Dietary intervention modifies DNA methylation age assessed by the epigenetic clock

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Received: 24/01/2018; Revised: 19/08/2018; Accepted: 25/08/2018

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/mnfr.201800092.

Short running head: Dietary intervention and epigenetic clock

Abbreviations

DNMT: DNA methyltransferases

MOF: monomeric and oligomeric flavanols

MTHFR: methylenetetrahydrofolate reductase

SAM: *S*-adenosyl-L-methionine

DAC: decitabine

Keywords: DNA methylation, MTHFR, folic acid, vitamin B_{12}, MOF, Infinium 450K, DNAm age

Abstract

Scope: Alterations in DNA methylation patterns are correlated with aging, environmental exposures and disease pathophysiology; the possibility of reverting or preventing these processes through dietary intervention is gaining momentum. In particular, methyl donors that provide *S*-adenosyl-methionine for one-carbon metabolism and polyphenols such as flavanols that inhibit the activity of DNA methyltransferases (DNMTs) could be key modifiers of epigenetic patterns.

Methods and results: We assessed DNA methylation patterns in publicly available Illumina Infinium 450K methylation datasets from intervention studies with either folic acid + vitamin B_{12} (GSE74548) or monomeric and oligomeric flavanols (MOF) (GSE54690) in 44 and 13 participants, respectively. Global DNA methylation levels increased in unmethylated regions such as CpG islands and shores following folic acid + vitamin B_{12} supplementation and de-

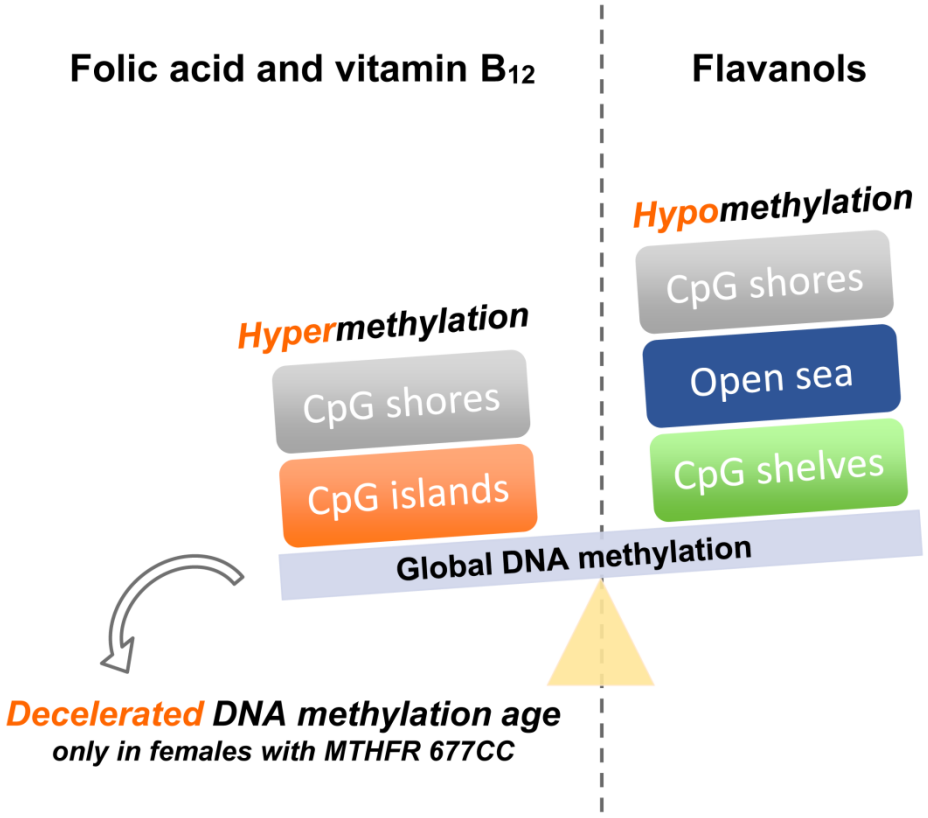
creased in highly methylated regions, including shelves and open-seas following intervention with MOF. After supplementation with folic acid + vitamin B_{12}, epigenetic age, estimated by the Horvath 'epigenetic clock' model, was reduced in women with the *MTHFR* 677CC genotype.

Conclusions: The effects of supplementation with folic acid + vitamin B_{12} and MOF on DNA methylation age are dependent upon gender and *MTHFR* genotype. Additionally, our findings demonstrate the potential for these dietary factors to modulate global DNA methylation profiles.

Graphical abstracts:

We examined the effects of dietary supplementation with folic acid+vitamin B_{12} and flavanols upon global epigenetic patterns and epigenetic age. We observed increased methylation at CpG islands and shores following folic acid+vitamin B_{12}, and decreased methylation at shelf and open sea regions after flavanols supplementation. Epigenetic age was decelerated by folic acid+vitamin B_{12}, but only in women with *MTHFR* 677CC.

Graphical Abstracts



Introduction

DNA methylation is an epigenetic mark that enables the regulation of gene expression and, thereby, diverse biological processes including embryonic development, genomic imprinting, and aging. Aberrant DNA methylation patterns are implicated in multiple common diseases such as lung cancer, cardiovascular diseases, and metabolic disorders [1-6]. This epigenetic mark involves the covalent addition of a methyl group from *S*-adenosyl-L-methionine (SAM), the universal methyl donor, to the C-5 position of cytosine bases by DNA methyl-transferases (DNMTs) [7]. SAM is generated via one-carbon metabolism comprising the fo-late and methionine cycles which includes the conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate that is catalyzed by methylenetetrahydrofolate reductase (MTHFR). The most well-characterized polymorphism in *MTHFR*, substitution of cytosine

by thymine at position 677 (C677T) in exon 4, leads to genotype and dose-dependent reduced activity of the enzyme, which causes increased homocysteine and decreased folate concentrations and these are associated with higher risk for age-associated diseases such as diabetes, vascular disease, and Down syndrome [8-10]. Although *MTFHR* genotype has been associated with changes in DNA methylation pattern in some studies [11], few effects were observed in a recent meta-analysis of pooled data from almost 10,000 individuals [12].

Nutrients and other food components can modify DNA methylation patterns [13] to modulate biological functions that influence health and aging. Higher intakes or status of several micronutrients participate in one-carbon metabolism, including folate, choline, betaine, methionine, vitamin B_6, and vitamin B_{12}, which contribute to SAM production are associated with increased DNA methylation [14, 15]. Other nutrients, including flavanols and (-)-epigallocatechin-3-gallate (EGCG), are competitive inhibitors of DNMTs through binding within the active site of the enzyme [16], and leading to decreased global DNA methylation. Many studies support the hypothesis that dietary compounds can modify DNA methylation patterns and influence the aging process [17]. An increasing body of evidence also suggests that the discrepancy between chronological age and DNA methylation age (DNAm age), that is an estimate of biological age determined by assessment of DNA methylation at 353 CpG sites across the genome [18], is associated with risk of age-related diseases such as diabetes, obesity, cancer, and cardiovascular disease [19, 20].

In this study, we investigated the effect of supplementation with folic acid + vitamin B_{12} as a methyl donor and flavanols as DNMT inhibitors upon global DNA methylation profiles, including estimates of DNA m age, using publicly available Illumina Infinium 450K methylation datasets. We observed that DNAm age estimated by Horvath's model was altered by gender and *MTHFR* 677CC genotype following dietary intervention.

Materials and Methods

Study design

We utilized publicly available Illumina Infinium 450K datasets from two separate dietary intervention studies. Ethical approval for the studies was granted by the institutional Medical Ethics Committee [21] and the Medical Ethical Committee of Maastricht University and Academic Hospital Maastricht [22]. In the first intervention study, 44 older participants (65-75 years) were randomized to supplementation with folic acid (400µg/day) and vitamin B_{12} (500µg/day) for two years. DNA methylation profiles before and after the intervention was measured in blood by Illumina Infinium 450K microarray and the dataset was made available through the Gene Expression Omnibus (GSE74548)[21]. *MTHFR* genotype was also available from the study. The second intervention study was a non-randomized trial in which 13 non-obese, healthy male smokers (aged 30-60 years) were supplemented with monomeric and oligomeric flavanols (MOF; 200µg/day) for 8 weeks. No genotype data was available from this study. DNA methylation profiles before and after the intervention were measured in blood by Illumina Infinium 450K microarray and the dataset was made available through the Gene Expression Omnibus (GSE54690)[22]. The characteristic of participants in both intervention studies are summarized in **Table 1**. For comparison with the effects of MOF, we also utilized a dataset of DNA methylation profiles from a study in which primary acute myeloid leukemia (AML) cells were treated with low-dose decitabine (DAC) (GSE40870) [23].

Data analysis

Datasets normalized by the SWAN procedure were used for our analysis [24]. Since we examined each intervention study separately, we did not normalize the two data sets together. Probes with detection *p*-value>0.05 were excluded [21-23], sex chromosomes and SNP10

probes [25] were also excluded. The annotations for TSS200, TSS1500, 5'UTR, 3' UTR, 1st exon, and gene body were acquired from the Illumina annotation file. For all datasets, global DNA methylation was determined by calculating the average across all interrogated CpG sites, and also in genomic regions (CpG islands, shores, shelves, and open-sea areas) with characteristically different CpG density. DNA methylation age was calculated by the Horvath 'epigenetic clock' model using the online tool (https://dnamage.genetics.ucla.edu/), and methylation data for 353 CpG loci as described previously [26].

Statistics

The effects of folic acid + vitamin B_{12} and of MOF on global DNA methylation were analyzed using paired-sample *t*-tests and ANOVA test. Both datasets were assessed for normality and revealed to be normally distributed, and therefore suitable for parametric testing (Supplemental Table 1). Paired sample *t*-tests were used to identify significant changes in global DNA methylation following MOF treatment, and age acceleration residuals among women with the *MTHFR* 677CC genotype. Repeated measures ANOVA was used to identify significant changes in global DNA methylation, DNA methylation age and age acceleration residuals of dietary interventions in relation to *MTHFR* genotype, gender and CpG density. Pearson's correlation coefficient was use to compare DNA methylation before and after the interventions. All analyses were performed using the IBM SPSS statistical software (version 24); data are presented as means ± SD, and p-values<0.05 were considered to be significant.

Results

Global DNA methylation changes by CpG density following supplementation with folic acid + vitamin B_{12} or with MOF

Global DNA methylation was calculated as the mean beta value across all loci on the Illumina Infinium microarray after excluding 47,788 loci from the sex chromosomes and SNP-associated loci. Global DNA methylation was not significantly changed after MOF supplementation (Δβ=-0.013±0.02, p=0.71, paired sample t-test) (Figure 1A). In contrast, folic acid + vitamin B_{12} supplementation increased global DNA methylation (Δβ=0.003±0.01; p=0.01, repeated measures ANOVA) (Figure 1B). Furthermore, the effect of supplementation with folic acid + vitamin B_{12} intervention displayed an interaction with *MTHFR* genotype (p=0.01), with increased methylation among those with the *MTHFR* 677CC genotype (Δβ=0.005±0.01) but not the 677TT genotype (Δβ<0.001±0.05) (Figure 1B).

Changes in DNA methylation following intervention were then analyzed at loci mapping to CpG islands, shores, shelves, and open-sea areas that have characteristically different CpG density. The mean baseline DNA methylation levels (®) were 0.24 and 0.24 at CpG islands, 0.49 and 0.50 at north shores, 0.48 and 0.49 at south shores, 0.74 and 0.76 at north shelves, 0.74 and 0.77 at south shelves, and 0.70 and 0.72 in open sea areas for samples from the MOF and folic acid + vitamin B_{12} intervention studies, respectively (Figure 2A). Following dietary intervention, DNA methylation was altered in a supplement-specific manner (p=0.01, repeated measures ANOVA). DNA methylation at north shelves decreased after MOF intervention (Δβ=-0.003±0.00006), but increased after folic acid + vitamin B_{12} supplementation (Δβ=0.001±0.00006) (Figure 2B). DNA methylation at CpG islands increased after folic acid + vitamin B_{12} supplementation (Δβ=0.003±0.00001), but decreased following MOF intervention (Δβ=-0.014±0.00007). However, these observed differences in DNA methylation change by genomic region were not statistically significant (p=1.00).

Interestingly, we observed a similar pattern of greater decreases in DNA methylation at regions with lower CpG density in cells from AML patients following treatment with

DAC, a DNA methyltransferase (DNMT) inhibitor (Figure 2C). The magnitude of change in DNA methylation was significantly greater with DAC treatment than with MOF supplementation (p<0.001, repeated measures ANOVA) and displayed an interaction with genomic region (p=0.01), but the patterns of change by genomic region were very highly correlated (R^2=0.99, p-value< 0.0001) (Figure 2D).

Change in DNA methylation age after supplementation with folic acid + vitamin B_{12} or with MOF

The effect of dietary intervention upon DNA methylation age was assessed using Horvath's 'epigenetic clock' model [26], for both intervention studies (Figure 3A and B). We calculated age acceleration residuals, defined as the regression of DNAm age on chronological age. Age acceleration did not significantly change following the interventions: the difference between means were -0.337±2.061 for MOF (p=0.87) and -0.765±1.435 for folic acid + vitamin B_{12} (p=0.60) (Figure 3 C and D). However, differential methylation at the 353 epigenetic clock loci was observed in an intervention-specific manner similar to that across the wider genome. Increased methylation was observed at the loci following folic acid + vitamin B_{12} supplementation, while reduced methylation was observed following MOF supplementation (p=0.01, repeated measures ANOVA). Similar effects were observed across all genomic regions, with no interaction between the form of dietary supplementation and CpG density (p=0.76) (Figure 3E).

DNA methylation age after folic acid +vitamin B_{12} supplementations by gender and MTHFR genotype

We analyzed the age acceleration residual by gender and *MTHFR* genotype for participants in the folic acid + vitamin B_{12} intervention study. Age acceleration residuals in females with the *MTHFR* 677CC genotype displayed the greatest change following dietary intervention (Δresidual=-2.70) in comparison to males with the same genotype (Δresidual=-0.75), and females and males with the 677TT genotype (Δresidual=0.16 and 0.76 respectively) (Figure 4). While the influence of genotype (p=0.16, repeated measures ANOVA), gender (p=0.41) and their interaction (p=0.67) upon response to intervention were not statistically significant across all participants, we observed a significant reduction in age acceleration solely among women with the *MTHFR* 677CC genotype (p=0.04, paired sample t-test). Furthermore, interestingly, we observed that age acceleration in males with the *MTHFR* 677TT genotype was higher than those with the *MTHFR* 677CC at baseline (Δmeans=6.014±2.422) and this relationship was unaltered following intervention (Δmeans=7.529±2.864) (Figure 4).

Discussion

Several dietary factors modify epigenetic patterns [13]. These include vitamins that contribute to the production of the methyl donor SAM via one-carbon metabolism e.g. polyphenols that inhibit of DNMT activity. Using publically-available datasets, our study has demonstrated that supplementation with folic acid + vitamin B_{12} alters global DNA methylation, with differential effects of intervention agents by genomic domain, *MTHFR* genotype and gender.

We observed that supplementation with folic acid + vitamin B_{12} increased global DNA methylation levels in individuals with the *MTHFR* 677CC but not the *MTHFR* 677TT genotype, and that this effect was greatest in CpG islands and shores that are characteristically CpG-dense regions. The T version of the *MTHFR* C677T polymorphism results in the substitution of an alanine residue with a valine, leading to a 50% reduction in enzymatic activity,

and 10-20% of individuals in Western Europe and North America have the 677TT genotype [27, 28]. Observational studies have reported that higher levels of folic acid and vitamin B_{12} in the diet are associated with higher global DNA methylation levels [14, 15]. However, contrary to our findings, Jung *et al* [29] reported no significant effect of supplementation with 0.8 mg folic acid/d on global DNA methylation even when *MTHFR* genotype was taken into account. We speculate that this discrepancy may be due to differences in methodology used for quantifying global DNA methylation. Jung *et al* used a liquid chromatography-tandem mass spectrometry approach that quantifies all methylated cytosines relative to total cytosine in the genome. In contrast, we used data from the Illumina Infinium DNA methylation microarray platform that interrogates more than 450,000 specific cytosine residues that are located predominantly in CpG-dense regions such as CpG islands that we observed were more susceptible to the effects of folic acid + vitamin B_{12}.

The 'epigenetic clock' model developed by Horvath [18] provides estimates of DNAm age – a putative measure of biological age. This model integrates DNA methylation at 353 CpG sites that map to genes associated with cell survival and development and that may measure the cumulative effect of an epigenetic maintenance system [18]. Age acceleration, where the calculated DNAm age exceeds chronological age, has been observed in the liver of obese individuals [30], is associated with the development of age-associated diseases such as Parkinson's disease [34] and coronary heart disease [31], and has demonstrated links with all-cause mortality in multiple studies [32-34]. Recent investigation of observational studies has shown that higher intakes of fish and vegetables are associated with reduced epigenetic aging using an enhanced version of an alternative model developed by Hannum *et al* [35]. Our study identified an association between dietary supplementation with folic acid + vitamin B_{12} and decreased epigenetic aging, but only in women with the *MTHFR* 677CC

genotype. Women might need less folate supplementation due to differences in body mass by gender [36], and therefore higher doses of folate may be required to produce the same biological effect in men. This is supported by the finding that, following folic acid + vitamin B_{12} intervention study, serum folate concentration was higher among women than men, and highest of all among women with the 677CC genotype (data not shown). We observed no significant correlation directly between age acceleration residuals and serum folate, serum vitamin B_{12} or plasma homocysteine levels (data not shown), thereby underlining the complex interaction between dietary intake, genotype and epigenetic aging. Interestingly, our study also revealed significantly higher DNAm among men with the *MTHFR* 677TT genotype in comparison to those with the CC genotype irrespective of dietary intervention, but this was not the case among women. To the best of our knowledge, our observation of reduced epigenetic aging following folic acid + vitamin B_{12} supplementation is the first reported effect of *MTHFR* genotype on DNA methylation in women, and may suggest that women with the more common 677CC genotype may benefit particularly from such supplementation. Notably, previous studies into genotype by gender interactions in response to folate have been conducted in populations with low folate intake, and we speculate that such gender and genotype effects may become apparent only when folate intake/ status is higher. Further work is required to elucidate how MTHFR activity may influence the epigenetic aging process.

Indeed, there has recently been tremendous interest in the previously described epigenetic models of ageing, such as the 'epigenetic clock' model by Horvath [18], that of Hannum *et al* [37], and the age-associated epigenetic drift described by Teschendorff and colleagues [38]. Importantly, there is already substantial evidence to suggest that epigenetic ageing accelerates in response to lifestyles and exposures, such as tobacco smoke [33], stress [39] and obesity [30], and also with outcomes such as all-cause mortality [40]. However, the

changes in DNA methylation at the loci incorporated into these models are predominantly small in effect size. Furthermore, a recent study by Maegawa *et al*. [41] reported that the rate of epigenetic drift is correlated with lifespan. Mice, with a maximum longevity of 4 years, show 1% drift in DNA methylation per year, while rhesus monkeys (maximum longevity: 40 years) show drift of 0.3% per year and humans (maximum longevity: 122.5 years) 0.1% per year. Maegawa *et al*. demonstrate that caloric restriction is able to reduce this DNA methylation drift, which may therefore help to explain the association of increased longevity after caloric restriction. As Breton *et al*. have discussed [42], many epigenetic epidemiology studies have reported 1-5% changes in DNA methylation in response to environmental exposures, whether measuring gene-specific or global DNA methylation (frequently using LINE-1 and *Alu* as surrogate markers). In the context of epigenetic drift with aging, it can be interpreted that a 1% change in DNA methylation in response to environmental exposures is equivalent to 10 years' worth of natural epigenetic drift. Most environmental epigenetic studies utilize blood as the tissue in which to measure DNA methylation, and Issa has hypothesized that it could be possible to use epigenetic drift in blood as a surrogate for other tissues [43]. This, of course, would need to be examined carefully, and further work is required to establish how changes observed in blood may correlate with life expectancy. Nonetheless, there is sufficient evidence to support the hypothesis that small magnitude changes in DNA methylation, particularly when occurring at the pre- or post-natal stage, could be biologically significant in the context of representing epigenetic drift and the health consequences later in life.

Our study has limitations. Firstly, the designs of the two independent intervention studies that we used differed in several respects including gender and age distribution of participants, and the duration of intervention. Because of this limitation, we chose to examine data from each independently, in parallel. Secondly, the number of participants receiving

folic acid + vitamin B_{12} supplementation was relatively small (n=44) and so our observation of effects of gender and *MTHFR* genotype on DNA methylation patterns and on epigenetic age acceleration will need to be confirmed in future studies. In addition, it is likely that we did not have sufficient statistical power to detect other interactions. We therefore took steps to minimize potential sources of variability in order to maximize our potential to observe biological changes. The intervention studies used for our analysis were performed in healthy, non-obese individuals who were non-smokers and did not drink alcohol excessively (folic acid + vitamin B12 intervention) or who were male smokers (MOF). The pairwise analysis of samples from participants before and after intervention enabled us to control for a number of potential confounding factors, and we stratified our analysis by gender, *MTHFR* genotype and by use of age acceleration residuals, which are not correlated with chronological age.

In summary, for the first time, we report that supplementation with folic acid + vitamin B_{12} or with monomeric and oligomeric flavanols can modify DNA methylation patterns and that these effects differ by intervention agent and by genomic location or CpG density. Further, the effect of folic acid + vitamin B_{12} supplementation appear to be gender- and *MTHFR* genotype-specific. If confirmed in future independent studies, these novel findings will strengthen the evidence base for personalized nutrition strategies which integrate genotypic data which may enhance the efficacy of dietary interventions [44]. Further work is required to determine the utility of these dietary compounds in the maintenance of normal epigenetic patterns for disease prevention.

Author contributions

HMB designed the research; CSL, SC, and TMB analyzed the data; CSL, SC, GGCK, VB, TMB, JCM, and HMB wrote the paper; HMB had primary responsibility for final content. All authors read and approved the final manuscript.

Acknowledgments

None to report.

Conflict of interest statement

The authors have no conflict of interest to report.

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Figure and table legends

Figure 1. Global DNA methylation between before and after dietary intervention. Mean global methylation levels following MOF (A) or folic acid + vitamin B_{12} (B) supplementation. Mean ± SD.

Figure 1

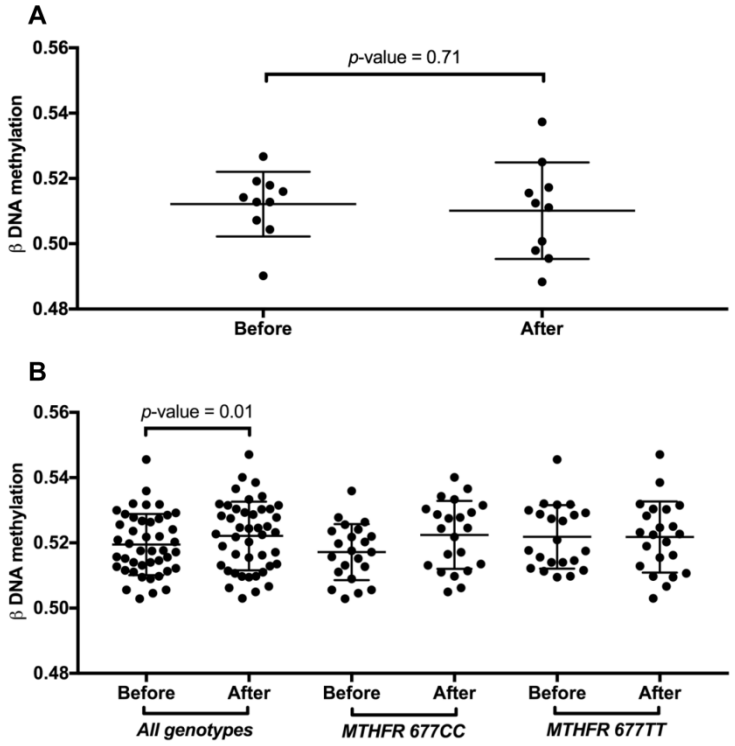


Figure 2. Global DNA methylation by CpG density after intervention. Global DNA methylation by CpG density before the interventions (A), mean of global DNA methylation changes after the interventions (B), mean of global DNA methylation changes by CpG density after decitabine (DAC) treatment in acute myeloid leukemia patients (C), and comparison of global DNA methylation changes by CpG density between DAC treatment and MOF intervention (D). Mean ± SD.

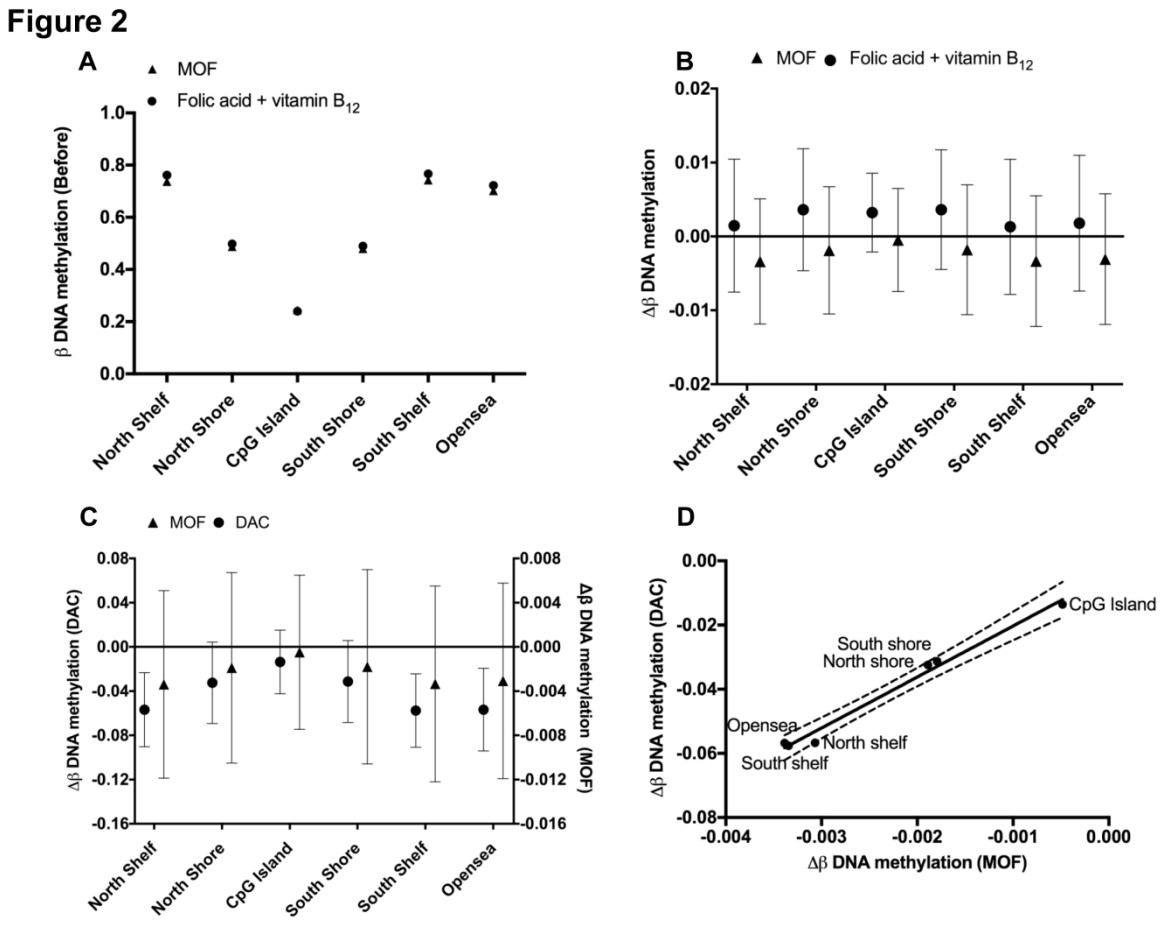


Figure 3. DNAm age following dietary interventions. The correlation of DNAm age before and after MOF supplementation (A) and folic acid + vitamin B_{12} supplementation (B). Age acceleration residuals before and after MOF supplementation(C) and after folic acid + vitamin B_{12} supplementation (D). (E) DNA methylation changes at epigenetic clock loci by CpG density, with each data point corresponding to the average methylation change across the study participants for each of the 353 clock loci. Mean ± SD. *** = p<0.001; **** = p<0.0001

Figure 3

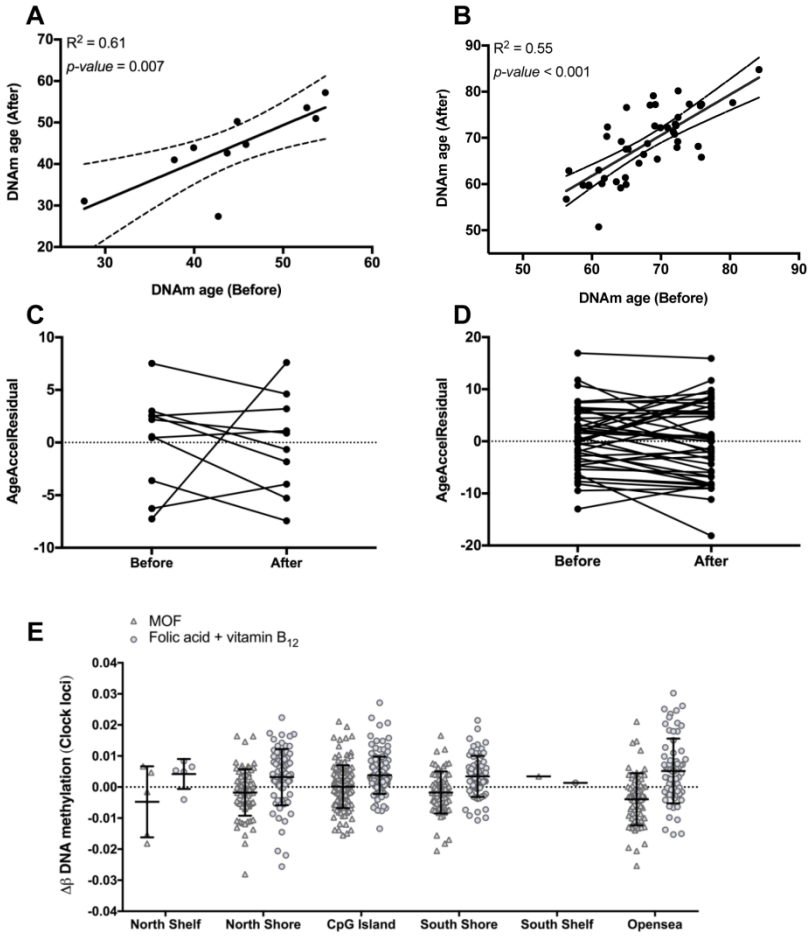
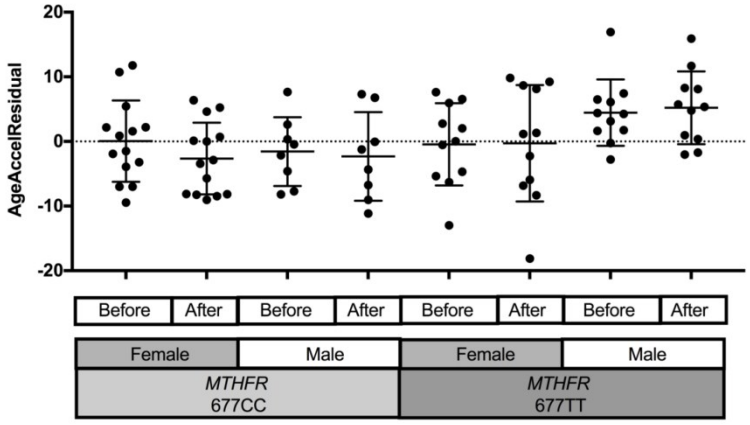


Figure 4. Age acceleration residuals before and after folic acid + vitamin B_{12} intervention by gender and *MTHFR* C677T genotypes. Mean ± SD.

Figure 4



Supplemental Figure 1. The correlation between epigenetic age and serum folate (nmol/L), serum vitamin B_{12} (pmol/L) and plasma homocysteine (μmol/L) by *MTHFR* genotype.

Table 1. The characteristics of participants in the MOF and folic acid + vitamin B_{12} intervention studies

Supplemental Table 1. Assessment of normality.

Table 1.

Study	**MOF**	**Folic acid and vitamin B_{12}**
Study design	Non-randomized trial	Double-blinded, randomized and placebo-controlled trial
Intervention	200mg monomeric and oligomeric flavanols (MOF)	400µg folic acid and 500µg vitamin B_{12}
N	13	44
Sex (male/female)	13/0	19/25
Duration of daily supplementation	8 weeks	2 years
Mean BMI (range)	25 (18-28)	27 (24-30)
MTHFR C677T (CC/TT)	NA	22/22