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To cite this article: Karin B. Michels & Alexandra M. Binder (2024) Impact of folic acid supplementation on the epigenetic profile in healthy unfortified individuals – a randomized intervention trial, *Epigenetics*, 19:1, 2293410, DOI: [10.1080/15592294.2023.2293410](https://doi.org/10.1080/15592294.2023.2293410)

To link to this article: <https://doi.org/10.1080/15592294.2023.2293410>



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Published online: 14 Dec 2023.



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Impact of folic acid supplementation on the epigenetic profile in healthy unfortified individuals – a randomized intervention trial

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ABSTRACT

Folate is an essential mediator in one-carbon metabolism, which provides methyl groups for DNA synthesis and methylation. The availability of active methyl groups can be influenced by the uptake of folic acid. We conducted a randomized intervention trial to test the influence of folic acid supplementation on DNA methylation in an unfortified population in Germany. A total of 16 healthy male volunteers (age range 23–61 y) were randomized to receive either 400 µg ($n = 9$) or 800 µg ($n = 7$) folic acid supplements daily for 8 weeks. Infinium Human Methylation 450K BeadChip Microarrays were used to assay site-specific DNA methylation across the genome. Microarray analyses were conducted on PBL DNA. We estimated several epigenetic clocks and mean DNA methylation across all autosomal probes on the array. AgeAccel was estimated as the residual variation in each metric. In virtually all participants, both serum and red blood cell (RBC) folate increased successively throughout the trial period. Participants with a larger increase in RBC folate had a larger increase in DNAmAge AgeAccel (Spearman Rho: 0.56, p -value = 0.03). No notable changes in the methylome resulting from the folic acid supplementation emerged. In this population with adequate folate levels derived from diet, an increase in RBC folate had a modest impact on the epigenetic clock predicting chronologic age.

ARTICLE HISTORY

Received 3 August 2023

Revised 7 October 2023

Accepted 5 December 2023

KEYWORDS

Epigenetics; DNA methylation; folic acid; red blood cell folate

Introduction

Folic acid fortification of staple foods has been introduced in many countries globally to prevent neural tube defects in newborns as this birth defect results from a folate deficiency in women around the time of conception. While the mechanisms underlying the link between low folate levels in the mother and failure to close the spina bifida in the foetus have not been fully clarified, it is likely that epigenetic mechanisms play a role [1]. Folate is an essential mediator in one-carbon metabolism, which provides methyl groups for DNA synthesis and methylation. The availability of active methyl groups can be influenced by diet and uptake of folic acid as well as other key players in one-carbon metabolism, such as choline and methionine. Folate deficiency may correlate with hypomethylation [2]. Conversely, high folate levels may be associated with hypermethylation; however, research in this area is lacking. If folic acid

fortification affects DNA methylation, this might be associated with disease initiation or promotion. Hence, it is important to understand the impact of folic acid intake on DNA methylation.

5,10-methylenetetrahydrofolate reductase (MTHFR) is a regulator enzyme of one-carbon metabolism and essential for conversion of folic acid, thus regulating folate bioavailability. The common variant (MTHFR)-C677T has reduced enzyme activity and therefore decreases blood folate concentrations, thus increasing intake requirement among carriers [3]. The MTHFR genotype affects the relation of folate concentration with both DNA replication (cell division/survival) and DNA methylation (cell regulation) [4].

European countries have generally not participated in fortification programmes such as fluoridation of drinking water, fortification of milk with vitamins A and D, or fortification of flour with

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micronutrients leaving decisions about supplementations to the consumer. Accordingly, European countries have not adopted folic acid fortification despite a high incidence of neural tube defects. In Germany, folate deficiency is common and folic acid supplementation is low. According to the German National Nutrition Survey II, the median dietary folate equivalents in Germany are 191 µg/d for men and 168 µg/d for

women [5]. More than half of all Germans consume less than 200 µg of folate. The German Society of Nutrition recommends a daily intake of 300 µg folate for adults [6]. All women planning to get pregnant are advised in addition to a diet rich in folate-containing foods to take a supplement containing at least 400 µg of folic acid daily at least 4 weeks prior to pregnancy and to maintain this intake at least until the end of the

Table 1. Study participant characteristics at blood draws.

Characteristic	Baseline (n = 16)	Follow-up (n = 16)
Baseline Age [years; Mean (SD)]	39.22 (10.99)	–
MTHFR Genotype [N (%)]		
C/C	9 (56.25)	–
C/T	7 (43.75)	–
Folic Acid Formulation [N (%)]		
400 µg	9 (56.25)	–
800 µg	7 (43.75)	–
RBC Folate [ng/ml; Mean (SD)]	644.20 (151.96)	800.67 (171.96)
Serum Folate [ng/ml; Mean (SD)]	9.64 (3.10)	18.01 (7.19)
Epigenetic Clock [Mean (SD)]		
DNAmAge	41.63 (10.13)	41.79 (11.25)
DNAmAgeHannum	37.89 (11.56)	39.46 (11.41)
DNAmDunedinPACE	0.70 (0.10)	0.73 (0.08)
DNAmGrimAge	39.57 (8.73)	39.11 (8.94)
DNAmGrimAge2	42.25 (8.70)	42.15 (9.11)
DNAmPhenoAge	24.40 (12.13)	23.26 (13.24)
DNAmTL	7.38 (0.29)	7.30 (0.28)
Genome-Wide Mean	0.53 (0.00)	0.53 (0.00)
DNAm-based Estimates of Cell Composition [Mean (SD)]		
CD8+ T Cells	0.23 (0.08)	0.23 (0.08)
CD4+ T Cells	0.31 (0.08)	0.29 (0.09)
Natural Killer Cells	0.13 (0.05)	0.15 (0.07)
B-Cells	0.11 (0.04)	0.11 (0.04)
Monocytes	0.21 (0.07)	0.23 (0.07)
Neutrophils	0.02 (0.03)	0.01 (0.01)

Table 2. Association between AgeAccel and folate levels.

AgeAccel Outcome	RBC Folate (per 100 ng/ml)		Serum Folate (per 10 ng/ml)	
	Estimate (95% CI)	P-value	Estimate (95% CI)	P-value
Baseline Associations^A				
DNAmAge	0.50 (–1.59, 2.60)	0.613	1.68 (–8.89, 12.25)	0.737
DNAmAgeHannum	0.62 (–1.27, 2.51)	0.490	1.62 (–8.01, 11.25)	0.722
DNAmDunedinPACE	0.01 (–0.02, 0.04)	0.438	0.01 (–0.16, 0.18)	0.897
DNAmGrimAge	0.53 (–0.15, 1.22)	0.115	1.89 (–1.72, 5.50)	0.279
DNAmGrimAge2	0.42 (–0.39, 1.23)	0.286	0.93 (–3.30, 5.15)	0.643
DNAmPhenoAge	0.75 (–0.56, 2.07)	0.239	4.51 (–1.92, 10.94)	0.154
DNAmTL	–0.01 (–0.08, 0.05)	0.740	–0.06 (–0.38, 0.27)	0.716
Genome-Wide Mean	0.00 (0.00, 0.00)	0.080	0.00 (–0.01, 0.00)	0.209
Associations of Change^B				
DNAmAge	2.33 (–0.35, 5.00)	0.083	–1.36 (–6.82, 4.10)	0.599
DNAmAgeHannum	0.31 (–1.09, 1.70)	0.644	–0.90 (–3.42, 1.62)	0.454
DNAmDunedinPACE	0.00 (–0.04, 0.05)	0.857	0.03 (–0.05, 0.10)	0.483
DNAmGrimAge	–0.50 (–2.12, 1.12)	0.515	1.34 (–1.56, 4.25)	0.335
DNAmGrimAge2	–0.66 (–2.32, 1.01)	0.411	1.45 (–1.56, 4.47)	0.316
DNAmPhenoAge	1.63 (–1.44, 4.70)	0.272	–0.36 (–6.26, 5.53)	0.896
DNAmTL	–0.05 (–0.14, 0.04)	0.290	0.06 (–0.11, 0.23)	0.442
Genome-Wide Mean	0.00 (0.00, 0.00)	0.525	0.00 (0.00, 0.00)	0.378

AgeAccel was modelled as a function of each folate measure separately, adjusting for baseline chronological age (years) using a linear model; providing association estimate (95% confidence interval (CI)) and Wald test *p*-value for the coefficient. ^AAgeAccel and folate levels evaluated at baseline; ^Bmodeling the change in AgeAccel and change in folate levels.

first trimester [6]. In the U.S., the respective recommended dietary allowances are 400 µg/d and, for pregnant women, 600 µg/d [7]. Therefore, a folic acid supplementation trial in Germany is feasible and informative.

We conducted an intervention study in Germany to explore the impact of folic acid supplementation on DNA methylation in an unfortified population stratified by MTHFR genotype. Specifically, we evaluated associations with DNA methylation-based estimates of characteristics related to biological age termed epigenetic clocks. Epigenetic clocks refer to models that predict characteristics such as chronological age, the pace of ageing, and mortality risk using specific DNA methylation signatures (collectively referred to here as ‘epigenetic age’). Higher epigenetic age relative to those of the same chronologic age is associated with increased rates of age-related disease and mortality [8]. Several large studies indicate that accelerated epigenetic age is associated with greater cardiovascular disease risk [9–13]. Both observational studies and randomized controlled trials suggest that folic acid supplementation and higher folate levels are associated with reduced risk of cardiovascular disease [14,15]. Folate is the cofactor for methionine synthase, which catalyzes the conversion of homocysteine, corresponding to in higher levels of homocysteine with lower folate levels. It is hypothesized that hyperhomocysteinemia may contribute to cardiovascular disease by causing damage to endothelial cells and leading to atherosclerosis [16]. Our study will evaluate whether changes in folate levels are correlated with changes in epigenetic age, both of which are indicators of cardiovascular disease risk. We expect that higher folate levels will be associated with lower epigenetic age, given their respective relations to cardiovascular health.

Methods

Study design and population

We conducted a randomized intervention trial to test the influence of folic acid supplementation on DNA methylation at the University of Freiburg, Germany. A total of 16 healthy male volunteers (age range 23–61 y) were randomized to receive

either 400 µg ($n=9$) or 800 µg ($n=7$) folic acid supplements daily for 8 weeks. Prior to the start of the intervention and after 2, 4, and 8 weeks of supplementation, blood and oral mucosa samples were collected and analysed. Blood samples were processed immediately following collection. Serum and red blood cells were isolated from EDTA blood samples and stored until further analyses. Peripheral blood leukocytes (PBLs) were isolated from the blood samples, and DNA was isolated from the PBLs and the mucosa samples. All samples yielded sufficient materials of good quality for further analyses. The study was approved by the committee for research ethics of the University of Freiburg.

Folate and vitamin B₁₂ assessment

Serum folate and red blood cell (RBC) folate were measured in hemolyzed EDTA blood samples using a chemiluminescent assay. Vitamin B₁₂ levels were assessed to exclude a vitamin B₁₂ deficiency. Vitamin B₁₂ was measured by an ElektroChemiLumineszenz ImmunoAssay “ECLIA“. Serum folate and vitamin B12 analyses were performed in the Clinical Laboratory of the University Medical Center, University of Freiburg, Germany. Red blood cell folate was measured at the Clotten Laboratory, Freiburg, Germany. Assessments of all analytes were adapted on the Roche Cobas e801, Germany.

MTHFR genotyping

MTHFR genotyping was performed in our laboratory using Realtime PCR via TaqMan (LC480 from Roche) following the PCR-RFLP method described by Frosst et al. [17].

Genome-wide methylation assessment

Infinium Human Methylation 450K BeadChip Microarrays were used to assay site-specific DNA methylation across the genome. Microarray analyses were conducted on PBL DNA. We analysed individual samples (1 µg) from each of the 16 participants collected at baseline prior to the intervention and at the end of the intervention at week 8 (week 4 for one participant). We also pooled

samples across all participants by combining 2.5 μ l DNA solution standardized at 25 ng/ μ l from each of the 16 participants at time point zero and vortexing the pooled sample for 2 minutes; a second pooled sample for time point week 8 was combined in the same way. The 32 individual samples and 2 pooled samples were shipped on dry ice to the University of Southern California Epigenome Center in Los Angeles, California, for Methylation Microarray Analysis. We pre-processed raw signal intensities by performing dye bias correction followed by single-sample background correction based on Normal-exponential convolution using out-of-band Infinium I probes (ssNoob) [18]. Site-specific DNAm levels (β -values) were estimated based on the ratio of methylated (M) and unmethylated (U) fluorescence intensities, given by $\beta = M / (M + U + 100)$, and ranging from 0 (unmethylated) to 1 (methylated).

DNA methylation-based biomarkers

We estimated several epigenetic clocks, including those trained to predict chronologic age (DNAmAge [19], DNAmAgeHannum [20]), telomere length (DNAmTL) [21], and characteristics associated with time-to-death (PhenoAge [22], GrimAge [23], and GrimAge2 [24]) and pace of ageing (DunedinPACE) [25]. Description of the training sets, the specific characteristics used to develop the model, and validation of the estimates are included in the specified references. We additionally estimated mean DNA methylation across all autosomal probes on the array ('Genome-Wide Mean'). AgeAccel was estimated as the residual variation in each metric after adjusting for participants' chronological age at sample collection and DNA methylation-based estimates of cell composition (CD8+ T cells, CD4+ T cells, natural killer cells, B-cells, monocytes, neutrophils). Cell composition was estimated using reference-based constrained projection [26], and the Identifying Optimal Libraries (IDOL) [27,28] probes to distinguish cell types for 450K arrays.

Statistical analysis

Linear mixed-effects models were used to model the change in serum and RBC folate over follow-up (week), incorporating a random intercept for

participant. Effect modification by folic acid supplement formulation (400 μ g vs 800 μ g) and MTHFR genotype was assessed based on the significance (Wald test) of a product term between time and each characteristic individually. For analyses integrating the DNA methylation data, the longitudinal change in AgeAccel was estimated as the difference between AgeAccel at follow-up (week 8; week 4 for one participant) and baseline (week 0). The longitudinal change in folate measures was similarly estimated. RBC and serum folate was missing at week 8 for one participant, and imputed with the values at week 4. A paired non-parametric Wilcoxon signed-rank test was used to evaluate longitudinal change in AgeAccel across individuals. The non-parametric Spearman's rank correlation was used to evaluate the relation between AgeAccel and folate measures at baseline, as well as the correlation between the longitudinal change in the AgeAccel and folate measures. The benefit of this approach is that our analysis is robust to influential observations, which are more likely to impact significance in a small study. As a supplementary analysis, we evaluate these associations using linear models, adjusting for age, to estimate effect size, corresponding 95% confidence interval (CI) and significance (Wald test). The alpha-level for all tests was 0.05. All analyses were performed in R v 4.2.0.

Results

Serum and RBC folate levels

Prior to intervention, all trial participants had normal serum and RBC folate level and normal vitamin B₁₂ levels (Table 1). In almost all participants, both serum and RBC folate increased successively throughout the trial period (Figure 1). There was a significant 0.98 ng/ml (95% confidence interval (CI): 0.67,1.29) mean increase in serum folate per week. There was a significant 22.16 ng/ml (95% CI: 16.81, 27.52) mean increase in RBC folate per week. The increase in serum and RBC folate over time was not significantly modified by MTHFR genotype or folic acid supplement formulation. RBC and serum folate were highly correlated at baseline (Spearman Rho: 0.83, $p < 0.001$). By week 8, this correlation was attenuated and no longer significant (Spearman Rho: 0.44, $p = 0.12$).

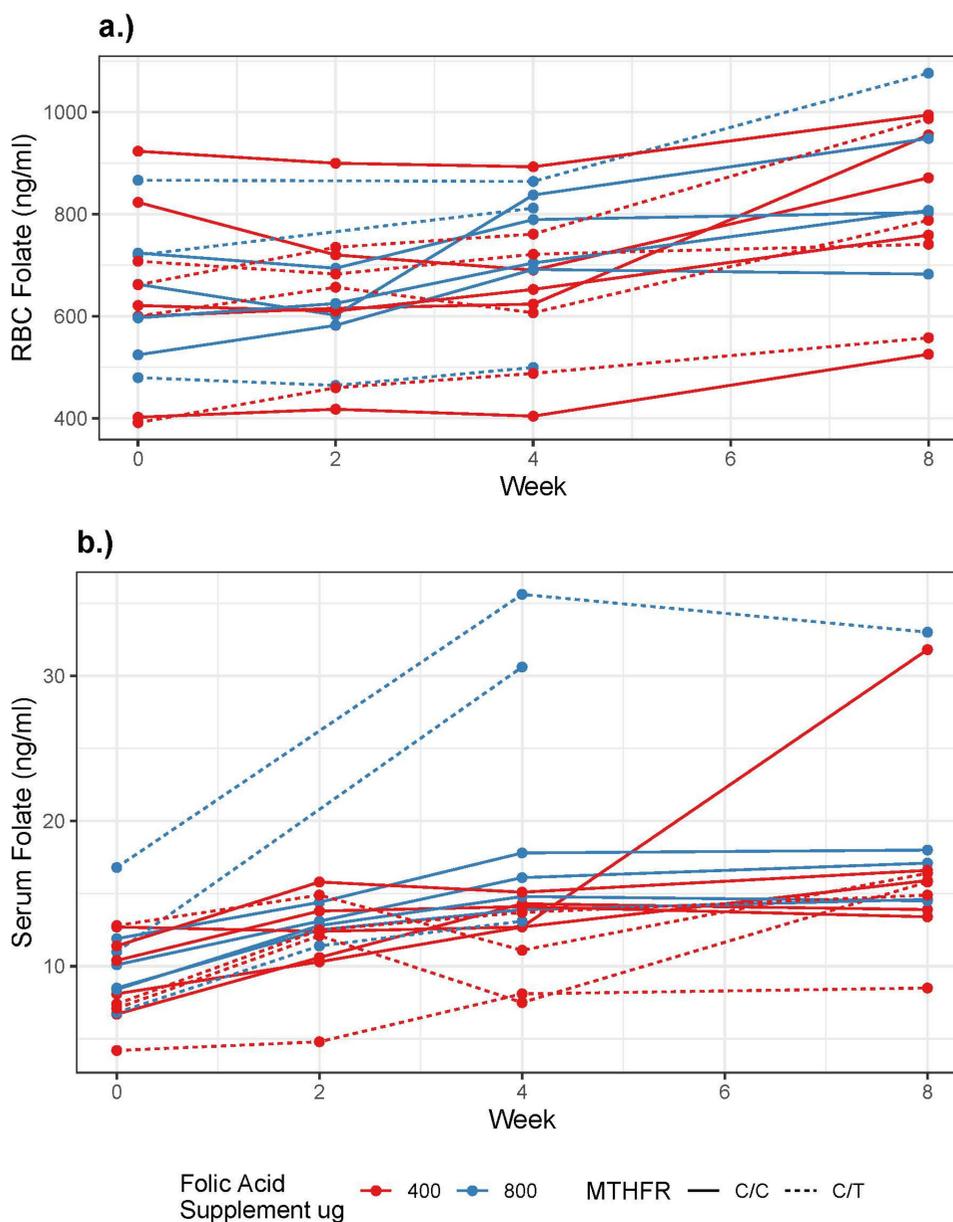


Figure 1. Graphic representation of a) RBC and b) serum folate levels of the 16 study participants after daily consumption of 400 or 800 μg folic acid. Normal range of RBC folate: 263–1028 ng/ml; serum folate: 4.6–18.7 ng/ml. Two participants had serum folate values >40 ng/ml, above the detection limit.

DNA methylation-based biomarkers

At baseline, RBC and serum folate were not correlated with any of our estimates of AgeAccel (Figure 2). The average change in AgeAccel between baseline and follow-up across participants was not significant for any measure (Figure 3a). However, the longitudinal change in DNAmAge estimates of AgeAccel was modestly correlated with the longitudinal change in RBC folate (Table 2, Figure 3b). Participants with a larger increase in RBC folate had a larger increase in DNAmAge

AgeAccel (Spearman Rho: 0.56, p -value = 0.03); this relation was not observed with serum folate. Based on linear models, a 100 ng/ml increase in RBC folate was associated with a 2.33 (95% CI: $-0.35, 5.00$) year increase in DNAmAge AgeAccel over follow-up. Increases in RBC and serum folate were not significantly correlated with changes in any other AgeAccel measures (Table 2, Figure 3b, c). Hence, no notable changes in the methylome resulting from the folic acid supplementation were observed.

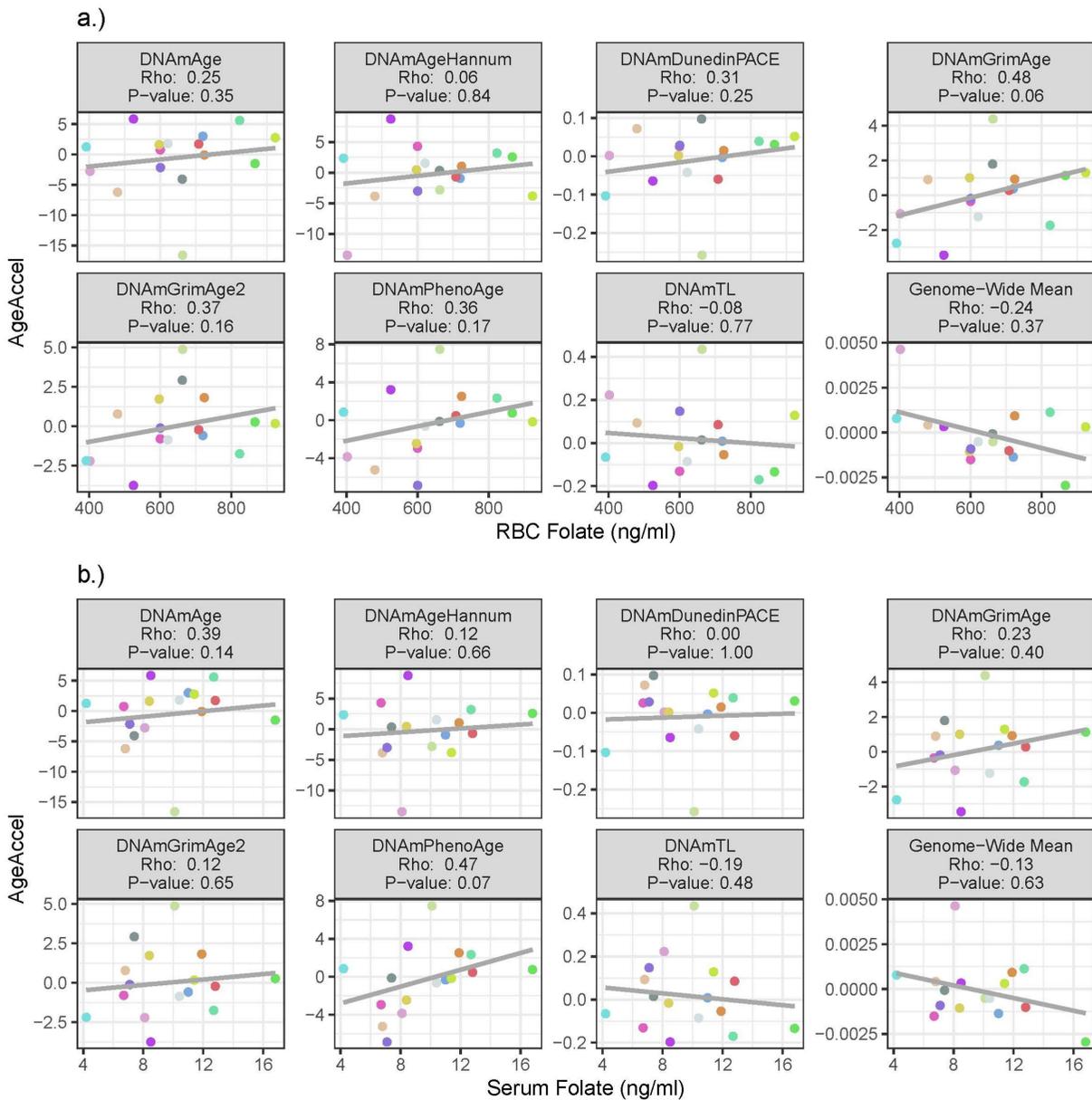


Figure 2. Correlation between AgeAccel and a) RBC folate, and b) serum folate at baseline. Point colour corresponds to participant.

Discussion

In this randomized intervention trial, we aimed to elucidate whether folic acid supplementation in an unfortified population with adequate folate blood levels affects DNA methylation status. The folic acid supplementation influenced RBC folate and serum folate levels over the course of 2 months in all participants; however, DNA methylation status remained unaffected. We observed a modest correlation between an increase in RBC folate levels and increase in DNAmAge AgeAccel.

Studies on the impact of folic acid supplementation are sparse but important in light of safety consideration given the folic acid fortification programmes in many countries around the globe. In the Aspirin-Folate Polyp Prevention Study in the U.S., folic acid supplementation with 1 mg/d for up to 6 y in subjects with previous colorectal adenomas increased the number of new adenomas [29]. In Norway, where there is no folic acid fortification, the combined supplementation of folic acid and vitamin B₁₂ resulted in an increase in cancer incidence

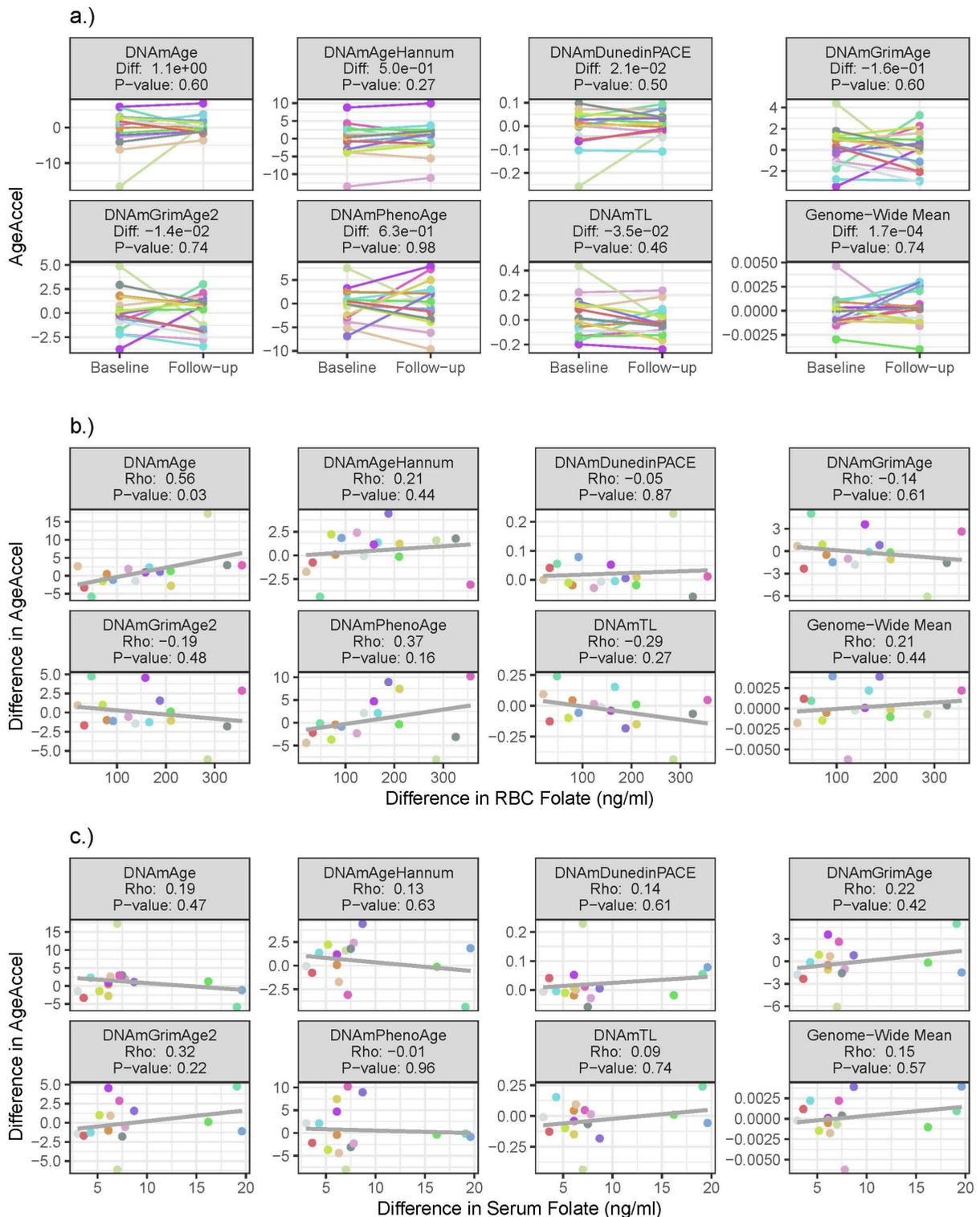


Figure 3. The longitudinal change in AgeAccel a) over follow-up, and in relation to the change in b) RBC folate, and c) serum folate. Point colour corresponds to participant.

among patients with ischaemic heart disease [30]. It has been suspected that folic acid supplementation might promote the progression of underlying preneoplastic lesions [29,31];

however, it remains unclear whether DNA methylation may play a role in this observation.

Previous studies have reported mixed results of folic acid intake on DNA methylation. A study in

colorectal cancer patients found an increase in global methylation [32], while another study observed hypomethylation [33], yet a third study found no methylation changes [34]. Van den Donk et al. [35] observed an increase in DNA methylation in promoters of six tumour suppressor genes after folic acid supplementation. Kok and colleagues examined the effects of long-term (104 weeks) daily folic acid (400 µg/d) and vitamin B 12 (500 µg/d) supplementation on genome-wide DNA methylation assessed with the 450K array in adult leukocyte samples of elderly subjects and found six significant DMRs between the intervention and placebo groups [36]. The amount of folic acid intake ranging from 400 µg/d to 5 mg/d as well as the duration of intervention ranging between 2 and 6 months varied considerably across studies. These differences may explain some of the diverging results observed since folate may have dual effects [28,37,38]. A recent review of studies addressing the association between nutrients involved in one-carbon metabolism and DNA methylation supported this heterogeneity of findings, which may also have been due to differences in the assessment of methylation status [39]. The majority of intervention studies examined the effect of one-carbon metabolism nutrients on global methylation, while only a few investigated gene-specific or genome-wide methylation [39].

The potential impact of folic acid supplementation on age acceleration assessed by the epigenetic clock has been little studied. Sae-Lee et al. used data from a folic acid intervention trial with publicly available Illumina Infinium 450K methylation datasets to explore the impact on the epigenetic clock [40]. In this study, 44 participants were randomized to supplementation with folic acid (400 µg/d) and vitamin B₁₂ (500 µg/d) for 2 y [36]. Age acceleration did not significantly change following the intervention, however, decreased epigenetic ageing was observed among women with the *MTHFR* 677CC genotype [40].

There are a number of possible explanations why we did not observe more substantial changes in DNA methylation despite continuously rising folate levels among all participants throughout the trial. Far and foremost, none of the participants had a folate deficit prior to supplementation. DNA

methylation may be relatively stable as long as one-carbon metabolism is sufficiently catalysed by all important mediators and therefore generates adequate methyl groups. Had we selected a deficient population, DNA methylation profiles may have been more variable. Moreover, while a deficit in the availability of methyl groups is strongly correlated with hypomethylation, excess availability does not necessarily translate into hypermethylation [41]. An alternative explanation may have been our intervention and observation period which spanned 2 months and may not have been sufficient to alter the methylation pattern more substantially. DNA methylation has been considered a relatively stable mechanism [42]. However, there may be differences in DNA methylation stability depending on the genomic location. Moreover, studies have identified certain stressors that may induce fairly rapid changes in DNA methylation. Incubation with a methyltransferase inhibitor may result in a rapid turnover of DNA methylation [43]. Immune response to infection also induces fast active demethylation of many CpG sites [44]. However, our intervention may not represent a sufficiently strong impact to influence DNA methylation. Furthermore, we only measure methylation in PBLs, hence changes in methylation patterns in other tissues would have escaped our assessment. Moreover, synthetic folic acid and folate from diet may have different effects.

Previous studies have pointed to an increased need for folate among carriers of the (*MTHFR*)-C677T variant. We did not detect differences according to the *MTHFR* variants; however, our study participants had adequate folate blood levels prior to intervention despite the lack of folic acid fortification programmes in Germany.

Our study was modest in sample size and duration limiting statistical power and generalizability of the results. However, we were able to test two regimens of folic acid interventions, 400 and 800 µg. Our modest study size precluded adjustment for potential confounding of the association between AgeAccel and folate levels, and between changes in these measures over follow-up. While the observed associations may be driven by common causes of change in AgeAccel and folate levels, our study does characterize shared

longitudinal variation. Future investigations may illuminate what factors contribute to the observed correlations.

In summary, we observe few and select differences in PBL DNA methylation patterns following supplementation with folic acid in a population with adequate folate levels.

Acknowledgments

We thank Mara Waldschmidt for help with conducting the trial and collecting the samples. We are grateful to the 16 trial participants for their time and compliance with the study protocol.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

This work was not supported by any funding.

Data availability statement

The data that support the findings of this study are available upon request from the corresponding author. The data are not publicly available due to privacy restrictions.

Author contributions

K.B. Michels designed the research; A.M. Binder analysed the data; K.B. Michels wrote the manuscript; A.M. Binder provided critical scientific input on the manuscript; both authors approved the final version of the manuscript.

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