Original Article

Associations of Plasma Folate and Vitamin B₆ With Blood DNA Methylation Age: An Analysis of One-Carbon Metabolites in the VA Normative Aging Study

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Abstract

One-carbon metabolism is an important contributor to aging-related diseases; nevertheless, relationships of one-carbon metabolites with novel DNA methylation-based measures of biological aging remain poorly characterized. We examined relationships of one-carbon metabolites with 3 DNA methylation-based measures of biological aging: DNAmAge, GrimAge, and PhenoAge. We measured plasma levels of 4 common one-carbon metabolites (vitamin B₆, vitamin B₁₂, folate, and homocysteine) in 715 VA Normative Aging Study participants with at least 1 visit between 1999 and 2008 (observations = 1153). DNA methylation age metrics were calculated using the HumanMethylation450 BeadChip. We utilized Bayesian Kernel Machine Regression models adjusted for chronological age, lifestyle factors, age-related diseases, and study visits to determine metabolites important to the aging outcomes. Bayesian Kernel Machine Regression models allowed for the estimation of the relationships of single metabolites and the cumulative metabolite mixture with methylation age. Log vitamin B₆ was selected as important to PhenoAge ($\beta = -1.62$ years, 95% CI: $-2.28, -0.96$). Log folate was selected as important to GrimAge ($\beta = 0.75$ years, 95% CI: $0.41, 1.09$) and PhenoAge ($\beta = 1.62$ years, 95% CI: $0.95, 2.29$). Compared to a model where each metabolite in the mixture is set to its 50th percentile, the log cumulative mixture with each metabolite at its 30th ($\beta = -0.13$ years, 95% CI: $-0.26, -0.005$) and 40th percentile ($\beta = -0.06$ years, 95% CI: $-0.11, -0.005$) was associated with decreased GrimAge. Our results provide novel characterizations of the relationships between one-carbon metabolites and DNA methylation age in a human population study. Further research is required to confirm these findings and establish their generalizability.

Keywords: DNA methylation age, Epigenetic clock, Folate, One-carbon metabolism, Vitamin B₆

An immense body of literature supports the notion that diet is among the most important factors in determining human health and life span (1,2). A notable amount of this literature has explored how nutrient status is linked to human health, and has identified DNA methylation as one potentially important process (3,4). DNA

methylation is a biological process whereby methyl groups ($-\text{CH}_3$) are covalently added to cytosine nucleotides on DNA and subsequently affect DNA expression (5). Critical to DNA methylation maintenance—and other processes like biosynthesis and amino acid homeostasis—is one-carbon metabolism. One-carbon metabolism

is characterized by biological reactions involving the transfer of one-carbon groups and generates the methyl groups the cell uses to methylate DNA. Dietary nutrients involved in the one-carbon metabolism pathway (eg, folate, vitamin B₆, and vitamin B₁₂) are particularly important to DNA methylation because they are coenzymes needed for one-carbon metabolism and ultimately affect the physiologic availability of methyl groups (6). Although previous studies have demonstrated that DNA methylation varies with one-carbon metabolite concentrations, data describing relationships with health and life span-relevant DNA methylation-based markers—like DNA methylation age—are lacking (7,8).

DNA methylation age metrics are novel measures of biological age calculated using DNA methylation values from specific combinations of age-correlated CpG dinucleotides. These metrics have garnered a great deal of attention due to their ability to predict chronological age, disease risk, and mortality risk. Notably, there are 3 principal DNA methylation age measures that have demonstrated high accuracy in chronological age prediction as well as robust chronological age-independent disease and mortality relationships in a plethora of human population studies (9,10). DNAmAge is the oldest of the 3 metrics, is calculated using 353 CpGs, and is primarily viewed as a predictor of chronological age (11). DNA Phenotypic Age (PhenoAge) is the second of the 3 metrics, and is primarily viewed as a predictor of disease risk (12–14). PhenoAge was built utilizing 9 clinical variables (ie, albumin, creatinine, glucose, C-reactive protein, lymphocyte percent, mean cell volume, red cell distribution width, alkaline phosphatase, and white blood cell count), and can be calculated using 513 CpGs—41 of which are shared with DNAmAge (15). DNA GrimAge is the most novel of the 3 metrics and is a predictor of mortality risk. GrimAge is exceptional in its ability to predict all-cause time-to-death (Cox regression $p = 2.0 \times 10^{-75}$). GrimAge is computed using 1030 unique CpGs, a DNA methylation surrogate of cigarette pack-years, and DNA methylation surrogates for 7 plasma protein markers (adrenomedullin, beta-2-microglobulin, cystatin C, growth differentiation factor-15, leptin, plasminogen activator inhibitor-1, and tissue inhibitor metalloproteinases 1) (16).

At present, few studies have examined the relationships between plasma levels of dietary factors with DNA methylation age measures. Three important works using samples from the Women's Health Initiative report significant relationships of DNAmAge (17), PhenoAge (15), and GrimAge (16) with macrodietary factors (eg, consumption of red meat, fruits, vegetables, whole grains, etc.), and also explore relationships of micro dietary factors. In particular, these studies report significant negative correlations of plasma levels of carotenes, xanthines, and tocopherols with DNAmAge, PhenoAge, and GrimAge (15–17). Despite this growing body of work, the degree to which existing studies address one-carbon metabolites remains limited. Given the importance of one-carbon metabolism in the processes of aging and aging-related diseases—particularly with respect to DNA methylation—better characterization of its relationship with DNA methylation age is necessary to better inform future population studies. Any distinct relationships that can be described with respect to age prediction, disease risk, or mortality risk would be particularly informative. In the present analysis, we address this research gap by investigating the relationships of 4 plasma one-carbon metabolites (vitamin B₆, vitamin B₁₂, folate, and homocysteine) with blood DNA methylation age measures. Specifically, we utilize Bayesian Kernel Machine Regression (BKMR), a Bayesian variable selection framework (18), to select metabolites most important to DNAmAge, PhenoAge, and GrimAge. From the same BKMR models we are then able to assess the associations of single metabolites and

the cumulative metabolite mixture with the respective aging metrics, as well as account for any nonlinearities in the associations. The 4 one-carbon metabolites were previously measured in the VA Normative Aging Study (NAS) and we use the NAS cohort for this present study (19). To evaluate the robustness of our findings in the context of normal physiological standards, we also perform sensitivity analyses that evaluate these same relationships when metabolite observations are limited to their clinical reference ranges.

Method

Study Population

The study sample used in this analysis was drawn from active participants in the VA NAS, a longitudinal cohort study of aging. Beginning in 1963, the NAS recruited healthy male participants from the Greater Boston/New England area (20). Although the NAS is now a closed cohort of older community-dwelling men, every 3–5 years since recruitment participants have returned for in-person, follow-up study visits. At these onsite visits, participants report detailed information about lifestyle factors that may affect their health, undergo comprehensive outpatient medical examinations, and provide biospecimens (including blood). In the NAS, dropout, which has been less than 1% per year (21), predominantly occurs when participants move out of the study area. Our study sample consisted of participants who had all available metabolite, outcome, and clinical data. We had a total of 715 participants with 1153 observations (ie, study visits) between the years 1999 and 2008. Of these 715 participants, 307 (43%) had 1 visit, 378 (53%) had 2 visits, and 30 (4%) had 3 visits. Characteristics were not different between participants who had 1 visit versus those with multiple visits. All participants provided written informed consent to the VA Institutional Review Board, and human participants approval was granted by the VA and Harvard T.H. Chan School of Public Health Institutional Review Boards (protocol 14027-102).

Plasma One-Carbon Metabolite Assessment

Concentrations for all 4 metabolites (vitamin B₆, vitamin B₁₂, folate, and homocysteine) in plasma were determined using methods previously described (22,23). In short, fasting blood plasma was collected from each participant at his study visit and frozen at -80°C . Plasma vitamin B₁₂ and folate concentrations were measured by radioassay using a commercially available kit from Bio-Rad (Hercules, CA). Vitamin B₆ (as pyridoxal 5 phosphate, the activated form of vitamin B₆) was measured using an enzymatic method involving tyrosine decarboxylase (24). Plasma homocysteine was measured via fluorescence detection with HPLC (25). Coefficients of variation for the biochemical assays of B₆, B₁₂, folate, and homocysteine were 5.0%, 4.7%, 4.3%, and 4.0%, respectively.

Measuring DNA Methylation and Calculating Measures of DNA Methylation Age

At each NAS follow-up visit, whole blood samples were collected from each participant. Bisulfite conversion (EZ-96 DNA Methylation Kit, Zymo Research, Orange, CA) was performed on extracted DNA from the buffy coat of whole blood. The Infinium HumanMethylation450 BeadChip was used to measure the DNA methylation of CpG probes. To minimize batch effects and achieve a similar age distribution across chips and plates, chips were randomized across plates. We then used a 2-stage age-stratified algorithm to randomize samples. To achieve quality control, we removed

samples where more than 5% of probes had a beadcount less than 3 or more than 1% of probes had a detection p -value $> .05$. Following preprocessing of the remaining samples with Illumina-type background correction without normalization and normalization of the samples with dye-bias and BMIQ3 adjustments, we generated methylation beta values. Beta values represent the percentage of methylation for each of the ~480 000 CpG sites in the BeadChip array. Specifically, $\beta = \text{intensity of the methylated signal (M)} / [\text{intensity of the unmethylated signal (U)} + \text{intensity of the methylated signal (M)} + 100]$. DNA methylation beta values range from 0 (completely unmethylated) to 1 (completely methylated).

All measures of DNA methylation age were calculated using Horvath's publicly available online calculator (<http://dnamage.genetics.ucla.edu>). Horvath's initial DNAmAge was derived from an elastic net penalized regression run on multiple data sets of different cell and tissue types. After 21 369 CpG probes—shared by both the Illumina HumanMethylation27 and HumanMethylation450 BeadChip platforms—were regressed on a calibrated version of chronological age, the elastic net selected 353 CpGs that correlated with age (193 positively and 160 negatively). The model coefficients from these 353 CpGs were used by the calculator to predict the age of each DNA sample (ie, DNAmAge). The calculator maintains predictive accuracy (age correlation 0.97, error = 3.6 years) across almost all body tissues including blood and brain (11).

DNA phenotypic age (PhenoAge) was created by employing a Cox penalized model where the hazard of mortality was regressed on 42 clinical markers and chronological age (15). From this penalized regression model, 9 clinical variables (ie, albumin, creatinine, glucose, C-reactive protein, lymphocyte percent, mean cell volume, red cell distribution width, alkaline phosphatase, and white blood cell count) and chronological age were selected to create a phenotypic age score. In test data, this score was highly correlated with chronological age ($r = 0.94$) and every 1-year increase in age score was associated with a 9% increase in all-cause mortality. The authors then used a different data set and elastic net penalized regression to determine methylation CpG sites related to their phenotypic score. Again they restricted their analyses to 20 169 CpGs shared across all 3 Illumina BeadChip platforms (27K, 450K, EPIC). In the end, they identified 513 CpGs to create the DNAm Phenotypic Age (PhenoAge) measure. Forty-one of these CpGs are shared with DNAmAge.

To calculate DNA GrimAge, the authors first determined DNA methylation values that could be used to estimate plasma protein levels. They first regressed 88 plasma protein variables on chronological age, sex, and ~450 000 CpGs (shared between the 450K and EPIC platforms) in elastic net regression models. Only 12 out of the 88 plasma proteins demonstrated moderately high ($r > 0.35$) correlations between their measured plasma levels and DNAm surrogate markers determined from the elastic net models. The authors also used 172 CpGs to establish a DNAm surrogate of cigarette smoking pack-years. They next regressed all-cause mortality time-to-death on: chronological age, sex, the pack-years DNAm surrogate marker, and the 12 plasma protein DNAm surrogate markers. The elastic net Cox regression model selected age, DNAm pack-years, sex, and 7 DNAm protein markers (adrenomedullin, beta-2-microglobulin, cystatin C, growth differentiation factor-15, leptin, plasminogen activator inhibitor-1, and tissue inhibitor metalloproteinases 1). After a linear transformation, the combination of these covariates was used to create DNAm GrimAge. Importantly, the CpGs used to create all DNAm surrogate markers are made up of 1030 unique CpGs. Out of all existing DNAm metrics, GrimAge is unparalleled in its

ability to predict all-cause time-to-death (Cox regression $p = 2.0 \times 10^{-75}$) (16).

The above prose provides information on the algorithms used to develop each methylation age measure. For the present study, DNAmAge, PhenoAge, and GrimAge were provided as outputs after uploading methylation beta values to Horvath's publicly available online calculator (<http://dnamage.genetics.ucla.edu>).

Statistical Analysis

Since we planned on examining relationships between 4 metabolites and 3 DNA methylation-based aging variables, we desired a robust methodology for determining biologically and statistically significant relationships. To achieve this goal, we employed BKMR—a Bayesian variable selection framework that accounts for any nonlinear relationships (18).

All metabolites were log-transformed to achieve normal distributions before beginning any analyses. We used the R “bkmr” package to create fully adjusted BKMR selection models for each of the 3 aging metrics (18). In previously published works, 10 000 iterations by Markov Chain Monte Carlo algorithm per BKMR model were deemed appropriate for selection (26). To increase the robustness of our results we ran 20 000 iterations per selection model. Specifically, DNAmAge, PhenoAge, and GrimAge, respectively, were regressed on each of the 4 metabolites and important covariates. Important covariates/lifestyle factors were determined a priori from the relevant literature (19,22,23,27). Covariates included chronological age (continuous), season of visit (spring [March–May], summer [June–August], fall [September–November], and winter [December–February]), body mass index (lean [<25], overweight [25–30], obese [>30]), alcohol intake (yes/no ≥ 2 drinks daily), cumulative cigarette pack-years (continuous), smoking status (never, former, or current), education (<12 years, 12–16 years, >16 years), glomerular filtration rate (continuous), and white blood cell proportions (CD8 naïve T cells, CD8pCD28nCD45Ra- T cells, plasma blasts, CD4T cells, NK cells, monocytes, and granulocytes; continuous) (11,28). Bayesian Kernel Machine Regression models were as follows:

$$\text{Aging Outcome}_i = b(B_6, B_{12}, \text{Folate}, \text{Homocysteine}) + \beta^T Z_i + e_i,$$

where the function $b()$ is a dose–response function containing nonlinear and/or interactions between the metabolites, and $Z = Z_1, \dots, Z_k$ are k potential confounders. Bayesian Kernel Machine Regression models also included a random intercept for participants to account for correlations between repeated measures (multiple visits) for some participants. Each model produced posterior inclusion probabilities (PIPs), which represent how important each metabolite was for each of the aging outcomes. Although a PIP > 0.50 threshold has been utilized in previously published studies (29,30), we decided to employ a more rigorous threshold (PIP > 0.60) for determining important independent variables (metabolites). The same BKMR models were able to determine the independent association of single metabolites and the cumulative metabolite mixture with the respective DNA methylation aging metrics. To assess the robustness of our findings independent of common aging-related diseases, we ran secondary analysis models that mirrored the fully adjusted linear models but included additional adjustments for diabetes (yes/no), hypertension (yes/no), and ischemic heart disease (yes/no).

We also performed 3 sets of sensitivity analyses to further interrogate the robustness of our findings. The first examined the single and

cumulative mixture metabolite relationships in BKMR models using only participant's first study visits with the primary model covariates ($N = 715$). The second examined the single and cumulative mixture metabolite relationships in BKMR models excluding observations that had plasma metabolite values outside of the reported normal clinical reference ranges (participants = 353, observations = 453) again using the primary model covariates (31–34). Third, we wanted to provide additional evidence that our findings were due to changes in the methylome uniquely captured by the composite DNA methylation age measures rather than due to changes that could be directly explained by the clinical variables used in calculating DNA methylation age. We had an opportunity to do this with PhenoAge because a number of its component clinical variables were independently measured from blood samples provided at the same study visits where DNA methylation age was measured. As mentioned, PhenoAge was built utilizing 9 clinical variables (ie, albumin, creatinine, glucose, C-reactive protein, lymphocyte percent, mean cell volume, red cell distribution width, alkaline phosphatase, and white blood cell count). We did not believe our findings were driven by creatinine, glucose, lymphocyte percent, or white blood cell count because our main models already adjusted for glomerular filtration rate (based on creatinine), diabetes, and white blood cell proportions. To assess the impact of the other clinical variables on our findings, we created an additional PhenoAge BKMR model adjusted for the aforementioned variables in addition to serum albumin, C-reactive protein, and alkaline phosphatase. Our cohort lacked data on mean cell volume and red cell distribution width, so we adjusted for imperfect alternatives of red cell physiology: hemoglobin and red blood cell count. Six hundred fifty-eight participants (956 observations) had all serum variables for this additional analysis. This type of analysis was not performed for DNAmAge because the measure is not based on clinical variables. It was not performed for GrimAge because our goal was to assess the methylome-independent impact of clinical variables on our analyses and our cohort lacked plasma measures of the GrimAge component proteins. All statistical analyses were performed using R Version 3.6.3 (R Core Team, Vienna, Austria). We considered a p -value $< .05$ to be statistically significant.

Results

Study Sample Characteristics and Clinical Data

Table 1 presents the biomarker, demographic, and clinical descriptive statistics for all observations included in the study sample. Data are presented for participant's first visits and for all study visits. In all study visits, participants had a mean (SD) chronological age of 73.7 (6.7) years. DNAmAge, GrimAge, and PhenoAge had mean (SD) values of 73.8 (7.3), 71.0 (6.6), and 68.3 (8.5) years, respectively. With respect to lifestyle variables, the majority of participants had at least 12 years of formal education (73%), consumed less than 2 alcoholic drinks per day (75%), and were former smokers (64%). The mean (SD) cumulative cigarette pack-years was 21.0 (25.2) years. With respect to health variables, participants had a mean (SD) glomerular filtration rate of 74.1 (17.8) mL/min/1.73 m². The majority of participants were overweight or obese (62%), were not diabetic (71%), had hypertension (73%), and did not have ischemic heart disease (69%).

Table 2 describes the plasma metabolite concentrations across study participants as well as published clinical reference ranges for each metabolite. Across all observations, the mean (SD) levels for B₆, B₁₂, folate, and homocysteine were 114.3 (108.4) nmol/L, 546.2

(348.9) pg/mL, 18.7 (15.9) ng/mL, and 11.0 (3.8) nmol/mL, respectively. Table 2 also lists the quintile values as well as the published clinical reference ranges for each metabolite. For all descriptive statistics, first visit values closely mirrored all visit values.

We also present the Pearson correlation matrices and data distributions of age variables and plasma metabolite concentrations across all observations in the study sample (Figure 1). GrimAge and DNAmAge shared the weakest correlation ($r = 0.60$). GrimAge and chronological age shared the strongest correlation ($r = 0.78$). Correlation coefficients between plasma metabolite concentrations ranged from -0.19 to 0.26 . The weakest correlation was between folate and homocysteine ($r = -0.08$) while the strongest was between B₁₂ and homocysteine ($r = 0.26$). Correlations between one-carbon metabolites and methylation age variables were weak with the strongest being between homocysteine and GrimAge ($r = 0.27$). To show unadjusted relationships of the one-carbon metabolites with chronological age, we provide LOESS regression lines from models where each one-carbon metabolite is regressed on chronological age (Supplementary Figure S1). Mirroring the correlations, vitamins B₆ and B₁₂ appeared to have the weakest relationships with chronological age.

Metabolite Selection by BKMR

Supplementary Table S1 presents the PIPs produced from fully adjusted BKMR models and models additionally adjusted for diabetes, hypertension, and ischemic heart disease. Given that PIP values represent the degree to which data support each metabolite being included in the final regression model, they can be utilized for variable selection. Posterior inclusion probabilities for DNAmAge ranged from 0.04 (folate) to 0.27 (B₆). No metabolites in the DNAmAge BKMR models met the 0.60 PIP threshold. Posterior inclusion probabilities for GrimAge ranged from 0.18 (homocysteine) to 0.64 (folate) with folate reaching the importance threshold for GrimAge. Posterior inclusion probability values for PhenoAge ranged from 0.36 (B₁₂) to 0.99 (folate). B₆ and folate met the importance threshold for PhenoAge. Importantly, PIP results were similar for all aging measures in the primary fully adjusted model framework, and the secondary model framework with additional adjustments for age-related diseases. Overall, PIPs for metabolites important to PhenoAge were greater than PIPs for metabolites important to GrimAge.

BKMR Single Metabolite Associations

Figure 2 depicts the relationships of single metabolites with the DNA methylation-based measures of methylation age in the fully adjusted model framework (Figure 2A), and the framework additionally adjusted for age-related diseases (Figure 2B). Relationships are shown for single log metabolites when the remaining 3 metabolites are set at their 10th, 50th, and 90th percentiles. No metabolites were consistently significantly associated with DNAmAge after adjusting for aging-related diseases. Log vitamin B₆ with the other metabolites at their 50th ($\beta = -0.46$, 95% CI: $-0.70, -0.13$) and 90th percentiles ($\beta = -0.80$, 95% CI: $-1.43, -0.17$) was associated with decreased GrimAge. Log folate with the other metabolites at their 10th ($\beta = 0.83$, 95% CI: $0.24, 1.42$) and 50th percentiles ($\beta = 0.75$, 95% CI: $0.41, 1.09$) was associated with increased GrimAge. Log homocysteine with the other metabolites at their 90th percentiles ($\beta = 0.78$, 95% CI: $0.29, 1.27$) was associated with increased GrimAge. Log vitamin B₆ with the other metabolites at their 10th ($\beta = -1.57$, 95% CI:

Table 1. Characteristics of Study Participants

	First Visit (N = 715)	All Visits (N = 1153)
Age variables		
Chronological age (years), mean (SD)	72.6 (6.7)	73.7 (6.7)
DNAmAge (years), mean (SD)	73.1 (7.4)	73.8 (7.3)
GrimAge (years), mean (SD)	70.3 (6.6)	71.0 (6.6)
PhenoAge (years), mean (SD)	67.5 (8.6)	68.3 (8.5)
Health and lifestyle variables		
Glomerular filtration rate (mL/min/1.73 m ²), mean (SD)	75.5 (18.2)	74.1 (17.8)
Pack-years, mean (SD)	21.2 (25.4)	21.0 (25.2)
Alcohol consumption, N (%)		
<2 drinks/day	582 (75)	936 (75)
≥2 drinks/day	133 (25)	217 (25)
BMI, N (%)		
Healthy/lean	143 (38)	239 (38)
Overweight	381 (35)	623 (35)
Obese	191 (27)	291 (27)
Diabetes, N (%)		
Yes	140 (29)	225 (29)
No	575 (71)	928 (71)
Education, N (%)		
≤12 years	192 (31)	312 (27)
12–16 years	351 (42)	560 (49)
>16 years	172 (27)	281 (24)
Hypertension, N (%)		
Yes	507 (67)	836 (73)
No	208 (33)	317 (27)
Ischemic heart disease, N (%)		
Yes	210 (31)	362 (31)
No	505 (69)	791 (69)
Season, N (%)		
Spring	177 (17)	286 (25)
Summer	186 (33)	299 (26)
Fall	216 (27)	352 (30)
Winter	136 (23)	216 (19)
Smoking status, N (%)		
Current	31 (2)	50 (4)
Former	463 (67)	740 (64)
Never	221 (31)	363 (32)

Notes: BMI = body mass index; SD = standard deviation.

Table 2. Plasma One-Carbon Metabolite Concentrations Across Study Participants and Clinical Reference Ranges

Metabolite	First Visit Mean (SD) N = 715	All Visits Mean ± SD N = 1153	Quantiles (All Visits)					Reference Ranges* (31–34)
			0%	25%	50%	75%	100%	
Vitamin B ₆ (nmol/L)	110.8 (103.9)	114.3 (108.4)	10.9	47.9	75.2	135.6	823.8	20.0–125.0
Vitamin B ₁₂ (pg/mL)	527.8 (363.9)	546.2 (348.9)	49.0	366.0	494.0	644.0	7252.0	160.0–950.0
Folate (ng/mL)	17.3 (13.9)	18.7 (15.9)	3.1	9.9	14.5	21.0	184.2	2.7–17.0
Homocysteine (nmol/mL)	11.0 (4.1)	11.0 (3.8)	4.1	8.8	10.2	12.4	69.0	5.0–15.0

Notes: SD = standard deviation.

*453 total observations from 353 participants had within reference range levels for all 4 metabolites.

–2.81, –0.33), 50th ($\beta = -1.62$, 95% CI: –2.28, –0.96), and 90th percentiles ($\beta = -1.69$, 95% CI: –2.92, –0.46) was associated with decreased PhenoAge. Log folate with the other metabolites at their 50th ($\beta = 1.62$, 95% CI: 0.95, 2.29) and 90th percentiles ($\beta = 1.76$, 95% CI: 0.46, 3.06) was associated with increased PhenoAge. Findings were consistent comparing models with and without age-related disease adjustments. Albeit not statistically

significant, models using only first visits or only including metabolite values within clinical reference ranges demonstrated similar trends and effect estimates for metabolites previously identified as important to the respective methylation age measures (Supplementary Figure S2). Finally, BKMR models additionally adjusted for the PhenoAge components of serum albumin, C-reactive protein, and alkaline phosphatase in addition to red

Figure 1. Correlation matrices of aging and one-carbon metabolite variables in the analyses. Pearson correlation matrices of age variables and one-carbon metabolites across all visits in the study sample ($N = 1153$). All metabolites were log-transformed to achieve normal distributions for the analyses. Asterisks denote statistical significance of correlations: * $p < .05$, ** $p < .01$, *** $p < .001$.

Figure 2. Single one-carbon metabolite associations ($N = 1153$). Change in DNA methylation age (95% CI) for single metabolites (vitamin B₆ [B₆], vitamin B₁₂ [B₁₂], folate [FA], or homocysteine [Hcy]) when the remaining 3 metabolites are fixed at their 10th, 50th, and 90th percentile levels in Bayesian Kernel Machine Regression models adjusted for chronological age, year of visit, pack-years, smoking status, season, alcohol consumption, education, glomerular filtration rate, body mass index, and white blood cell proportions (A) and additionally adjusted for diabetes, hypertension, and ischemic heart disease (B). All metabolites were log-transformed to achieve normal distributions for the analyses. Full color version is available within the online issue.

blood cell count and hemoglobin, demonstrated statistically significant negative associations of vitamin B₆ with PhenoAge and statistically significant positive associations of folate with

PhenoAge. These findings were in alignment with the results from our main analyses (Supplementary Figure S3).

BKMR Cumulative Metabolite Mixture Associations

Figure 3 describes the relationships of the cumulative metabolite mixture with the DNA methylation-based measures of methylation age in the fully adjusted model framework (Figure 3A), and the framework additionally adjusted for age-related diseases (Figure 3B). The cumulative mixture was not associated with DNAmAge or PhenoAge. However, compared to a model where each metabolite in the mixture is set to its 50th percentile, when every metabolite was at its 30th ($\beta = -0.13$, 95% CI: -0.26 , -0.005) and 40th percentile ($\beta = -0.06$, 95% CI: -0.11 , -0.005), the cumulative mixture was associated with decreased GrimAge. Similar trends were observed in the first visit analyses, but these results did not meet statistical significance (Supplementary Figure S4A). The reference range analyses demonstrated statistically significant positive associations of the cumulative metabolite mixture with DNAmAge and GrimAge when all metabolites were above their 50th percentile (Supplementary Figure S4B). A statistically significant negative association of the cumulative metabolite mixture was also observed with GrimAge when all metabolites were at their 40th percentile (Supplementary Figure S4B).

Discussion

In this study of older community-dwelling men, we identified novel age-related disease-independent associations of plasma one-carbon metabolites with DNA methylation-based measures of biological aging. Utilizing BKMR selection models, we identified vitamin B₆ as important to PhenoAge and folate as important to both GrimAge and PhenoAge. Examination of single metabolite relationships in the same BKMR models revealed statistically significant associations of plasma vitamin B₆ levels with decreased PhenoAge and statistically significant relationships of plasma folate levels with increased GrimAge and PhenoAge. Cumulative mixture results from BKMR models demonstrated a statistically significant association of the 4-metabolite mixture with decreased GrimAge.

One-carbon metabolism is a process characterized by one-carbon transfer reactions and ultimately plays a role in DNA synthesis and DNA methylation. It is divided into 2 main pathways (ie, remethylation and transsulfuration) and several enzymes and cofactors are involved in these pathways. Vitamin B₆, vitamin B₁₂, and folate are among these important cofactors. Homocysteine is one of the important intermediaries of these pathways (35,36). Given previously reported relationships of one-carbon metabolites with DNA methylation, we expected to observe statistically significant relationships of all metabolites with our DNA methylation-based measures of biological age. Moreover, existing data exploring relationships of dietary nutrients with DNA methylation age led us to further hypothesize that many of these nutrients would have protective, negative relationships with methylation age. For instance, results from a study of 120 older Europeans taking part in the NU-AGE project observed decreases in DNA methylation age acceleration following a 1-year Mediterranean-like diet intervention (37). Another important study was based on 51 overweight/obese African Americans with low vitamin D status and randomized to placebo or 1 of 4 treatments of vitamin D₃ supplementation (38). Findings from this study demonstrated negative associations of vitamin D₃ supplementation with DNAmAge (38). Specifically, they report that vitamin D₃ supplementation of 4000 IU/day for 16 weeks was associated with

Figure 3. Relationships between one-carbon metabolite mixtures and DNA methylation age variables ($N = 1153$). Change in DNA methylation age variables (95% CI) for the cumulative one-carbon metabolite mixture from Bayesian Kernel Machine Regression (BKMR) models adjusted for chronological age, year of visit, pack-years, smoking status, season, alcohol consumption, education, glomerular filtration rate, body mass index, and white blood cell proportions (A) and additionally adjusted for diabetes, hypertension, and ischemic heart disease (B). In each BKMR model, all metabolites at the specified percentiles (x-axis) were compared to a model with all metabolites at their 50th percentile. Full color version is available within the online issue.

a 1.85-year decrease in DNAmAge. Work in the Women's Health Initiative has further demonstrated significant negative correlations of plasma levels of carotenes, xanthines, and tocopherols with DNAmAge, PhenoAge, and GrimAge (15–17).

Of all the metabolites in our study sample, vitamins B₆ and B₁₂ were the only metabolites negatively correlated with DNA methylation age. As mentioned, both vitamins B₆ and B₁₂ are important cofactors in one-carbon metabolism. They also cannot be synthesized by the human body and must be derived from dietary sources. Vitamin B₆ was negatively correlated with all 3 methylation aging measures while B₁₂ was negatively correlated with PhenoAge and GrimAge. Furthermore, vitamin B₆ was selected as important to PhenoAge and was found to have a negative association with PhenoAge even after adjusting for age-related diseases. One reason why we see less robust relationships with DNAmAge could be due to its utility—especially in comparison to PhenoAge and GrimAge—as a predictor of chronological age. As previously mentioned, PhenoAge and GrimAge represent measures of disease and mortality risk, respectively. One impetus for studying PhenoAge and GrimAge measures is not simply to provide a means of predicting disease or mortality risk, but to offer a way to track these risks in the event that interventions to mitigate them are available. Although these findings need to be independently replicated and then tested in a randomized control trial, our data suggest that vitamin B₆ may be one potential mitigator of disease risk assessed via PhenoAge.

Of the existing methylation one-carbon metabolite studies, a double-blinded placebo-controlled trial of 44 individuals in the Dutch B-vitamins for the PRevention Of Osteoporotic Fractures (B-PROOF) study specifically examines relationships with some of the methylation age variables used in the present analysis (39).

This work randomized participants to receive a treatment of 400 µg folic acid plus 500 µg of vitamin B₁₂ for 2 years. Results from that study demonstrated that the treatment significantly reduced the DNAmAge of women with the methylenetetrahydrofolate reductase—an enzyme involved in one-carbon metabolism—677CC genotype by 2.70 years (39). There are 2 notable differences between this study and our own. First, we observe significant relationships with PhenoAge and GrimAge, but not DNAmAge. Although we have a much larger sample size and use longitudinal data, our study is still observational in nature. A “signal-to-noise” ratio lower than that of a randomized trial with a prescribed multiweek treatment arm may explain why we only observe relationships with the 2 markers most sensitive to health and life span (ie, PhenoAge and GrimAge), but not with DNAmAge.

Secondly, participants in the randomized study received both folate and vitamin B₁₂. We only observe a significant association of folate with our aging measures, and that association is positive not negative. As previously noted, we observe significant negative age associations with vitamin B₆, but none with vitamin B₁₂. Given that folate is almost always nontoxic to humans, folate deficiency has been the frequent culprit in disease processes including those that are age-related like diabetes and cataracts (40,41). Our observed positive association of folate with GrimAge and PhenoAge seems contrary to this relationship, but the literature contains similar paradoxical folate relationships with other aging markers like mitochondrial DNA copy number and telomere length (42,43). Our observed positive association may simply reflect that higher folate levels increase the availability of methyl groups for DNA methylation processes. Notably, our folate results also demonstrate potentially important interactions between the examined metabolites.

For instance, when the remaining metabolites are set to their 90th percentile, the positive association between folate and GrimAge is no longer statistically significant. The opposite is observed with PhenoAge. When the remaining metabolites are set to their 10th percentile, the positive association between folate and PhenoAge is no longer statistically significant. These findings suggest that there may be a context-dependent effect of folate on these 2 measures of aging and further highlights the merits of mixture analysis methods for uncovering such complex relationships (18,44).

With respect to our cumulative 4-metabolite mixture findings, our results are somewhat in concordance with results of the B-PROOF study (39). In particular, in the main analyses, we observe a negative association of the mixture with GrimAge—the methylation age variable most closely related to mortality (16). These results suggest that even though individual metabolites may have positive relationships with methylation age, the combination of metabolites is associated with reductions in methylation age. Notwithstanding, in the main analyses, we did not observe GrimAge associations with our 4-metabolite mixture across a wide range of percentiles (ie, we observed negative associations with GrimAge only at the 30th and 40th percentiles). Moreover, results from the reference range sensitivity analyses demonstrated positive associations of the cumulative metabolite mixture at its upper percentiles with both DNAmAge and GrimAge. This may suggest unique physiological relationships of these metabolites when all are within their reference ranges, but this assertion merits further study. In all, these cumulative mixture findings require further characterization.

The strengths of the present study include the adoption of data from a well-established longitudinal cohort, the incorporation of 3 novel methylation-based markers of aging, and the utilization of a robust selection model framework. Other strengths include our multiple sensitivity analyses. In particular our analysis demonstrating statistically significant associations of PhenoAge with vitamin B₆ and folate after additionally adjusting for serum components of PhenoAge further suggests that the relationships of one-carbon metabolites with methylation age measures are different from the relationships of one-carbon metabolites with established plasma proteins. Given the growing ability of researchers to conduct studies of human DNA methylation age in vitro (45,46), evidence from our study may help to catalyze future investigations that can provide mechanistic insights to better understand the unique relationship of one-carbon metabolites with DNA methylation age measures.

Nevertheless, our study does have a few limitations. First, our study does not speak to the sources of participants' one-carbon metabolite levels (eg, dietary, supplements, etc.). However, this does not minimize the significance of our findings. On the contrary, existing research has found food questionnaires and other dietary assessment methods to be subjective and limited with respect to actual measures of nutrient absorption and bioavailability (47). By using plasma metabolite concentrations, we provide objective measures that do speak to metabolite bioavailability, which is important for hypotheses pertaining to physiologic processes. Secondly, albeit not a unique practice in major longitudinal cohort studies, we use plasma one-carbon metabolites that were initially measured for other studies. Still, these metabolites are among those most studied in the relevant literature and these data help make innovative studies like our own feasible (8). Although presently unavailable in our cohort, future studies including plasma methionine, betaine, S-adenosylmethionine,

S-adenosylhomocysteine, and other important one-carbon metabolites will be essential for further characterizing DNA methylation age relationships. Third, although our study sample is from a longitudinal cohort with considerable data on participant lifestyle/demographic factors and we identified important covariates a priori using relevant studies (19,27), we cannot fully dismiss the possibility of residual or unknown confounding in our analyses. Importantly, we conducted 2 other sets of sensitivity analyses to test the robustness of our results. These analyses using first visits and reference range metabolite values made use of a smaller portion of our study sample. Although some of the results from the sensitivity studies varied from our main analyses, the estimates for metabolites selected as important from BKMR models remained predominantly consistent in direction and magnitude. This suggests that the lack of statistical significance—particularly for the important BKMR metabolites—is likely an issue of statistical power. Lastly, our findings are based on an older cohort of Caucasian males that reside in the Northeastern United States. Additional research in other demographic groups and in other environments is needed to confirm our findings more broadly.

In conclusion, our study provides novel characterizations of one-carbon metabolites with DNA methylation aging biomarkers. Moreover, these characterizations are independent of common age-associated diseases (ie, diabetes, hypertension, and ischemic heart disease). In particular our findings demonstrate that plasma vitamin B₆ levels are associated with decreased PhenoAge and that plasma folate levels are associated with increased PhenoAge and GrimAge. Our study also describes associations of the cumulative 4-metabolite mixture with decreased GrimAge. Together, these data suggest that DNA methylation age variables may have different nutrient sensitivities even within a singular pathway like that of one-carbon metabolism. Future research is needed to confirm these findings, but if they do indeed hold true, this knowledge will greatly inform future work involving DNA methylation age variables, nutrition, and human disease.

Supplementary Material

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

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Author Contributions

J.C.N. conceived and designed the study. J.C.N. performed the data analyses and drafted the manuscript. E.C., X.G., C.W., P.V., E.W.B., A.A.B., and J.S. contributed to the analyses. All authors revised and approved the manuscript.

Conflict of Interest

None declared.

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