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RESEARCH ARTICLE

Plasma B vitamins and LINE-1 DNA methylation in leukocytes of patients with a history of colorectal adenomas

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Scope: Low concentrations of folate, other B vitamins, and methionine are associated with colorectal cancer risk, possibly by changing DNA methylation patterns. Here, we examine whether plasma concentrations of B vitamins and methionine are associated with methylation of long interspersed nuclear element-1 (LINE-1) among those at high risk of colorectal cancer, i.e. patients with at least one histologically confirmed colorectal adenoma (CRA) in their life.
Methods and results: We used LINE-1 bisulfite pyrosequencing to measure global DNA methylation levels in leukocytes of 281 CRA patients. Multivariable linear regression was used to assess associations between plasma B vitamin concentrations and LINE-1 methylation levels. Plasma folate was inversely associated with LINE-1 methylation in CRA patients, while plasma methionine was positively associated with LINE-1 methylation.
Conclusion: This study does not provide evidence that in CRA patients, plasma folate concentrations are positively related to LINE-1 methylation in leukocytes but does suggest a direct association between plasma methionine and LINE-1 methylation in leukocytes.

Keywords:
B vitamins / Colorectal adenomas / DNA methylation / Folate / Humans / Methionine

1 Introduction

Changes in DNA methylation appear to be crucial in colorectal carcinogenesis. Both an increase of DNA methylation at CpG islands in or near the promoters of specific genes [1, 2] and a global loss of methylated cytosines [3] have been implicated in colorectal cancer (CRC) development. DNA hypermethylation contributes to the silencing of certain genes such as tumor suppressor genes [4], while global DNA hypomethylation is thought to contribute to carcinogenesis by affecting chromosomal stability, telomeric regulation, transposable element reactivation, and gene expression regulation [5]. Methylation of long interspersed nuclear element (LINE-1) repeats is often used as a surrogate for genome-wide DNA methylation. LINE-1 repeats comprise approximately 18% of the human genome and are heavily methylated in normal somatic cells [6].

Owing to the current challenges of obtaining target tissues in humans such as that in the large bowel, DNA methylation in blood is gaining widespread use as a proxy for DNA methylation in specific tissues, including colorectal tissue, in epidemiological studies [7–10]. A decrease in LINE-1

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Abbreviations: **CRA**, colorectal adenoma; **CRC**, colorectal cancer; **FAD**, flavin adenine dinucleotide; **FFQ**, food frequency questionnaire; **IQR**, interquartile range; **MMA**, methylmalonic acid; **MTHFR**, methylenetetrahydrofolate reductase; **PA**, 4-pyridoxic acid; **PL**, pyridoxal; **PLP**, pyridoxal 5′-phosphate; **SAM**, *S*-adenosylmethionine

methylation in leukocytes has been associated with an increased risk for CRC [11]; likewise, a decrease in global DNA methylation in leukocytes has been associated with risk for colorectal adenoma (CRA) [7, 8], which are established precursors for CRC [12–14].

Folate, vitamins B2 (riboflavin), B6 (pyridoxal 5′-phosphate (PLP) is the active form), B12 (cobalamin), and methionine are essential in one-carbon metabolism [15]; B vitamins are needed for DNA synthesis, and are vital to the production of *S*-adenosylmethionine (SAM), the universal methyl donor required for DNA methylation. Alcohol may limit methyl group availability by interfering with folate absorption and folate-metabolizing enzymes [16]; cigarette smoking reduces systemic circulating levels of B vitamins, possibly by increasing the activity of antioxidant defense enzymes in tissues, which require folate, B6 species, and riboflavin [17]. Furthermore, the common 677 C → T polymorphism in the methylenetetrahydrofolate reductase (MTHFR) gene has been shown to increase the risk of CRC [18] and CRAs [19] when folate status is low, although this association has not been consistently observed [20–22]. Riboflavin in its coenzymatic form of flavin adenine dinucleotide (FAD) is an essential cofactor for MTHFR. For those who have the MTHFR 677TT genotype, MTHFR enzyme activity decreases as a result of its inability to retain the FAD cofactor. Folate may protect against activity loss by increasing the affinity of MTHFR for FAD in the presence of folate [23, 24]. Folate intake in combination with a low or medium intake of vitamin B2 was shown to be a risk factor for CRAs in a previous study in the same population [25].

The relationships between B vitamin concentrations and global DNA methylation in leukocytes are unclear. Results from several feeding trials in healthy subjects have been inconclusive [26–29]. Additionally, to our knowledge, data describing the relationships between circulating levels of B vitamins and global DNA methylation in leukocytes of subjects at relatively high risk for CRC, i.e. those with previous adenomas [30, 31] and/or those with a family history of CRC in a first-degree relative [32], do not yet exist.

Similarly, randomized controlled trials studying folic acid supplementation on global DNA methylation in leukocytes and colon tissue both with healthy subjects and with adenoma patients yielded equivocal findings [33–40]. There is emerging evidence about the dual role of folate, where high levels of folate may protect against development of colorectal neoplasia, but could promote established CRAs [41, 42]. Additionally, results from a recent meta-analysis showed that the number of adenomas is strongly associated with risk of developing advanced and nonadvanced metachronous neoplasms [31].

We investigated the associations between circulating levels of (combined) B vitamins, methionine, and LINE-1 methylation in leukocytes of CRA patients. We also examine whether these relationships were influenced by number of lifetime adenomas, history of CRC among first-degree relatives, MTHFR C677T genotype, smoking status, and alcohol intake.

2 Materials and methods

2.1 Study design and population

The POLIEP follow-up study is a cohort study of the cases ($n = 767$), who had formerly participated in the POLIEP study [43], a Dutch case–control study comprising ten different hospitals in the Netherlands and conducted between 1997 and 2002. The study was designed to investigate gene–environment interactions and risk of CRAs. All participants in the study had at least one histologically confirmed CRA ever in their life. Additional inclusion criteria are that participants were Dutch speaking, of European origin, between the ages of 18–75 years at the time of an endoscopy for eligibility into the case–control study, had no hereditary CRC syndromes, no chronic inflammatory bowel disease, no history of CRC, and no (partial) bowel resection. All cases who agreed to be involved in the follow-up study and those for whom we had a recent address were invited ($n = 519$) to complete a validated semiquantitative food frequency questionnaire (FFQ) [44], a lifestyle questionnaire, and to give a blood sample. The FFQ was used to ascertain dietary intake and supplement use. Supplement use included any multivitamins, folic acid supplements, and B vitamin complex supplements. All participants who gave a blood sample, in which LINE-1 methylation levels could be assessed, were included in our analyses ($n = 281$). Self-reported family history of CRC is defined as having at least one first-degree family member diagnosed with this cancer. Participants had a full colonoscopy or a sigmoidoscopy at entry into the case–control study and medical and pathology information from past and subsequent colonoscopies from the earliest colonoscopy up to and including the most recent colonoscopy were collected. Adenoma occurrence was ascertained by colonoscopy and pathology reports. Written informed consent was obtained from each participant. This study was approved by the Radboud University Nijmegen Medical Centre Ethics Committee (CMO-nr: 2005/283) and by the review board of every participating outpatient clinic.

2.2 Biochemical analyses

Blood samples were collected from nonfasting participants in EDTA tubes and put directly on ice and protected from sunlight. Within 30 min of collection, the samples were centrifuged at 1000 rpm for 3 min followed by 2000 rpm for 5 min at 4°C. Plasma and buffy coat were pipetted separately into cryogenic vials and stored at −80°C. Plasma concentrations of folate and cobalamin were determined by microbiological assays using a colistin-sulfate resistant strain of *Lactobacillus leichmannii* and a chloramphenicol-resistant

strain of *Lactobacillus casei*, respectively [45, 46]. Plasma concentrations of methionine, riboflavin, and vitamin B6 species (PLP, pyridoxal (PL), and 4-pyridoxic acid (PA)) were measured using LC-MS/MS [47, 48]. Samples were analyzed in batches of 86 and quality control included six calibration samples, three control samples, and one blank sample in each batch. Coefficients of variation were 4–5% (folate), 0.7–2.9% (methionine), 5.5–13.2% (riboflavin), 2.6–11.1% (vitamin B6 species), 4–5% (cobalamin), and 1.1–8.1% (methylmalonic acid (MMA)). Samples were analyzed in random order. The laboratory staff was blinded to the clinical outcome status of the patients donating the blood samples. All biomarkers were analyzed at BEVITAL AS, Norway (http://www.bevital.no).

2.3 Genotyping assay

DNA was isolated from whole blood, and the MTHFR C677T polymorphism was genotyped using a PCR-RFLP method [49]. PCR was performed with internal negative controls. Reproducibility was confirmed by analyzing 20% of the samples in duplicate. There was also an external quality control program, and results showed a 100% match with expected genotype.

2.4 LINE-1 analysis

2.4.1 DNA extraction and bisulfite conversion of DNA

DNA was isolated from buffy coat using the Hamilton STAR workstation, as described by the manufacturer (Hamilton Robotics, Bonaduz, Switzerland). Magnetic beads that bind DNA were added to the samples. A magnetic box was then used to separate the DNA from other components of blood. DNA was eluted in an elution buffer. The amount of DNA in each sample was determined using a Caliper automation system (Caliper Life Sciences, Hopkinton, MA, USA). One hundred nanograms of DNA (concentration 25 ng/μL) were bisulfite converted using EZ DNA Methylation Gold™ Kit (Zymo Research, Orange, CA, USA) according to the manufacturer's protocol. Bisulfite-converted DNA was used for LINE-1 PCR immediately following conversion.

2.4.2 LINE-1 PCR and pyrosequencing

The use of pyrosequencing to measure LINE-1 methylation is a validated method and previous studies have demonstrated that LINE-1 methylation is a good indicator of cellular 5-methylcytosine levels [6]. We used and adapted the method developed by Yang et al. Global DNA methylation was quantified using bisulfite-PCR and pyrosequencing [6]. Repetitive elements primers were designed toward a consensus LINE-1 sequence (positions 331–318 for LINE-1 (GenBank accession number X58075)). Analysis of DNA methylation in LINE-1 repetitive elements was performed (Qiagen N.V., Germany). PCR conditions consisted of an initialization step of 95°C for 15 min followed by touchdown PCR with denaturation at 95°C for 30 s, annealing at 53°C for 30 s (−1°C each cycle), and extension at 72°C for 40 s for a total of five cycles then 35 cycles of 95°C for 30 s, 50°C for 30 s, 72°C for 40 s, followed by a final extension at 72°C for 10 min. The PCR product was bound to Sepharose HP (Amersham Biosciences, Uppsala, Sweden) and the Sepharose beads containing the immobilized PCR product were prepared for pyrosequencing according to the manufacturer's instructions (Qiagen N.V.). Pyrosequencing was performed on PCR product with bound LINE-1 sequencing primer and using the Pyromark Q24 System (Qiagen N.V.). The nucleotide dispensation order was GCTCGTGTAGTCAGTCG. Methylation quantification was performed using the provided software, Pyromark. The degree of methylation was expressed as the percentage of 5-methylated cytosines (%5mC) over the sum of methylated and unmethylated cytosines. Reproducibility was confirmed by analyzing 10% of the samples in duplicate. The within-sample coefficients of variation in duplicate runs were 4.3–7.3%.

2.5 Statistical analyses

Statistical analyses were performed using SAS version 9.2. Natural logarithmic transformations were applied to normalize distributions of all plasma metabolites, alcohol intake, and BMI because the original distributions were skewed toward higher values. Median and corresponding interquartile range (IQR) were calculated for all continuous variables with the exception of age and number of endoscopies, which were presented as a mean with the corresponding SD. Categorical variables were expressed in numbers and corresponding percentages. Results are presented separately for number of lifetime adenomas (one adenoma versus at least two adenomas). We also tested for significant differences in dietary and lifestyle characteristics between cases with one adenoma and cases with at least two adenomas. For categorical variables, we used the chi-square test, and for none of the variables were there cells with less than five observations. For nonparametric variables, the Wilcoxon–Mann–Whitney test was applied, and a Student's unpaired *t*-test was used for parametric data.

To evaluate the associations between log-transformed B vitamins and LINE-1 methylation, we used unadjusted and multivariable least squares linear regression analyses. We estimated individual associations of plasma folate, methionine, riboflavin, PLP, PL, PA, and cobalamin with LINE-1 methylation in separate models. We also constructed separate models for those with one adenoma and for those with ≥2 adenomas. We calculated beta estimates (β) and corresponding 95% confidence intervals (95% CIs) to determine

the associations between the B vitamins and LINE-1 methylation levels. To test for confounding in the association between plasma B vitamins and LINE-1 methylation, we added potential confounders to the models and checked whether crude estimates changed by more than 10% when added to the linear regression models. Potential confounders that were selected a priori were smoking status, family history of CRC, BMI, alcohol intake, age, sex, and number of endoscopies. Correlation between the analytes was checked using Spearman correlation coefficients. The final linear regression models included the covariates age, sex, BMI, alcohol intake, smoking status, family history of CRC, and other analytes (mutual B vitamins and methionine). The assumption of linearity between LINE-1 methylation in leukocytes and independent variables was confirmed using graphic methods.

In order to examine if a family history of CRC in a first-degree relative would change associations between B vitamins and LINE-1 methylation, we performed a stratified analysis. We also examined whether the associations between each B vitamin and LINE-1 methylation were modified by MTHFR C677T genotype, smoking status, alcohol intake, and sex. Interaction between plasma folate and plasma vitamin B2 was studied by stratification by tertiles of plasma vitamin B2. Given that folate intake in combination with a low or

Table 1. Dietary and lifestyle characteristics by number of lifetime adenomas

	One adenoma	≥2 adenomas
Demographics		
Number of patients (%)	149 (53.0)	132 (47.0)
Age at blood draw (years), mean (SD)[c)]	64.4 (9.4)	67.4 (7.7)
Sex, female (%)	59 (40.7)	53 (41.7)
Education, *n* (%)		
High	39 (26.2)	30 (22.7)
Medium	64 (43.0)	61 (46.2)
Low	37 (24.8)	36 (27.3)
Dietary intake, median (IQR)		
Folate, μg/day	192.9 (154.5–240.7)	185.5 (152.4–230.0)
Vitamin B2 (riboflavin)	1.6 (1.2–1.9)	1.5 (1.2–2.0)
Vitamin B6	1.7 (1.4–2.0)	1.7 (1.5–2.1)
Vitamin B12 (cobalamin)[c)]	3.9 (3.1–5.1)	4.3 (3.3–5.6)
Alcohol intake, g/day	10.0 (2.8–25.0)	9.8 (1.7–22.1)
Plasma concentrations, median (IQR)		
Folate (nmol/L)	8.1 (5.49–13.47)	7.6 (5.2–11.5)
Methionine (μmol/L)	28.5 (24.6–34.1)	28.3 (24.6–32.9)
Riboflavin (nmol/L)	17.7 (11.4–30.6)	16.02 (10.2–30.2)
Vitamin B6 sum (nmol/L)	120.6 (90.1–186.3)	116.8 (85.5–177.2)
PLP (nmol/L)	78.1 (50.6–113.8)	72.5 (53.8–111.5)
PL (nmol/L)	16.6 (12.2–25.4)	16.4 (11.6–25.7)
PA (nmol/L)	25.7 (19.0–45.5)	26.6 (19.9–39.7)
Cobalamin (pmol/L)	337.9 (278.8–411.2)	356.0 (284.0–431.4)
MMA (μmol/L)	0.2 (0.2–0.3)	0.2 (0.2–0.3)
Lifestyle characteristics		
BMI (kg/m^2), median (IQR)	25.7 (23.5–27.7)	25.8 (24.2–28.3)
Supplement use, yes (%)[a)]	39 (26.2)	28 (21.2)
Smoking habits, *n* (%)		
Current	21 (14.5%)	14 (11.0%)
Ever	80 (55.2%)	80 (63.0%)
Never	44 (30.3%)	31 (24.4%)
Other characteristics		
MTHFR C677T genotype, *n* (%)		
CC	72 (49.7%)	53 (40.5%)
CT	62 (42.8%)	64 (48.9%)
TT	11 (7.6%)	14 (10.7%)
First-degree family history of colorectal cancer, *n* (%)	42 (28.2%)	39 (29.6%)
Number of endoscopies, mean (SD)[c)]	2.9 (1.5)	4.7 (2.2)
Advanced adenomas, *n* (%)[b),c)]	69 (46.3%)	94 (71.2%)
LINE-1 methylation (%), median (IQR)	73.3 (70.91–74.8)	72.7 (68.9–75.0)

a) Includes multivitamins, folic acid supplements, and B vitamin complex supplements.
b) Advanced adenomas are defined as villous or tubulovillous adenomas and/or size ≥10 mm and/or high-grade dysplasia and/or three or more adenomas detected during a colonic examination.
c) *p*-Value < 0.05 for difference between one adenoma and ≥2 adenomas.

medium intake of vitamin B2 was a risk factor for CRA in earlier analyses within the same population [25], it would be worthwhile to explore this interaction in relation to LINE-1 methylation by stratifying by vitamin B2; in addition to stratification by vitamin B2, we also stratified by methionine, the sum of vitamin B6, cobalamin, and MMA. Because the independent variables were log-transformed, the resulting regression coefficient, β, should be interpreted as a 1% change in the independent variable corresponds to a β/100 change in the LINE-1 methylation. Statistical significance was tested at the 0.05 level.

3 Results

3.1 Characteristics of the study population

Dietary and lifestyle characteristics of the population can be found in Table 1. Two hundred eighty-one patients were included in our analyses. Patients with at least two lifetime adenomas were older (mean age = 67.38 years, SD = 7.71) than those with one adenoma (mean age = 64.37 years, SD = 9.44). The proportion of females was similar between the two groups (about 41%). Use of supplements containing B vitamins was higher in those with only one adenoma (26.2%) compared to those with at least two adenomas (21.2%). With the exception of PA and cobalamin, B vitamin plasma concentrations were all lower in the ≥2 adenoma group compared to the one adenoma group (Table 1). LINE-1 methylation in leukocytes was also slightly lower in those with recurrent adenomas (72.71%, IQR 68.87–74.96) compared to those with one adenoma (73.34% IQR 70.91–74.77).

Plasma analytes were not correlated (Spearman rank correlation coefficients between analytes $-0.25 < \rho < 0.56$) with the exception of the plasma B6 vitamers PLP, PL, and PA, which were highly correlated (PLP and PL $\rho = 0.86$, PLP and PA $\rho = 0.68$, PL and PA $\rho = 0.82$). Therefore, we excluded the B6 vitamers as covariates, using instead the sum of these vitamers in the multivariable regression model.

3.2 B vitamins and LINE-1 methylation levels according to number of lifetime adenomas

The overall multivariable-adjusted β-estimate (95% CI) between plasma folate and LINE-1 methylation for the entire population was −1.46 (−2.65, −0.27). The results from multivariable linear regression analyses according to number of lifetimes adenomas are shown in Table 2. Plasma folate was inversely associated with LINE-1 methylation for those with one lifetime adenoma (multivariable adjusted β-estimate (95% CI) of −2.01 (−3.85, −0.19) as well as for patients with at least two lifetime adenomas (multivariable adjusted β of −1.70 (−3.35, −0.06). The significant inverse relationship between plasma folate and LINE-1 methylation means that for

Table 2. Associations between plasma B vitamins and LINE-1 methylation according to number of lifetime adenomas using multivariable linear regression

	One adenoma ($n = 149$)		≥2 adenomas ($n = 132$)	
	Unadjusted β[c] 95% confidence interval	Multivariable adjusted[a] β[c] 95% confidence interval	Unadjusted β[c] 95% confidence interval	Multivariable adjusted[a] β[c] 95% confidence interval
Folate[b]	−0.86	−2.01	−1.17	−1.70
	−1.96, 0.24	−3.85, −0.19	−2.40, −0.06	−3.35, −0.06
Methionine[b]	3.36	3.47	−0.33	−0.47
	0.01, 6.72	−0.40, 7.33	−4.24, 3.57	−4.87, 3.94
Riboflavin[b]	−0.46	−0.60	−0.20	0.46
	−1.53, 0.61	−2.08, 0.87	−1.31, 0.91	−1.04, 1.95
Vitamin B6 sum[b]	0.12	1.68	−1.24	−1.28
	−1.01, 1.25	−0.27, 3.63	−2.73, 0.25	−3.51, 0.94
PLP[b]	0.29	1.79	−1.00	−0.94
	−0.95, 1.53	−0.27, 3.85	−2.67, 0.67	−3.36, 1.49
PL[b]	−0.20	−0.84	−1.22	−1.27
	−1.17, 0.78	−0.86, 2.54	−2.41, −0.02	−3.00, 0.46
PA[b]	−0.10	1.00	−1.11	−0.98
	−1.05, 0.85	−0.49, 2.49	−2.27, 0.06	−2.66, 0.69
Cobalamin[b]	0.35	1.09	0.12	0.82
	−1.94, 2.64	−1.62, 3.79	−2.49, 2.74	−2.34, 3.99
MMA[b]	−0.92	0.29	0.70	−0.56
	−2.87, 1.03	−2.19, 2.76	−2.06, 3.47	−3.88, 2.77

a) Adjusted for age, sex, BMI, alcohol intake, smoking status, family history of colorectal cancer, and other analytes.
b) These variables were log-transformed.
c) The independent variables were log-transformed before inclusion into the models. A 1% change in the independent variable corresponds to a β/100 change in the dependent variable (LINE-1 methylation).

Table 3. Associations between plasma B vitamins and LINE-1 methylation according to family history using multivariable linear regression

	Family history of CRC ($n = 81$) Multivariable adjusted[a)] β[c)] 95% confidence interval	No family history of CRC ($n = 200$) Multivariable adjusted[a)] β[c)] 95% confidence interval
Folate[b)]	−2.77	−1.02
	−5.57, 0.02	−2.32, 0.29
Methionine[b)]	−2.37	3.91
	−8.03, 3.30	0.39, 7.42
Riboflavin[b)]	−1.30	−0.17
	−3.66, 1.06	−1.36, 1.02
Vitamin B6 sum[b)]	1.88	−0.27
	−1.42, 5.17	−1.93, 1.39
PLP[b)]	1.84	0.20
	−1.79, 5.47	−1.52, 1.91
PL[b)]	0.53	−0.83
	−1.95, 3.01	−2.27, 0.61
PA[b)]	1.15	−0.69
	−1.15, 3.44	−1.99, 0.61
Cobalamin[b)]	4.90	0.35
	−1.03, 10.82	−2.22, 2.92
MMA[b)]	2.26	−0.93
	−1.90, 6.43	−3.31, 1.45

a) Adjusted for age, sex, BMI, alcohol intake, smoking status, and other analytes.
b) These variables were log-transformed.
c) The independent variables were log-transformed before inclusion into the models. A 1% change in the independent variable corresponds to a β/100 change in the dependent variable (LINE-1 methylation).

patients with ≥2 adenomas, a 1% increase in plasma folate is associated with a decrease of $-1.70/100 = -0.017$ (β/100) units in LINE-1 methylation (%5-methylated cytosines/total amount of cytosines). There were null associations between other B vitamins and LINE-1 methylation according to number of lifetime adenomas.

3.3 B vitamins and LINE-1 methylation levels according to family history of CRC

The associations between B vitamins and LINE-1 methylation stratified by family history of CRC can be found in Table 3. In patients with a family history of CRC, we found a borderline significant inverse association between plasma folate and LINE-1 methylation (β of −2.77 (5.57, 0.02)), adjusted for age, sex, BMI, alcohol intake, smoking status, and other analytes. There was a positive association between plasma methionine and LINE-1 methylation (multivariable-adjusted β-estimate of 3.91 (0.39, 7.42)), but only in patients with no family history. No other statistically significant associations were observed.

3.4 B vitamin and LINE-1 methylation levels according to MTHFR C677T genotype

The associations between plasma B vitamins and LINE-1 methylation levels according to MTHFR C677T genotype are shown in Table 4. We found an inverse association between plasma folate and LINE-1 methylation for those with the MTHFR 677CC genotype (β of −2.01 (−3.77, −0.25)). For those with the MTHFR 677TT genotype, there was an inverse association between plasma riboflavin and LINE-1 methylation (multivariable adjusted β of −9.08 (−15.47, −2.69)) and a positive association between the sum of B6 vitamers and LINE-1 methylation (multivariable adjusted β of 12.00 (0.85, 23.15)). No interaction terms reached statistical significance (data not shown).

3.5 Plasma folate and LINE-1 methylation levels according to plasma riboflavin, smoking status, and alcohol intake

The associations between plasma folate and LINE-1 methylation levels stratified by plasma riboflavin, smoking status, and alcohol intake are shown in Table 5. Plasma riboflavin and alcohol intake were each divided into tertiles while smoking status was categorized as current, former, or never. We found inverse associations of LINE-1 methylation levels with plasma folate in the lowest tertile of plasma riboflavin (β of −3.84 (−6.27, −1.40)) and in never smokers (β of −3.19 (−6.23, −0.15)), and only in the second tertile of alcohol consumption (β of −3.56 (−5.88, −1.04)). There were inverse associations between plasma folate and LINE-1 methylation for all levels of plasma methionine, cobalamin, sum of vitamin B6, and MMA but none were significant (data not shown).

4 Discussion

In the present cohort study with patients, who have had a history of at least one CRA ever in their life, we observed inverse associations between plasma folate and LINE-1 methylation from peripheral blood leukocytes. An increase in plasma folate concentrations was related to a decrease in LINE-1 methylation in those with one adenoma and in those with at least two lifetime adenomas. Our results are somewhat unexpected, as we hypothesized that higher plasma folate concentrations would be associated with higher LINE-1 methylation, as folate is important for the availability of SAM, the universal methyl donor required for DNA methylation. On the other hand, there is growing evidence that high levels of folate may be protective in early carcinogenesis but may accelerate the growth of established colorectal neoplasia, as mentioned earlier. The complex dual role of folate in colorectal carcinogenesis depends on the timing of exposure in addition to dose and duration [41, 42, 50, 51]. In our population of

Table 4. Associations between plasma B vitamins and LINE-1 methylation according to MTHFR C677T genotype

	MTHFR C677T genotype		
	CC ($n = 125$) Multivariable adjusted[a)] β[c)] 95% confidence interval	CT ($n = 126$) Multivariable adjusted[a)] β[c)] 95% confidence interval	TT ($n = 25$) Multivariable adjusted[a)] β[c)] 95% confidence interval
Folate[b)]	−2.01 −3.77, −0.25	−1.56 −3.57, 0.46	5.48 −0.60, 11.56
Methionine[b)]	2.13 −2.93, 7.18	0.49 −4.20, 5.18	1.29 −11.46, 14.05
Riboflavin[b)]	−0.75 −2.52, 1.01	−0.12 −1.72, 1.48	−9.08 −15.47, −2.69
Vitamin B6 sum[b)]	0.97 −1.29, 3.23	−0.09 −2.66, 2.47	12.00 0.85, 23.15
PLP[b)]	1.32 −1.09, 3.72	0.29 −2.49, 3.08	9.63 −2.76, 22.01
PL[b)]	0.13 −1.77, 2.03	−0.73 −2.79, 1.34	7.25 −2.50, 16.99
PA[b)]	0.33 −1.29, 1.95	−0.56 −2.45, 1.33	4.54 −6.14, 15.22
Cobalamin[b)]	0.01 −4.00, 4.03	2.46 −0.90, 5.82	−5.68 −20.67, 9.31
MMA[b)]	0.08 −3.09, 3.24	−0.28 −3.32, 2.77	−9.22 −19.51, 1.07

a) Adjusted for age, sex, BMI, alcohol intake, smoking status, family history of colorectal cancer, and other analytes.
b) These variables were log-transformed.
c) The independent variables were log-transformed before inclusion into the models. A 1% change in the independent variable corresponds to a β/100 change in the dependent variable (LINE-1 methylation).

exclusively adenoma patients, it may not be too surprising that plasma folate was inversely related to LINE-1 methylation in leukocytes, as increasing levels of plasma folate could promote adenoma growth, where increased levels of global DNA hypomethylation exist.

Results from a cross-sectional study with healthy Dutch participants showed no association between plasma folate and global DNA methylation, as measured by LC-MS/MS [29], and plasma folate concentrations in this study were similar to those measured by us. In controlled folate feeding trials with healthy, postmenopausal women, folate status was positively associated with global DNA methylation levels in leukocytes [26, 27]. In these feeding trials, female volunteers consumed low-folate diets for 7 weeks [26] or 91 days [27] showing a concomitant gradual loss of global DNA methylation.

Furthermore, five randomized controlled trials have explored the effects of folic acid, the synthetic form of folate, on global DNA methylation in colorectal tissues [33–36, 52] and leukocytes [52] of CRA patients. While the aim of these studies was not to examine the relationship between plasma folate and global DNA methylation, they are relevant, as they demonstrate the influence of folic acid on changes in global DNA methylation levels. Following folic acid supplementation, three of these studies observed an increase in global DNA methylation [33, 36, 52], while results were null in others [34, 35].

Possible reasons for inconsistent results include differences in study design. Selection biases may occur in case–control studies but are unlikely in cohort studies, where loss to follow-up is not related to exposure. There are also differences in methods of measuring DNA methylation, type of tissue studied (colon versus leukocytes), study participants (healthy versus cancer patients), and adjustments for confounding factors. Furthermore, many of the aforementioned studies were conducted in the United States where folic acid fortification is mandatory, and subsequently plasma concentrations of study participants were relatively high.

The small randomized controlled trial performed by Pufulete et al. in the United Kingdom, where folic acid fortification is not compulsory, is interesting because global DNA methylation was measured in both leukocytes as well as colon tissue in adenoma patients [52]. Subjects were randomized to receive 400 μg/day folic acid for a short period of time (10 weeks), and there was a borderline significant increase in global DNA methylation in leukocytes ($p = 0.05$), measured using [^{3}H] methyl incorporation assay, in the intervention group compared to those in the placebo group following supplementation. There was also a borderline significant increase in global DNA methylation in colon tissue following folic acid intervention ($p = 0.09$), and the weaker increase in colon tissue may be a consequence of blood being "closer" than colon tissue to environmental exposures [53]. Additionally, the absorption and metabolism of natural folates and

Table 5. Associations between plasma folate and LINE-1 methylation stratified by plasma riboflavin, smoking status, and alcohol intake using a multivariable regression model

	Association between plasma folate[b] and LINE-1 methylation Multivariable adjusted[a] β[c] 95% confidence interval
Plasma riboflavin	
1 (2.48–12.34 nmol/L) ($n = 87$)	−3.84
	−6.27, −1.40
2 (12.35–24.19 nmol/L) ($n = 88$)	−1.16
	−3.16, 0.85
3 (24.20–329.76 nmol/L) ($n = 87$)	−0.24
	−2.10, 2.57
Smoking status	
Never ($n = 75$)	−3.19
	−6.23, −0.15
Former ($n = 160$)	−1.38
	−2.91, 0.16
Current ($n = 35$)	−0.14
	−2.82, 2.53
Alcohol intake	
1 (0.00–4.38 g/day) ($n = 90$)	−0.41
	−2.73, 1.90
2 (4.39–17.90 g/day) ($n = 90$)	−3.56
	−5.88, −1.04
3 (17.91–143.50 g/day) ($n = 90$)	−1.32
	−3.37, 0.73

a) Adjusted for age, sex, BMI, alcohol intake, smoking status, family history of colorectal cancer, and other analytes.
b) This variable was log-transformed.
c) The independent variables were log-transformed before inclusion into the models. A 1% change in the independent variable corresponds to a β/100 change in the dependent variable (LINE-1 methylation).

synthetic folates, i.e. folic acid, are different, and may therefore have dissimilar effects on one carbon metabolism [54,55].

Methionine is an essential amino acid, and like folate, it is required for the synthesis of SAM, and as such, we would expect a positive relationship between methionine and LINE-1 methylation. Indeed, we found a positive association between plasma methionine and LINE-1 methylation in patients without a family history of CRC. For those with a family history of CRC, there was no significant association between plasma methionine and LINE-1 methylation perhaps indicating that family history of CRC may modulate the associations between plasma methionine and LINE-1 methylation, although it may be premature to draw definitive conclusions based on a small subgroup analysis.

The significant inverse relationship in the current study between plasma folate and LINE-1 methylation in patients with the MTHFR 677CC genotype is in contrast to those with the MTHFR 677CT or MTHFR 677TT genotype, where there was no association. Based on the premise that the C to T base transition resulting in an alanine to valine substitution impairs enzyme activity, and decreases plasma folate concentrations [23, 56] and that folate is required for the production of SAM for DNA methylation, we had expected direct associations between plasma folate and LINE-1 methylation in persons with the TT genotype. Our results are inconsistent with the results from an observational Italian study using cross-sectional data, which revealed lower global DNA methylation in subjects with the TT genotype compared to those with the CC genotype when plasma folate concentrations were low [57]. However, results from controlled folate feeding trials have also been unexpected. No changes in global leukocyte DNA methylation following short-term folate restriction in healthy women with the MTHFR 677CC genotype have been reported [28], while other studies in women have demonstrated changes in global DNA methylation in those with the TT genotype following folate repletion [58, 59]. Results from the feeding trials and our own results in the current study should be interpreted with prudence, as there were relatively few patients with the MTHFR 677TT genotype.

Results from additional exploratory analyses indicated significant inverse associations between plasma folate and LINE-1 methylation for those in the lowest category of plasma riboflavin, those who had never smoked and those in the middle category for alcohol intake. We had expected that smoking and alcohol would decrease concentrations of B vitamins and subsequently decrease LINE-1 methylation.

Our study possesses several strengths. It is, to our knowledge, the first to provide a snapshot of the relationship between plasma B vitamins and global DNA methylation in patients with a history of adenomas. Our study population is fairly large and exclusively includes subjects with a history of CRAs, and is therefore at higher risk for developing CRC. We were able to measure plasma B vitamins rather than assessing exposure using recall methods such as an FFQ.

This study also has some limitations. Cross-sectional data does not allow us to make causal inferences from associations between plasma analytes and LINE-1 methylation. It may be of interest for future studies to include measurements of SAM and *S*-adenosylhomocysteine, and to take repeated blood measurements at different time points. Red blood cell folate concentrations may represent a better long-term measure of folate status, but a single measurement of plasma folate per subject seems to be sufficient in large epidemiological studies [60]. Although we have measured LINE-1 methylation, we do not have information about specific promoter methylation, and we cannot eliminate the possibility that the correlations between plasma B vitamins and promoter methylation may differ in those with different number of adenomas, family history of CRC, MTHFR C677T genotype, smoking, and alcohol intake. CpG island promoter methylation could, in fact, be more frequent among individuals with lower LINE-1 methylation, a proxy for global DNA methylation [61]. Furthermore, to date, while we are unaware of studies having definitively proven the relationship between leukocyte methylation and methylation in colon tissue, the

results from Pufulete et al. [52] do suggest similarities in DNA methylation between the two tissues in response to folic acid. Additionally, because obtaining target tissues in humans is so often problematic and complicated, global DNA methylation in leukocytes is commonly used as a substitute for target tissues in epidemiological studies [7–10].

For our analyses, we used the follow-up FFQ and blood sample, so indeed, some CRA patients could have been diagnosed over a decade prior to measurement of B-vitamin status and LINE-1 methylation. However, in the original case–control study, few cases could confirm that they changed their diets when asked about dietary changes after diagnosis of CRA [62], and certainly while LINE-1 methylation levels in leukocytes have changed since diagnosis of CRA, we were interested in capturing the relationships between plasma B vitamins and methionine and LINE-1 methylation in adenoma patients during follow-up. We would reason then that the fact that measurements of B-vitamins and LINE-1 methylation were done after diagnosis of CRA would have little implication for the interpretation of our findings.

It is unclear why there is an inverse association between plasma folate and LINE-1 methylation. Patients with CRAs may experience changes in their one-carbon metabolism and may be more sensitive to these nutrients. Nevertheless, in light of the current unexpected results, more research is warranted to further elucidate the true relationships between B vitamins and global DNA methylation in healthy individuals as well as in individuals at high risk for developing CRC.

In conclusion, our study suggests that plasma folate is inversely associated with LINE-1 methylation in leukocytes of CRA patients. Plasma methionine was positively associated with LINE-1 methylation. We also observed indications that these relationships may be potentially modified by family history of CRC, and MTHFR 677 C → T genotype although drawing definitive conclusions may be difficult due to relatively small sample sizes in our subgroup analyses.

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