Dietary intake of one-carbon metabolism nutrients and DNA methylation in peripheral blood

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ABSTRACT

Background: Folate and other one-carbon metabolism nutrients are essential to enable DNA methylation to occur, but the extent to which their dietary intake influences methylation in adulthood is unclear.

Objective: We assessed associations between dietary intake of these nutrients and DNA methylation in peripheral blood, overall and at specific genomic locations.

Design: We conducted a cross-sectional study using baseline data and samples from 5186 adult participants in the Melbourne Collaborative Cohort Study (MCCS). Nutrient intake was estimated from a food-frequency questionnaire. DNA methylation was measured by using the Illumina Infinium HumanMethylation450 BeadChip array (HM450K). We assessed associations of intakes of folate, riboflavin, vitamins B-6 and B-12, methionine, choline, and betaine with methylation at individual cytosine-guanine dinucleotides (CpGs), and with median (genome-wide) methylation across all CpGs, CpGs in gene bodies, and CpGs in gene promoters. We also assessed associations with methylation at long interspersed nuclear element 1 (LINE-1), satellite 2 (Sat2), and Arthrobacter luteus restriction endonuclease (Alu) repetitive elements for a subset of participants. We used linear mixed regression, adjusting for age, sex, country of birth, smoking, energy intake from food, alcohol intake, Mediterranean diet score, and batch effects to assess log-linear associations with dietary intake of each nutrient. In secondary analyses, we assessed associations with low or high intakes defined by extreme quintiles.

Results: No evidence of log-linear association was observed at $P < 10^{-7}$ between the intake of one-carbon metabolism nutrients and methylation at individual CpGs. Low intake of riboflavin was associated with higher methylation at CpG cg21230392 in the first exon of *PROM1* ($P = 5.0 \times 10^{-8}$). No consistent evidence of association was observed with genome-wide or repetitive element measures of methylation.

Conclusion: Our findings suggest that dietary intake of one-carbon metabolism nutrients in adulthood, as measured by a food-frequency questionnaire, has little association with blood DNA methylation. An association with low intake of riboflavin requires replication in independent cohorts. This study was registered at http://www.clinicaltrials.gov as NCT03227003. *Am J Clin Nutr* 2018;108:611–621.

Keywords: B vitamins, DNA methylation, epigenetics, folate, one-carbon metabolism

INTRODUCTION

DNA methylation is an epigenetic mechanism used by cells to regulate gene expression. It involves the addition of a methyl (-CH3) group to 5-carbon cytosine residues, predominantly

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Supplemental Methods, Supplemental Tables 1–11, and Supplemental Figures 1–4 are available from the "Supplementary data" link in the online posting of the article and from the same link in the online table of contents at https://academic.oup.com/ajcn/.

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Abbreviations used: Alu, arthrobacter luteus restriction endonuclease; CpG, cytosine-guanine dinucleotide; FFQ, food-frequency questionnaire; HM450K, Illumina Infinium HumanMethylation450 BeadChip array; LINE-1, long interspersed nucleotide element 1; MCCS, Melbourne Collaborative Cohort Study; *MTHFR*, 5,10-methylenetetrahydrofolate reductase; Sat2, satellite 2; UCC, urothelial cell carcinoma.

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within cytosine-guanine dinucleotides (CpGs). Although much emphasis has been placed on the key role played by folate (sometimes also referred to as vitamin B-9) to produce *S*-adenosylmethionine, a methyl group donor in the DNA methylation process, other nutrients involved in one-carbon metabolism, such as choline, betaine, riboflavin, vitamins B-6 and B-12, and the amino acid methionine, are also necessary (**Figure 1**) (1–3). Disruption of the one-carbon metabolism pathway can interfere with DNA replication, DNA repair, and regulation of gene expression and could be related to carcinogenesis (2, 4, 5). This has motivated a large body of literature investigating potential associations between one-carbon metabolism nutrients and cancer risk (6–8).

One-carbon metabolism nutrients are not naturally produced by the body, so they need to be obtained from food. Previous studies of one-carbon metabolism nutrient intake and DNA methylation, particularly epidemiologic studies in humans (1), have been limited in sample size and have produced inconsistent, most often weak, evidence of associations (4). For example, although one study reported that lower dietary intake of folate was associated with higher global methylation (9), another observed an association in the opposite direction (10), and a third, larger study, identified no association (11); for 2 of these studies (10, 11), the intake of other one-carbon metabolism nutrients was not associated with global methylation levels.

Most studies investigating the association between one-carbon metabolism nutrients and blood DNA methylation have used measures based on repetitive genomic sequences such as long interspersed nucleotide element 1 (LINE-1), arthrobacter luteus restriction endonuclease (Alu), and Satellite 2 (Sat2) to assess global methylation (12). Recent improvements in technology allow quantification of DNA methylation at hundreds of thousands of CpGs, but few studies, if any, have examined associations between adult intake of these nutrients and DNA methylation using high-throughput microassays. Joubert et al. (13) recently identified, using the Illumina HumanMethyl450K Beadchip, 44 CpG sites at which DNA methylation in newborns was associated with maternal plasma folate concentrations.

The main aim of this study was to assess whether dietary intakes of nutrients involved in one-carbon metabolism were associated with DNA methylation in peripheral blood, globally and at individual CpG sites, using cross-sectional data for 5186 participants in the Melbourne Collaborative Cohort Study (MCCS). The secondary aims were to examine associations with methylation measured at LINE-1, Sat2, and Alu repetitive DNA elements in a smaller subset of MCCS participants and to assess associations with the 44 CpGs identified by Joubert et al. (13).

METHODS

The MCCS is a prospective cohort study of 41,513 individuals (17,044 men and 24,469 women) recruited between 1990 and 1994 (8). The age range at recruitment was 27–80 y (99.3% were aged 40–69 y). Southern European migrants to Australia were oversampled to extend the range of exposures, including dietary patterns. Information on country of birth, highest level of education, smoking status, physical activity, alcohol consumption, and dietary intake was collected from questionnaires. Height and weight were measured by trained staff using standard methods and a blood sample was collected.

The participant selection process for the present study is summarized in **Figure 2**. Methods relating to DNA extraction and bisulfite conversion, and DNA methylation data processing, are detailed in **Supplemental Methods**.

To assess associations with DNA methylation at individual CpGs and median methylation across CpGs, the study sample comprised MCCS participants selected for inclusion in 1 of 6 nested case-control studies of DNA methylation and each of colorectal, prostate, lung, B-cell, kidney, and urothelial cell carcinoma (UCC) (14–18). DNA methylation was measured using the Illumina Infinium HumanMethylation450 BeadChip array (HM450K). For each nested case-control study, all incident cases of the cancer of interest were selected, each with a control individually matched using incidence density sampling with age as the time scale and year of birth, country of birth, and type of blood specimen (peripheral blood mononuclear cell, buffy coat, or dried blood spot) as matching variables. For the colorectal cancer study, year of baseline attendance was used as a matching variable instead of year of birth. For the lung cancer study, controls were also matched on smoking status at the time of blood collection. Participants were free of any invasive cancer at blood draw, except for participants in the UCC study, who only had to be free of UCC.

After the exclusion of 111 samples (1.8%) with sex-discrepant methylation measures, HM450K data were available for 5983 samples (Figure 2). We further excluded 665 (11%) samples from participants who were duplicated within or between studies: where a participant was included as both a control and a case, the case data was excluded ($n = 271$; 4.5%); for participants with 2 measurements remaining ($n = 394$; 6.6%), the sample with the larger number of failed CpGs was excluded. We excluded 98 (1.8%) participants who reported extreme values of total energy intake (less than the first percentile or >99th percentile), 10 (0.2%) participants with incomplete food-frequency questionnaire (FFQ) data, and 26 (0.5%) participants with missing covariate data. After these exclusions, 5186 participants remained with complete data.

We assessed associations with methylation at LINE-1 repetitive DNA elements using data from participants from separate nested case-control studies of breast and colorectal cancer using a similar protocol for the selection of samples (19, 20). In the breast cancer study, we also measured Alu and Sat2 repetitive elements. After exclusions for extreme energy intake ($n = 54$), incomplete FFQ data ($n = 1$), and missing covariate data ($n = 9$; smoking status), there were 1091 and 1613 participants for the analysis of these repetitive DNA elements from the breast and colorectal cancer studies, respectively (Figure 2).

Dietary nutrient intake

Participants completed a 121-item FFQ specifically developed for the MCCS using weighed food records from equal numbers of people born in Australia, Italy, and Greece and answered additional questions about milk consumption and alcohol intake (21). Sex-specific average portion sizes were assigned to each food item, and daily frequencies of some fruits were seasonally

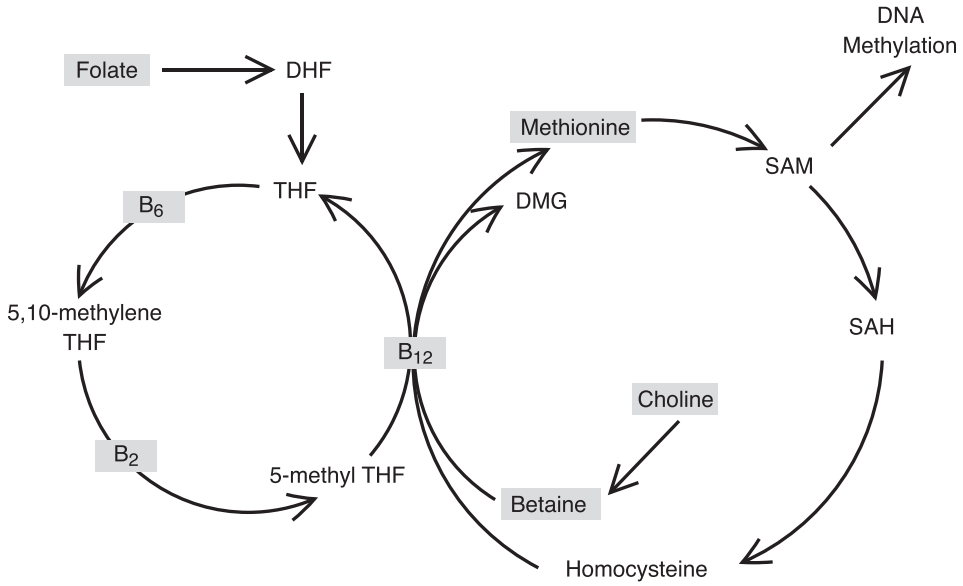

FIGURE 1 One-carbon metabolism. Shading indicates the substrate is obtained via the diet. DHF, dihydrofolate; DMG, dimethyl glycine; SAH, *S*-adenosylhomocysteine; SAM, *S*-adenosylmethionine; THF, tetrahydrofolate. Reproduced from reference 1 with permission.

adjusted. Nutrient-composition data were derived from the Australian NUTTAB 2006 database (22) for the B vitamins and methionine, and from the USDA database for choline and betaine (23, 24). Mean daily intakes for riboflavin, vitamin B-6, folate, vitamin B-12, choline, betaine, and methionine were obtained by multiplying the daily frequency of each food item by the nutrient composition for an average sex-specific portion size. For folate intake, we used the dietary folate equivalent, which combines intakes of folate from food and folic acid fortification (because fortification was relatively rare in Australia at the time of the FFQ, these measures were very similar to intakes of folate from food in our study) (25). Because these nutrients have all been considered to contribute to DNA methylation (26), a composite "methyl donor index" measure was derived by summing standardized intake values on the log scale [(value − mean)/SD] across the 7 individual nutrients.

Ethics

All of the participants provided informed consent, and the study was approved by the Cancer Council Victoria's Human Research Ethics Committee. This study was registered at http://www.clinicaltrials.gov as NCT03227003.

Statistical analysis

Following the method described previously (18), the reliability of methylation measures across studies was examined at each CpG site using intraclass correlation coefficients, based on 1049 duplicate samples; CpG variability was defined as the SD of methylation values across all samples (cases and controls).

Epigenome-wide association study

For the analysis of associations with methylation at individual CpGs, controls and cases were first analyzed separately. Linear mixed models were used to analyze the association between the methylation M-value for each CpG and the log of the dietary intake of each one-carbon metabolism nutrient. As a secondary analysis, we also assessed associations with low or high intakes of individual nutrients and the methyl donor index by categorizing nutrient intake variables into quintiles and comparing the first and fifth (**Supplemental Table 1**) with the middle 3 quintiles. The selection of potential confounders was made using a directed acyclic graph (**Supplemental Figure 1**). All of the models were thus adjusted for age, sex, country of birth (Greece, Italy, United Kingdom/Malta, Australia/New Zealand/other), smoking status [never, former (quit ≥15 or <15 y ago), current (<15 or ≥15 cigarettes/d)], alcohol intake (lifetime abstainers, ex-drinkers, low intake [1–39 g/d (men) and 1–19 g/d (women)], medium intake [40–59 g/d (men) and 20–39 g/d (women)], high intake [≥60 g/d (men) and ≥40 g/d (women)]), energy intake from food, and the Mediterranean diet score (values 0–9, with higher scores reflecting stronger adherence to a Mediterranean-style diet) (27) as a measure of overall diet quality.

We also adjusted for sample type (dried blood spot, peripheral blood mononuclear cells, buffy coats), and cell counts [estimated by using the Houseman algorithm (28)], and batch effects were accounted for by modeling study, plate, and chip as random effects. The potential for collider bias due to the inclusion in the analysis of participants selected because they subsequently developed cancer (cases) (29) was assessed by comparing regression coefficients from the case and control data sets (**Supplemental Figure 2**). Because we did not detect evidence of inflation in the coefficients for cases, the results were then combined using fixed-effects meta-analysis.

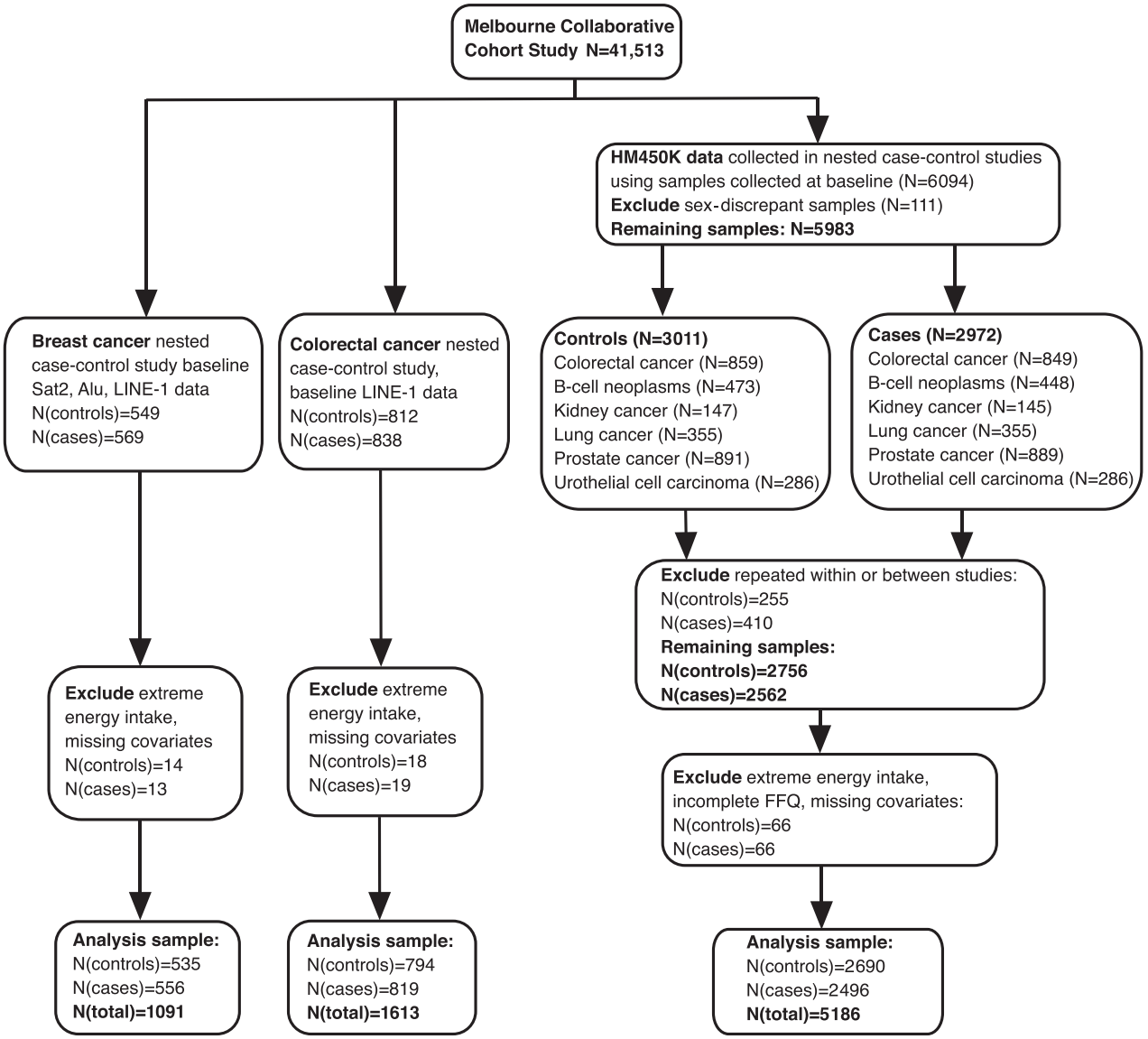

FIGURE 2 Flowchart of the participant selection process used to pool data from several methylation studies nested in the Melbourne Collaborative Cohort Study. Alu, Arthrobacter luteus restriction endonuclease; FFQ, food-frequency questionnaire; HM450K, Illumina Infinium HumanMethylation450 BeadChip array; LINE-1, long interspersed nuclear element 1; Sat2, satellite 2.

Genome-wide measures of methylation

We calculated, as a genome-wide measure of DNA methylation, the median M-value (log-transformed percentage of methylation) across HM450K CpGs (30). We excluded CpGs for which measures were reported to be technically unreliable (31). Three genome-wide measures were considered using median M-values across *1*) all 294,561 remaining CpGs, *2*) the 102,073 CpGs located in gene bodies, and *3*) the 143,259 CpGs located in gene promoters (32), the latter including CpGs annotated to promoters in the first exon, TSS200, TSS1500, and 5′UTR regions according to the Illumina annotation file (version 2) (33). Sensitivity analyses were conducted, restricting to more reliable and variable CpGs, defined as those with an intraclass correlation coefficient >0.1 and an SD >0.05.

For each subset of the HM450K CpGs, a linear mixed model was used to analyze the association between the median methylation measure and the log of the dietary intake of each one-carbon metabolism nutrient. As a secondary analysis, we also assessed associations with low or high intakes of individual nutrients and the methyl donor index, as described above. Linear regression was used to analyze LINE-1, Alu, and Sat2 data. All of the models were adjusted for age, sex, country of birth, smoking status, alcohol intake, energy intake from food, and the Mediterranean diet score. Regression coefficients were expressed per 1 SD of the log nutrient intake, where appropriate. This SD was computed on the basis of all participants included in the study (Supplemental Table 1). For the HM450K-based genome-wide measures, we also adjusted for sample type (dried

blood spot, peripheral blood mononuclear cells, buffy coats) and cell counts [estimated by using the Houseman algorithm (28)], and batch effects were accounted for by modeling study, plate, and chip as random effects. All of the analyses that fit log-linear effects for nutrient intake were undertaken separately for controls and for cases and the results were combined by using fixed-effects meta-analysis (34). For the secondary analyses, similar models were fit for controls and cases combined with additional adjustment for case-control status. Where visual inspection of quantile-quantile plots suggested systematic inflation of P values, analyses were repeated with additional adjustment for the first 10 principal components derived from 250,000 randomly selected CpGs (35, 36). Analyses were performed using Stata/MP 14.2 (StataCorp) and R version 3.4.0.

RESULTS

The baseline characteristics and one-carbon metabolism nutrient intakes of study participants are summarized in **Table 1**. These were similar for cases and controls. For the B vitamins, the median intakes were above the recommended dietary intake for Australian adults (37). We observed strong correlations (**Supplemental Table 2**) between intakes of vitamin B-12 and vitamin B-6 (Spearman's $\rho = 0.69$), between intakes of choline and methionine ($\rho = 0.85$), and between intakes of riboflavin and folate ($\rho = 0.79$), and a moderate correlation between intakes of methionine and each of riboflavin and vitamin B-12 ($\rho = 0.52$ and 0.64, respectively) and of choline and each of riboflavin, vitamin B-6, folate, and vitamin B-12 ($\rho = 0.64$, 0.53, 0.52, and 0.54, respectively).

Epigenome-wide association study

No evidence of a log-linear association with dietary intake of one-carbon metabolism nutrients, or with the composite methyl donor index, was observed with any individual CpG at $P < 10^{-7}$. These results are summarized in quantile-quantile plots in **Figure 3**. For each nutrient, the 5 CpG sites with the smallest P values are shown in **Supplemental Table 3**. The results for 44 CpGs with a significant association between DNA methylation in newborns and maternal plasma folate concentrations, as reported in Joubert et al. (13), are shown in **Supplemental Table 4**. For 2 of these, cg05665581 in *FAM24A* and cg00426709, an intergenic CpG, the associations were nominally significant ($P = 0.009$ and 0.038, respectively), and the regression coefficients were consistent in the direction with that previously reported. Neither of these associations was significant after Bonferroni correction for multiple testing.

The analysis of low or high intakes of individual nutrients and the methyl donor index identified at $P < 10^{-7}$ 3 CpGs associated with low intake of riboflavin and 1 CpG associated with high intake of each of vitamins B-6 and B-12 (**Supplemental Table 5**). There appeared to be systematic inflation of test statistics for these 3 epigenome-wide association studies, which was largely removed by further adjustment for 10 principal components (**Supplemental Figures 3** and **4**); after this adjustment, only the association of cg21230392 on exon 1 of *PROM1* with low concentrations of riboflavin remained significant at $P < 10^{-7}$ (coefficient: 0.082; 95% CI: 0.052, 0.111; $P = 5.0 \times 10^{-8}$).

Genome-wide measures of methylation

Overall levels of blood DNA methylation, as measured by our genome-wide measures, were similar for cases and controls (**Supplemental Tables 6** and **7**). We observed no consistent evidence of an association between one-carbon metabolism nutrient intakes and median HM450K methylation, overall, in gene bodies, or in gene promoters (**Table 2**). A nominally significant association was observed between the intake of vitamin B-6 and methylation at gene promoters (scaled regression coefficient, calculated as the coefficient multiplied by 1000 times the SD of the log of nutrient intake: 4.62; 95% CI: 0.26, 8.99; $P = 0.04$; see Supplemental Table 1 for the scaling SDs), but this was not observed after restricting the analysis to more reliable and variable CpGs (coefficient: 0.54; 95% CI: −2.53, 3.62; $P = 0.73$; Table 2). A similar result was observed for vitamin B-12: the regression coefficient was 4.93 (95% CI: 0.49, 9.37; $P = 0.03$) including all CpGs at gene promoters, but was 0.55 (95% CI: −2.57, 3.68; $P = 0.73$) after restricting to more reliable and variable CpGs. The apparent association between methylation at CpGs in gene promoters and the methyl donor index (coefficient: 6.97; 95% CI: 0.61, 13.3; $P = 0.03$) was attenuated and no longer significant (coefficient: 3.35; 95% CI: −1.12, 7.83; $P = 0.14$) when only reliable and variable CpGs were considered. There were no nominally significant results for cases or controls separately (**Supplemental Table 8**). The findings from the analysis of low or high intakes of these nutrients (**Supplemental Table 9**) were consistent with those described above, with the exception of low consumption of vitamin B-6, for which weak evidence of association was observed with higher median methylation across all CpGs (coefficient: 7.91; 95% CI: 1.10, 14.7; $P = 0.02$) and across reliable and variable CpGs (coefficient: 7.77; 95% CI: 0.62, 14.9; $P = 0.03$).

Associations of nutrient intakes with DNA methylation at LINE-1, Sat2, and Alu repetitive elements are shown in **Table 3** and **Supplemental Table 10**. An inverse association was observed between the intake of riboflavin and LINE-1 methylation in the colorectal cancer LINE-1 methylation study (coefficient: −0.30; 95% CI: −0.57, −0.04; $P = 0.02$), but this association was not observed in the breast cancer LINE-1 methylation data set (coefficient: −0.23; 95% CI: −5.66, 5.20; $P = 0.93$). Results from the analysis of low or high intakes defined by extreme quintiles (**Supplemental Table 11**) were consistently null, with the exception of weak evidence that a high intake of choline was associated with higher methylation at Alu repetitive elements (coefficient: 12.8; 95% CI: 1.73, 23.9; $P = 0.02$).

In the primary analyses of genome-wide and repetitive element DNA methylation log-linear effects, we performed 80 tests for genome-wide measures of methylation and observed 4 (5%) results with $P < 0.05$. In the corresponding secondary analyses, fitting effects for extreme quintiles of nutrient intakes, we performed 160 tests for genome-wide measures of methylation and observed 12 (7.5%) results with $P < 0.05$. Neither of these

TABLE 1
Participant characteristics at baseline blood collection, and nutrient intakes and RDIs for participants with HM450K data[1]

	Controls ($n = 2690$)	Cases ($n = 2496$)
Age, y	60.7 (53.8, 65.4)	60.8 (54.1, 65.5)
Male sex, *n* (%)	1842 (69)	1679 (67)
Country of birth, *n* (%)		
Australia/New Zealand/other	1834 (68)	1704 (68)
United Kingdom/Malta	218 (8.1)	190 (7.6)
Italy	372 (14)	356 (14)
Greece	266 (9.9)	246 (9.9)
Smoking, *n* (%)		
Never	1241 (46)	1101 (44)
Former		
Quit ≥15 y ago	880 (33)	860 (34)
Quit <15 y ago	180 (6.7)	161 (6.5)
Current		
<15 cigarettes/d	103 (3.8)	90 (3.6)
≥15 cigarettes/d	286 (10.6)	284 (11)
Alcohol consumption, *n* (%)		
Lifetime abstainer	620 (23)	569 (23)
Ex-drinker	104 (3.9)	108 (4.3)
Low intake	1573 (58)	1422 (57)
Medium intake	244 (9.1)	242 (9.7)
High intake	149 (5.5)	155 (6.2)
Mediterranean diet score[2]	5 (4, 6)	5 (4, 6)
Energy intake, kJ/d	8600 (6843, 10,863)	8666 (6853, 10,945)
Riboflavin intake,[3] mg/d	2.38 (1.81, 3.04)	2.39 (1.80, 3.03)
Vitamin B-6 intake,[4] mg/d	1.82 (1.37, 2.65)	1.84 (1.36, 2.73)
Folate intake,[5] μg/d	404 (292, 540)	405 (290, 539)
Vitamin B-12 intake,[6] μg/d	3.15 (2.25, 4.44)	3.15 (2.27, 4.52)
Methionine intake, mg/d	1618 (1294, 2034)	1657 (1315, 2075)
Choline intake,[7] mg/d	373 (301, 466)	379 (303, 470)
Betaine intake, mg/d	356 (247, 511)	368 (249, 520)
Methyl donor index[8]	0.02 (−1.52, 1.53)	0.07 (−1.56, 1.65)

[1] Values are medians (IQRs) for continuous variables or *n* (%) for categorical variables. RDIs are for persons aged 51–70 y, which is comparable to age at baseline for our sample. AI, adequate intake; EAR, estimated average requirement; HM450K, Illumina Infinium HumanMethylation450 BeadChip array; RDI, recommended dietary intake (not available for methionine or betaine).

[2] Range of values: 0–9; higher scores reflect stronger adherence to a Mediterranean-style diet (more fruit, vegetables, legumes, cereals, and fish; less red meat and dairy foods; and a moderate amount of alcohol).

[3] RDI: 1.3 mg/d (men), 1.2 mg/d (women); EAR: 1.0 mg/d (men), 0.9 mg/d (women).

[4] RDI: 1.7 mg/d (men), 1.5 mg/d (women); EAR: 1.4 mg/d (men), 1.3 mg/d (women).

[5] RDI: 400 μg/d; EAR: 320 μg/d (men and women).

[6] RDI: 2.4 μg/d; EAR: 2.0 μg/d (men and women).

[7] AI: 550 mg/d (men); 425 mg/d (women).

[8] Sum over the 7 nutrients of *z* scores of log of nutrient intake.

percentages is significantly greater than those expected if there were no associations ($P = 1.0$ and 0.15, respectively).

DISCUSSION

Our cross-sectional study of >5000 adults found no consistent evidence of an association between DNA methylation measured in peripheral blood and dietary intakes of one-carbon metabolism nutrients. This was the case for individual CpGs on the HM450K array, for genome-wide measures derived from the array, and for DNA methylation measured at DNA repetitive elements LINE-1, Alu, and Sat2. These findings are generalizable to men and women of European origin aged >40 y.

The limitations of our study are common to those that use FFQs to estimate dietary intakes. The estimation of intake of one-carbon nutrients using FFQs has considerable error (38). Furthermore, some misclassification of nutrient intake is inevitable because we did not have data on vitamin B supplement use; however, because only 16% of the MCCS cohort reported using multivitamins at baseline, it is unlikely that this lack of information would have substantially influenced our results. Finally, the reliability of LINE-1 measures was found to be low (39), and the extent to which repetitive elements accurately reflect global methylation has been questioned (12). The strengths of our study include the large sample size compared with previous studies and the range of methylation measures considered, including several genome-wide methylation measures based on

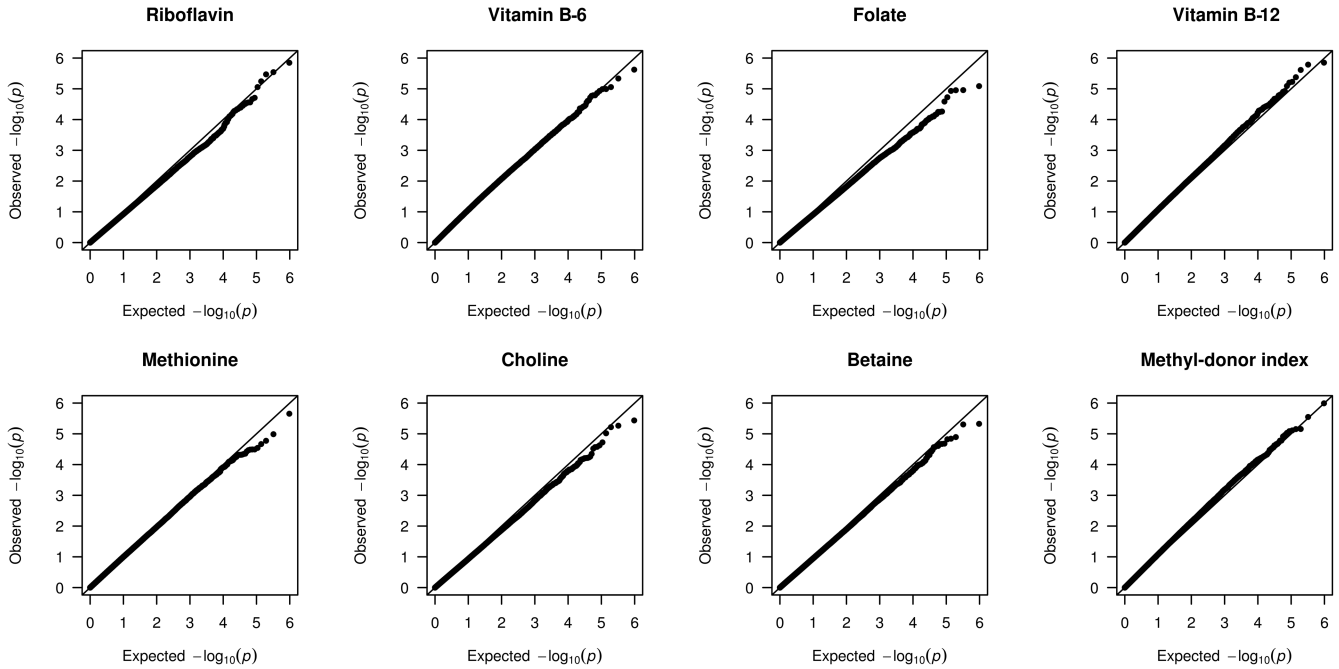

FIGURE 3 Quantile-quantile plots for associations between one-carbon metabolism nutrient intake and methylation M-value at 484,993 CpG sites. Cases and controls were combined by using fixed-effects meta-analysis; $n = 5186$ participants. CpG, cytosine-guanine dinucleotide.

the HM450K array and 3 measures of methylation at repetitive DNA elements. These measures have previously been shown to be associated with the risk of cancer (14–16, 18, 20, 40). Using the same data, we detected >300 associations with BMI at $P < 10^{-7}$ (41).

The lack of evidence of log-linear associations between nutrient intake and DNA methylation might be explained in several ways. Given that DNA methylation occurs in a highly tissue-specific manner, it may be that blood DNA methylation is not strongly influenced by dietary intake of one-carbon metabolism nutrients in adulthood. Alternatively, the FFQ might not have measured dietary intakes sufficiently accurately to detect associations; measurement error is known to reduce statistical power and bias regression estimates toward the null (42). Another potential explanation for our findings is that dietary intakes of one-carbon metabolism nutrients are not strongly correlated with bioavailable nutrient concentrations, which may be what influences DNA methylation (43). Measuring the concentration of these micronutrients in plasma or serum samples might help address this issue. The relation between nutrient intake, blood concentrations of nutrients, and DNA methylation is complex (44, 45). It could be affected by alcohol consumption, smoking, gut flora, and absorption characteristics, medications such as proton pump inhibitors, antidepressants and diuretics, or by genetic factors [e.g., polymorphisms in 5-methyltetrahydrofolate-homocysteine methyltransferase (*MTR*) and 5,10-methylenetetrahydrofolate reductase (*MTHFR*)] (46). We were only able to account for alcohol consumption and smoking in our analysis. Blood concentrations may reflect more accurately the consumption and assimilation of one-carbon metabolism nutrients, and therefore might be more relevant to DNA methylation.

It might be that any influence of one-carbon nutrients on DNA methylation is only apparent when there is an important deficiency in dietary intake, so that we may not have detected an association because our study participants had generally adequate nutrient intakes (47). In 2 studies of healthy volunteers, a diet low in folate (sometimes also referred to as vitamin B-9; 50–120 μg/d; the current recommended dietary intake for Australia is 400 μg/d) over 7–9 wk was shown to induce DNA hypomethylation in blood mononuclear cells, which returned toward normal once dietary folate concentrations were restored (48). We assessed in secondary analyses whether low or high dietary intakes of one-carbon metabolism nutrients defined by extreme quintiles were associated with the various DNA methylation measures considered, but found little evidence that this was the case after considering the large number of additional statistical tests performed. The potential association between a low dietary intake of riboflavin and methylation at cg21230392 in *PROM1* requires replication in an independent dataset.

Overall, previous studies that have examined associations between one-carbon nutrients and DNA methylation in the blood or other tissues of human adults have used small sample sizes and reached inconsistent conclusions (47). A study in 384 healthy Japanese women aged 20–74 y reported an inverse association between dietary folate intake and global leukocyte DNA methylation ($P = 0.03$) measured by using the Luminometric Methylation Assay (9). Agodi et al. (10) also reported weak evidence of an association ($P = 0.04$) between folate deficiency (defined as <320 μg/d) estimated from an FFQ and LINE-1 blood-derived DNA methylation in 177 Italian women (≤50 y). In both studies, many tests were performed. In a larger study that included 987 participants aged 45–84 y (11), no consistent associations were found between dietary intakes of folate, vitamin B-12, vitamin

TABLE 2
Linear model for intakes of one-carbon metabolism nutrients and HM450K-derived genome-wide median measures of blood DNA methylation[1]

	High-quality probes[2]			High-quality and ICC >0.1 and SD >0.05		
Nutrient and genomic region	Coefficient[3]	(95% CI)	*P*	Coefficient[3]	(95% CI)	*P*
Riboflavin						
All CpGs	1.26	(−2.11, 4.62)	0.46	0.80	(−2.74, 4.34)	0.66
Body CpGs	−1.11	(−6.33, 4.10)	0.68	0.19	(−3.54, 3.93)	0.92
Promoter CpGs	1.14	(−4.26, 6.55)	0.68	1.93	(−1.88, 5.74)	0.32
Vitamin B-6						
All CpGs	−0.26	(−2.98, 2.46)	0.85	−0.57	(−3.43, 2.29)	0.69
Body CpGs	−0.63	(−4.85, 3.58)	0.77	−1.06	(−4.07, 1.96)	0.49
Promoter CpGs	4.62	(0.26, 8.99)	0.04	0.54	(−2.53, 3.62)	0.73
Folate						
All CpGs	1.14	(−2.00, 4.28)	0.48	0.85	(−2.45, 4.15)	0.61
Body CpGs	−2.23	(−7.10, 2.64)	0.37	0.50	(−2.98, 3.98)	0.78
Promoter CpGs	0.66	(−4.37, 5.70)	0.80	1.83	(−1.72, 5.38)	0.31
Vitamin B-12						
All CpGs	−0.31	(−3.07, 2.46)	0.83	−0.61	(−3.52, 2.30)	0.68
Body CpGs	−0.84	(−5.13, 3.44)	0.70	−1.03	(−4.10, 2.04)	0.51
Promoter CpGs	4.93	(0.49, 9.37)	0.03	0.55	(−2.57, 3.68)	0.73
Methionine						
All CpGs	−0.60	(−4.22, 3.01)	0.74	−0.96	(−4.76, 2.84)	0.62
Body CpGs	−2.70	(−8.29, 2.90)	0.35	−1.81	(−5.82, 2.19)	0.37
Promoter CpGs	1.07	(−4.73, 6.88)	0.72	0.59	(−3.50, 4.68)	0.78
Choline						
All CpGs	1.70	(−2.26, 5.67)	0.40	1.53	(−2.64, 5.70)	0.47
Body CpGs	−1.63	(−7.77, 4.51)	0.60	1.12	(−3.27, 5.52)	0.62
Promoter CpGs	2.56	(−3.81, 8.93)	0.43	2.79	(−1.69, 7.28)	0.22
Betaine						
All CpGs	2.19	(−0.73, 5.10)	0.14	1.85	(−1.22, 4.92)	0.24
Body CpGs	−1.22	(−5.74, 3.29)	0.60	1.15	(−2.08, 4.38)	0.48
Promoter CpGs	2.41	(−2.27, 7.09)	0.31	2.93	(−0.37, 6.23)	0.08
Methyl donor index[4]						
All CpGs	1.44	(−2.52, 5.40)	0.48	0.68	(−3.48, 4.84)	0.75
Body CpGs	−2.97	(−9.10, 3.16)	0.34	−0.55	(−4.94, 3.84)	0.81
Promoter CpGs	6.97	(0.61, 13.3)	0.03	3.35	(−1.12, 7.83)	0.14

[1] $n = 5186$ participants. *P* values are from Wald tests for the corresponding coefficients. CpG, cytosine-guanine dinucleotide; HM450K, Illumina Infinium HumanMethylation450 BeadChip array; ICC, intraclass correlation coefficient.

[2] As filtered by the procedure implemented by Naeem et al. (31).

[3] Modeled median methylation per 1-SD increase in log of nutrient intake, adjusted for age, sex, country of birth, alcohol intake, energy intake from food, Mediterranean diet score, smoking status, sample type, and cell counts, with random effects for study, plate, and chip; results using case and control data were combined using fixed-effects meta-analysis; coefficients and 95% CIs were multiplied by 1000.

[4] Sum over the 7 nutrients of *z* scores of log of nutrient intake.

B-6, or methionine and DNA methylation at LINE-1 and Alu repetitive elements. The study by Vineis et al. (49) included 196 blood samples from lung cancer cases and matched controls and tested associations between blood concentrations of one-carbon nutrients and blood DNA methylation at promoters of cancer-associated genes [cyclin-dependent kinase inhibitor 2A (*CDKN2A*)/*p16*, Ras association domain member 1 (*RASSF1A*), glutathione S-transferase pi 1 (*GSTP1*), *MTHFR*, and O6-methylguanine DNA methyltransferase (*MGMT*)]. The authors observed weak evidence that folate was positively associated with methylation at *RASSF1A* and that methionine was inversely associated with methylation at *MTHFR*, but did not adjust the *P* values for multiple testing (49). In a small randomized, double-blind, placebo-controlled intervention trial, healthy, non–folate-deficient young Australians were administered large doses of folate and vitamin B-12 supplements (ranging from 3.5 to 10 times the recommended dietary intake, in the form of food or tablets) over a 12-wk course, but no changes were detected in lymphocyte DNA methylation measured by using an in vitro methyl acceptance assay (50). A small ($n = 31$) randomized controlled trial (51) of folic acid supplementation (400 μg/d for 10 wk) conducted in the United Kingdom reported an associated 39% ($P = 0.05$) and a 25% ($P = 0.08$) increase in blood and colon DNA methylation (also measured using an in vitro methyl acceptance assay), respectively. However, participants receiving folic acid supplementation were more likely to be nonsmokers and to have lower alcohol intakes and had different *MTHFR* and *MS* genotypes compared with those in the placebo group. We are not aware of other epigenome-wide association studies of dietary intakes of one-carbon nutrients in adulthood.

Other evidence that one-carbon nutrients may affect methylation levels comes from studies in rural African women,

TABLE 3
Intake of one-carbon metabolism nutrients and global measures of blood DNA methylation (LINE-1, Alu, Sat2)[1]

Nutrient and measure	Coefficient[2]	(95% CI)	*P*
Riboflavin			
BC Alu	−1.76	(−6.65, 3.13)	0.48
BC Sat2	0.50	(−2.19, 3.18)	0.72
BC LINE-1	−0.23	(−5.66, 5.20)	0.93
CRC LINE-1	−0.30	(−0.57, −0.04)	0.02
Vitamin B-6			
BC Alu	−2.09	(−6.54, 2.35)	0.36
BC Sat2	−1.26	(−3.66, 1.15)	0.31
BC LINE-1	−0.23	(−5.17, 4.70)	0.93
CRC LINE-1	−0.10	(−0.33, 0.12)	0.37
Folate			
BC Alu	−2.26	(−6.87, 2.34)	0.34
BC Sat2	1.59	(−0.93, 4.11)	0.22
BC LINE-1	−0.32	(−5.44, 4.79)	0.90
CRC LINE-1	−0.08	(−0.33, 0.17)	0.52
Vitamin B-12			
BC Alu	−0.92	(−4.91, 3.08)	0.65
BC Sat2	−0.79	(−2.95, 1.37)	0.47
BC LINE-1	0.72	(−3.72, 5.16)	0.75
CRC LINE-1	−0.02	(−0.24, 0.21)	0.88
Methionine			
BC Alu	2.22	(−3.02, 7.46)	0.41
BC Sat2	−0.81	(−3.66, 2.03)	0.58
BC LINE-1	2.90	(−2.92, 8.72)	0.33
CRC LINE-1	0.17	(−0.12, 0.47)	0.25
Choline			
BC Alu	1.44	(−4.35, 7.23)	0.63
BC Sat2	−0.76	(−3.89, 2.38)	0.64
BC LINE-1	4.49	(−1.93, 10.9)	0.17
CRC LINE-1	0.16	(−0.15, 0.47)	0.30
Betaine			
BC Alu	0.00	(−4.14, 4.13)	1.00
BC Sat2	−0.42	(−2.67, 1.84)	0.72
BC LINE-1	−0.41	(−5.00, 4.18)	0.86
CRC LINE-1	0.14	(−0.08, 0.37)	0.22
Methyl donor index[3]			
BC Alu	−0.46	(−3.15, 2.24)	0.74
BC Sat2	−0.42	(−1.88, 1.04)	0.57
BC LINE-1	1.14	(−1.85, 4.13)	0.45
CRC LINE-1	−0.05	(−0.36, 0.26)	0.75

[1] *P* values are based on a *t* test for the corresponding coefficient. Alu, arthrobacter luteus restriction endonuclease; BC, breast cancer study ($n = 1091$); CRC, colorectal cancer study ($n = 1613$); LINE-1, long interspersed nuclear element 1; Sat2, satellite 2.

[2] Modeled methylation per 1-SD increase in log of nutrient intake, adjusted for age, sex, country of birth, alcohol intake, energy intake from food, Mediterranean diet score, and smoking status; cases and controls were combined by using fixed-effects meta-analysis.

[3] Sum over the 7 nutrients of *z* scores of log of nutrient intake.

whose dietary intake of one-carbon metabolites and cofactors fluctuated seasonally. Concentrations of maternal biomarkers of one-carbon metabolism nutrients at conception were associated with methylation at metastable epi-alleles in DNA from infants' lymphocytes or hair follicles at birth. Specifically, plasma concentrations of riboflavin and vitamin B-6, but not folate, vitamin B-12, or methionine, showed this association (52). Previous work from the same study reported positive associations between these 2 biomarkers and carefully measured dietary intakes. The measured average intakes of folate, riboflavin, and vitamin B-6 in this population were lower than the estimated average requirement, especially during the "hungry" season (26). In contrast, the cutoffs for the 20th percentiles of intakes (defined as "low;" Supplemental Table 1) in our study were not substantially lower than the corresponding recommended dietary intakes/adequate intakes, suggesting that nutrient deficiencies were rare in MCCS participants. Drawing a parallel with the observation that maternal smoking affects infant blood DNA methylation at many of the loci also detected in methylation association studies of current smoking in adulthood (53, 54), we

assessed, using our adult samples, 44 associations reported in a recent large study between maternal plasma folate concentrations and blood DNA methylation in newborns (13). We found only weak evidence, and small effect sizes, for 2 of them; this finding is consistent with the fact that methylation levels at most CpGs are established during the periconceptional period and are more stable in adulthood (55). It should be noted that associations observed for maternal and adulthood smoking were of substantially greater magnitude (53, 54).

In conclusion, we did not find any consistent evidence of log-linear associations between dietary intakes of one-carbon metabolism nutrients and blood DNA methylation in adults. This finding may have implications for the interpretation of studies assessing associations between one-carbon nutrient intakes and risk of disease, because it would suggest that any associations between intakes of these nutrients and cancer risk might not be mediated through DNA methylation. A possible association of CpG cg21230392 with a low intake of riboflavin requires replication in independent cohorts. Further work should assess associations of DNA methylation with concentrations of these nutrients in plasma.

Cancer cases were ascertained through the Victorian Cancer Registry (VCR) and the Australian Cancer Database hosted by the Australian Institute of Health and Welfare (AIHW). Vital status was ascertained through the VCR and the National Death Index, also hosted by AIHW.

The authors' responsibilities were as follows—JAC, P-AD, AMH, JKB, and RLM: drafted the manuscript; JAC, P-AD, and C-HJ: analyzed the data; JAC, P-AD, JKB, AMH, DRE, JEJ, MTB, EM, and RLM: designed the study; JEJ, C-HJ, EM, DFS, JLH, DDB, DRE, MCS, MTB, GGG, and RLM: participated in the acquisition of the data; and all authors: participated in the interpretation of data, critically revised the manuscript for important intellectual content, and read and approved the final manuscript. None of the authors had a conflict of interest to declare.

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