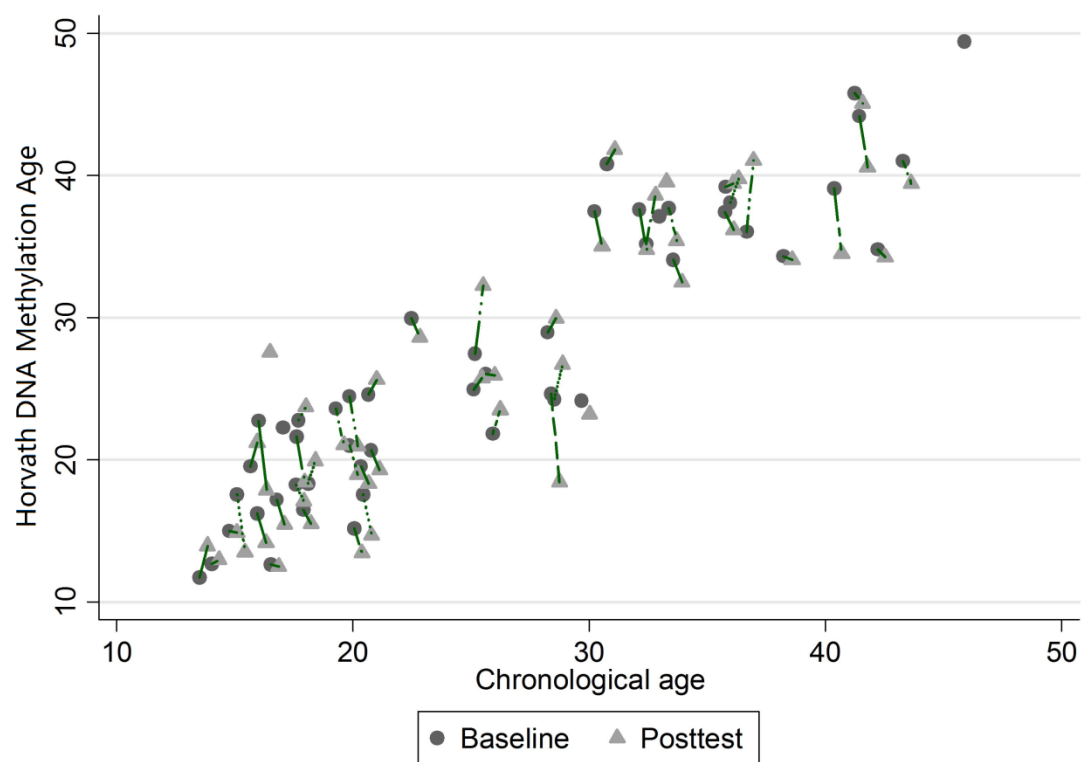
Note: Totally 99 observations of 51 participants. Model 1 was unadjusted, model 2 was adjusted for sex, BMI and CD4%. 25(OH)D concentration was natural log transformed.

Table 5. Associations between the changes of Horvath epigenetic aging and the changes of 25(OH)D concentration induced by vitamin D₃ supplementation based on instrumental variable models

	Model 1		Model 2	
	β (95% CI)	p	β (95% CI)	p
25(OH)D	-0.04 (-0.08, -0.01)	0.019	-0.04 (-0.08, -0.00)	0.038
Female	-2.89 (-4.96, -0.81)	0.006	-2.89 (-4.93, -0.85)	0.005
BMI	-0.02 (-0.14, 0.10)	0.764	-0.02 (-0.13, 0.10)	0.781
Baseline 25(OH)D	-0.02 (-0.11, 0.07)	0.672	-0.02 (-0.10, 0.07)	0.661
CD4 percent	0.05 (-0.08, 0.17)	0.476	0.05 (-0.07, 0.17)	0.440

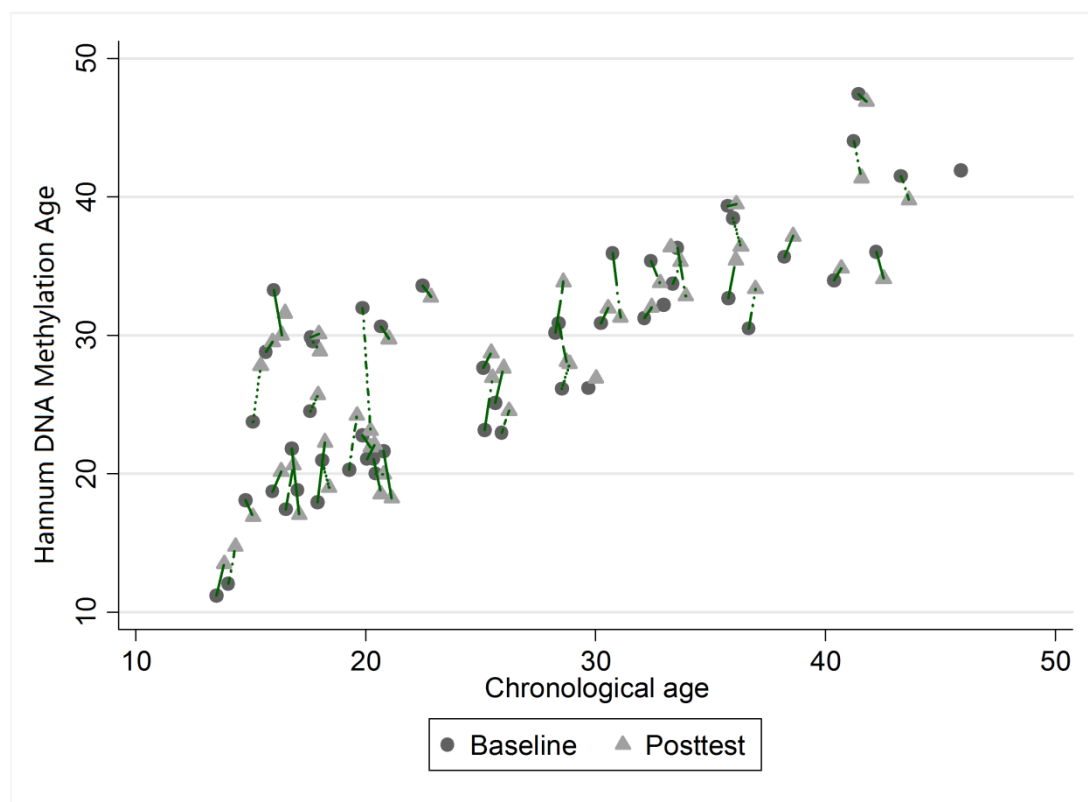
Note: Model 1 used assignment 1 as instrumental variable that reassigned participants into two groups, which were group 1 including placebo and 600 IU/day, and group 2 including 2000 IU/day and 4000 IU/day. Model 2 used assignment 2 as instrumental variable, which was the original design of this study. 25(OH)D concentration was natural log transformed.

Figure 1



Accepted

Figure 2



Accepted