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ORIGINAL PAPER

Dietary methyl donors, methyl metabolizing enzymes, and epigenetic regulators: diet–gene interactions and promoter CpG island hypermethylation in colorectal cancer

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Abstract Dietary methyl donors might influence DNA methylation during carcinogenesis of colorectal cancer (CRC). Among 609 CRC cases and 1,663 subcohort members of the Netherlands Cohort Study on diet and cancer ($n = 120{,}852$), we estimated CRC risk according to methyl donor intake across genotypes of folate metabolizing enzymes and methyltransferases.

Although diet–gene interactions were not statistically significant, methionine intake was inversely associated with CRC among subjects having both common rs2424913 and rs406193 *DNMT3B* $C > T$ genotypes (highest versus lowest tertile: RR = 0.44; $p_{trend} = 0.05$). Likewise, vitamin B2 was modestly inversely associated among individuals with the *MTHFR c.665CC* (rs1801133) genotype (RR = 0.66; $p_{trend} = 0.08$), but with a significant reduced risk when ≤ 1 rare allele occurred in the combination of folate metabolizing enzymes *MTHFR*, *MTRR* and *MTR* (RR = 0.30; $p_{trend} = 0.005$). Folate or vitamin B6 were neither inversely associated with CRC nor was methyl donor intake associated with the CpG island methylator phenotype (CIMP).

Despite the absence of heterogeneity across genotypes, might an effect of methyl donors on CRC be more pronounced among individuals carrying common variants of folate metabolizing enzymes or DNA methyltransferases. Combining genotypes may assist to reveal diet associations with CRC, possibly because rare variants of related genes may collectively affect specific metabolic pathways or enzymatic functions.

Keywords Methyl donors · Diet–gene interactions · Promoter hypermethylation · CRC

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Introduction

Hypermethylation of CpG islands in gene promoters is an important epigenetic alteration involved in carcinogenesis [1]. In colorectal cancer (CRC), the CpG island methylator phenotype (CIMP) is characterized by frequent promoter CpG island hypermethylation [2]. However, little is known about potential determinants of this type of aberrant DNA methylation in CRC.

Folate and methionine are dietary methyl group donors that may be hypothesized to influence DNA methylation, whereas vitamins B2 and B6 potentially modulate the bioavailability of methyl groups [3, 4]. Low folate status or intake was suggested to decrease genomic methylation [5–8], while folate supplementation resulted in increased

global DNA methylation in the colonic mucosa [9]. Adequate methyl donor intake possibly also prevents aberrant CpG island promoter hypermethylation. In this respect, a weak inverse association with gene promoter hypermethylation was suggested [10], although methyl donor intake and alcohol consumption, which may reduce the bioavailability of folate, were not associated with CIMP in CRC [11]. Conversely, folate supplementation was suggested to increase promoter hypermethylation of multiple genes in colorectal mucosa [12], and circulating folate concentration was associated with increased gene promoter hypermethylation in colorectal tumors [13]. In addition, high vitamin B6 intake may be associated with increased *MutL homologue 1 (MLH1)* promoter methylation in CRC [14]. Apparently, the precise effect of methyl group bioavailability on gene promoter hypermethylation is still unclear and should be investigated further.

A potential effect of methyl donor intake on DNA methylation may be modified by polymorphisms in folate metabolizing enzymes. For example, the catalytic activity of the methylene tetrahydrofolate reductase (MTHFR) enzyme may be reduced in individuals carrying rare variants of the *MTHFR* $c.665C > T$ (rs1801133) and $c.1286A > C$ (rs1801131) polymorphisms [15, 16], which were also associated with the CIMP phenotype in colorectal cancer [17–19]. We previously observed inverse associations between *methionine synthase* (*MTR*) $c.2756A > G$ (rs1805087) and CIMP, and between *methionine synthase reductase* (*MTRR*) $c.66A > G$ (rs1801394) with *MLH1* hypermethylation [20]. Other enzymes involved in epigenetic regulation of gene expression are DNA methyltransferases (DNMTs) and histone methyltransferases (HMTs). However, whether an influence of methyl donor intake is modified by polymorphisms in such epigenetic regulators has not previously been studied in relation to CRC.

Here, we aimed to investigate associations between dietary folate, methionine, vitamins B2 and B6 with overall CRC, and risk of CRCs harboring CIMP, accounting for the occurrence of any, or combinations of rare variants of folate metabolizing enzymes *MTHFR, MTR* and *MTRR*, the DNA methyltransferase *DNMT3B*, and histone methyltransferases *Euchromatin histone methyltransferase 1 (EHMT1), Euchromatin histone methyltransferase 2 (EHMT2)* and *PR domain zinc finger protein 2 (PRDM2)* in the Netherlands Cohort Study on diet and cancer.

Methods

Study population

The participants of this study were incident CRC patients from the Netherlands Cohort Study on diet and cancer (NLCS), which has been described in detail elsewhere [21]. Briefly, this prospective cohort study was initiated in September 1986 and includes 58,279 men and 62,573 women aged 55–69 years and free of disease at baseline. The cohort is followed for cancer occurrence by annual record linkage to the Netherlands Cancer Registry (NCR) and to the Pathologisch Anatomisch Landelijk Geautomatiseerd Archief (PALGA), a nationwide network and registry of histopathology and cytopathology reports [22, 23]. A subcohort of 5,000 subjects was randomly selected after baseline exposure measurement, to estimate accumulation of person-time in the cohort through biennial follow-up of vital status. Cases with prevalent cancer other than non-melanoma skin cancer were excluded from this subcohort, which left 4,774 men and women eligible for analysis.

Food frequency questionnaire

At baseline, participants filled out a self-administered, 150-item semi-quantitative food frequency questionnaire (FFQ), which concentrated on habitual consumption of food and beverages during the year preceding the start of the study, and also contained questions about age, sex, body weight and length, smoking status and family history of CRC. Daily mean nutrient intakes were calculated as the cumulated product of the frequencies and portion sizes of all food items and their tabulated nutrient contents from the Dutch Food Composition Table (NEVO table, 1986) [24]. The questionnaire was validated through comparison with a 9-day diet record [25]. Reproducibility and stability of dietary habits were determined by five annually repeated measurements [26]. In order to minimize observer bias in coding and interpretation of the data, questionnaire data were key-entered twice for all incident cases in the cohort and for all subcohort members in a blinded manner with respect to case/subcohort status.

Folate data were derived from a validated liquid chromatography trienzyme method [27] used to analyze the 125 most important Dutch foods contributing to folate intake [28]. Dietary supplement data were also obtained via the food frequency questionnaire. However, the use of B-vitamin supplements was low (7%) and folic acid was generally not included in these supplements in the Netherlands in the late 1980s. Therefore, folic acid supplement use most likely plays a very minor role in our study population, and supplement use was not further accounted for in the analyses.

Sample collection

Subcohort members still alive in December 2000 ($n = 3,579$) were contacted and asked to collect mouth swabs, of whom 1,929 (54%) responded and returned the

mouth swab with informed consent. In total, DNA could successfully be isolated of 1,829 subcohort members who also had complete follow-up information [20].

Tumor material of the CRC patients was collected after approval by the ethical review boards of Maastricht University, the NCR and PALGA. During a follow-up period of 7.3 years after baseline, 734 incident CRC patients were identified who had an available PALGA report of the lesion as well as a sufficient amount of isolated DNA needed for molecular analyses.

Genotyping analyses

MTHFR (rs1801133 and rs1801131), *MTR* (rs1805087), *MTRR* (rs1801394), *DNMT3B* (rs2424913 and rs406193), *EHMT1* (rs4634736), *EHMT2* (rs535586) and *PRDM2* (rs2235515) genotypes were determined using multiplex polymerase chain reaction (PCR) amplification and single base extension (SBE) reactions as described previously [20, 29]. Genotype data were validated by sequencing of fragments containing specific SNPs, which were similar to the main results for all but one (99.6%) of the 9 SNPs within a subset of 30 samples [20]. Reproducibility of the analysis was established among 93 samples, and we observed that the analyses could be reproduced in 99.5% of these cases [20]. In total, genotyping analyses were successful from 1,736 subcohort members and 659 CRC patients.

Promoter methylation analyses

The CpG island methylator phenotype (CIMP) was defined by promoter hypermethylation of at least 3 out of 5 methylation markers (*CACNA1G, IGF2, NEUROG1, RUNX3*, and *SOCS1*), as suggested by Weisenberger et al. [2]. Hypermethylation of the CpG islands of these five CIMP markers and of the *MLH1* gene was determined by Methylation Specific PCR (MSP) [30] and described in detail by de Vogel et al [20]. The MSP analyses were successful of 81, 79, 79, 90, 83, and 93% out of the 734 patients for *CACNA1G, IGF2, NEUROG1, RUNX3, SOCS1*, and *MLH1*, respectively.

Microsatellite instability

MSI was determined by a pentaplex PCR, using the MSI markers BAT-26, BAT-25, NR-21, NR-22 and NR-24, as described in detail by Suraweera et al. [31]. MSI analyses were successful on 662 (90%) out of the 734 available samples.

Statistical analyses

Cox proportional hazards regression models were used to estimate multivariate-adjusted incidence rate ratios (RR) and corresponding 95% confidence intervals (CI) over tertiles of dietary folate, methionine, vitamins B2 and B6, using the lowest tertiles as reference. Tests for dose response trends over the tertiles of intake were estimated by fitting the ordinal exposure variables as continuous variables and evaluated using the Wald test. Standard errors of the RR were estimated using the robust Huber-White sandwich estimator to account for additional variance introduced by sampling from the cohort [32]. The proportional hazards assumption was tested using the scaled Schoenfeld residuals [33] and by fitting the main determinants as time-dependent variables. The dietary variables were adjusted for total energy intake by calculating nutrient residuals from the regression of nutrient intake on total energy intake, as described by Willett et al. [34]. The analyses were stratified according to genetic status of individuals, i.e. among those homozygous to common genetic variants and among subjects carrying rare alleles. Interactions were tested between dietary folate, methionine, vitamins B2 and B6, and each of the genetic variants. Associations between dietary factors and CRC were also estimated for combinations of genotypes per functional group (i.e. based on the number of rare alleles in any of the folate metabolizing enzymes *MTHFR*, *MTR* and *MTRR*, in the DNA methyltransferase *DNMT3B*, or in any of the histone methyltransferases *EHMT1*, *EHMT2* and *PRDM2*.

To investigate whether dietary methyl donors have an effect on promoter hypermethylation in CRC, associations of folate, methionine, vitamins B2, and B6 with the CIMP phenotype were estimated. The associations with *MLH1* hypermethylation and MSI were reported previously [14]. Furthermore, it was investigated whether the associations with *MLH1* hypermethylation, MSI, or CIMP would be modified by genetic status, by estimating the associations with methylation endpoints within genotypes of folate metabolizing enzymes, *DNMT3B* and histone methyltransferases.

All models included the co-variates dietary folate, methionine, vitamin B2 and B6 and were additionally adjusted for age, sex, family history of CRC, smoking status, body mass index (BMI), alcohol consumption, and energy intake. After excluding subjects with missing information on these covariates or subjects who did not completely filled out the questionnaire, 1,663 subcohort members and 609 CRC cases remained for statistical analyses. All analyses were performed with the Stata statistical software package (version 10).

Results

CRC risk was estimated over tertiles of folate intake, methionine, vitamins B2 and B6, among subjects homozygous for common alleles and among carriers of rare alleles.

Table 1 Intake of folate and methionine and CRC risk stratified by genetic status

Gene and SNP (rs number, MAF)*	Tertile of intake	Folate†					Methionine‡				
		Common homozygotes		Heterozygotes and rare homozygotes			Common homozygotes		Heterozygotes and rare homozygotes		
		n§	RR (95% CI)	n	RR (95% CI)	$p_{interaction}$	n	RR (95% CI)	n	RR (95% CI)	$p_{interaction}$
MTHFR c.665C > T (rs1801133, 0.30)	1	90	Ref.	133	Ref.	*0.31*	86	Ref.	123	Ref.	*0.85*
	2	83	0.80 (0.52–1.21)	78	0.51 (0.35–0.75)		101	1.43 (0.96–2.13)	101	0.84 (0.57–1.24)	
	3	100	1.22 (0.81–1.85)	123	0.82 (0.56–1.21)		86	0.92 (0.61–1.41)	110	1.08 (0.70–1.67)	
	p_{trend}**		*0.28*		*0.30*			*0.63*		*0.90*	
MTHFR c.1286A > C (rs1801131, 0.37)	1	103	Ref.	116	Ref.	*0.83*	95	Ref.	113	Ref.	*0.33*
	2	72	0.61 (0.40–0.92)	89	0.73 (0.50–1.07)		85	1.00 (0.65–1.54)	115	1.14 (0.80–1.64)	
	3	98	1.03 (0.68–1.56)	124	1.02 (0.69–1.51)		93	1.02 (0.65–1.60)	101	0.92 (0.62–1.38)	
	p_{trend}		*0.97*		*0.86*			*0.99*		*0.58*	
MTR c.2756A > G (rs1805087, 0.19)	1	136	Ref.	89	Ref.	*0.81*	128	Ref.	83	Ref.	*0.42*
	2	107	0.55 (0.39–0.79)	57	0.84 (0.53–1.33)		141	1.22 (0.87–1.71)	62	0.87 (0.55–1.38)	
	3	154	0.99 (0.70–1.39)	71	0.94 (0.57–1.54)		128	1.03 (0.71–1.48)	72	0.94 (0.57–1.54)	
	p_{trend}		*0.90*		*0.83*			*0.98*		*0.69*	
MTRR c.66A > G (rs1801394, 0.56)	1	56	Ref.	172	Ref.	*0.24*	49	Ref.	164	Ref.	*0.09*
	2	24	0.29 (0.14–0.59)	140	0.78 (0.58–1.05)		38	1.00 (0.52–1.93)	165	1.08 (0.80–1.46)	
	3	49	0.62 (0.32–1.22)	177	1.06 (0.78–1.45)		42	1.22 (0.53–2.78)	160	0.96 (0.70–1.33)	
	p_{trend}		*0.23*		*0.69*			*0.94*		*0.72*	
DNMT3B C > T (rs2424913, 0.42)	1	89	Ref.	139	Ref.	*0.17*	84	Ref.	129	Ref.	*0.23*
	2	60	0.60 (0.38–0.95)	106	0.70 (0.49–0.99)		61	0.61 (0.37–1.00)	144	1.37 (0.99–1.90)	
	3	68	0.73 (0.44–1.20)	159	1.12 (0.80–1.57)		72	0.66 (0.39–1.11)	131	1.20 (0.83–1.74)	
	p_{trend}		*0.20*		*0.40*			*0.15*		*0.43*	
DNMT3B C > T (rs406193, 0.14)	1	170	Ref.	56	Ref.	*0.20*	162	Ref.	49	Ref.	*0.26*
	2	123	0.67 (0.48–0.93)	41	0.56 (0.32–0.98)		157	1.10 (0.80–1.51)	46	1.05 (0.60–1.82)	
	3	175	1.04 (0.75–1.44)	51	0.72 (0.39–1.31)		149	0.91 (0.64–1.28)	53	1.43 (0.78–2.59)	
	p_{trend}		*0.72*		*0.34*			*0.45*		*0.32*	
EHMT1 G > A (rs4634736, 0.10)	1	187	Ref.	38	Ref.	*0.30*	172	Ref.	39	Ref.	*0.99*
	2	129	0.62 (0.45–0.84)	34	0.95 (0.49–1.82)		167	1.09 (0.81–1.48)	35	1.12 (0.57–2.22)	
	3	186	0.99 (0.73–1.35)	40	0.97 (0.48–1.97)		163	0.95 (0.69–1.32)	38	1.24 (0.54–2.86)	
	p_{trend}		*0.97*		*0.90*			*0.58*		*0.63*	
EHMT2 G > A (rs535586, 0.35)	1	103	Ref.	122	Ref.	*0.86*	89	Ref.	122	Ref.	*0.20*
	2	73	0.55 (0.35–0.84)	89	0.73 (0.50–1.05)		83	1.17 (0.76–1.81)	119	0.99 (0.69–1.42)	
	3	89	0.88 (0.57–1.35)	135	1.09 (0.76–1.59)		93	1.24 (0.80–1.91)	105	0.80 (0.53–1.22)	
	p_{trend}		*0.50*		*0.59*			*0.48*		*0.25*	

Table 1 continued

Gene and SNP (rs number, MAF)*	Tertile of intake	Folate†					Methionine‡				
		Common homozygotes		Heterozygotes and rare homozygotes		$p_{interaction}$	Common homozygotes		Heterozygotes and rare homozygotes		$p_{interaction}$
		n§	RR (95% CI)	n	RR (95% CI)		n	RR (95% CI)	n	RR (95% CI)	
PRDM2 $G > A$ (rs2235515, 0.23)	1	116	Ref.	106	Ref.	*0.98*	120	Ref.	85	Ref.	*0.19*
	2	93	0.67 (0.46–0.96)	65	0.61 (0.39–0.96)		107	0.95 (0.66–1.35)	90	1.29 (0.83–2.01)	
	3	121	0.95 (0.65–1.39)	95	0.93 (0.61–1.43)		103	0.97 (0.65–1.43)	91	1.02 (0.64–1.64)	
	p_{trend}		*0.81*		*0.86*			*0.69*		*0.91*	

* SNP: Single Nucleotide Polymorphism, MAF: Minor Allele Frequency among subcohort members

† Among subcohort members within tertiles: median folate intake: 162, 200 and 255 μg/day; accumulated time at risk: 4131, 4091 and 4093 person years

‡ Among subcohort members within tertiles: median methionine intake: 1316, 1583 and 1881 mg/day; accumulated time at risk: 4110, 4105 and 4100 person years

§ Number of colorectal cancer cases

¶ RRs based on a model containing the variables folate, methionine, vitamin B2, vitamin B6, and further adjusted for age, sex, family history of colorectal cancer, body mass index, smoking status, alcohol consumption and total energy intake

** p-value for linear trend

Folate or methionine intakes were not associated with CRC within either common homozygotes or within heterozygotes and rare homozygotes of any of the genotypes (Table 1). However, we observed a non-significant inverse association between vitamin B2 intake and CRC risk among subjects with the *MTHFR c.665CC* (rs1801133) common genotype (RR for the highest versus the lowest tertile of intake = 0.66, $p_{trend} = 0.08$, Table 2), and an inverse association among subjects with the common GG genotype of *PRDM2* $G > A$ (rs2235515, RR = 0.67, $p_{trend} = 0.05$). In addition, vitamin B2 was associated with reduced CRC risk in individuals carrying the variant allele of *DNMT3B* $C > T$ (rs2424913, RR = 0.69, $p_{trend} = 0.05$). Conversely, subjects in the third tertile of vitamin B6 intake were at increased CRC risk when they carried the rare allele of *DNMT3B* $C > T$ (rs406193, RR = 1.90, $p_{trend} = 0.04$), or the common allele of *PRDM2* $G > A$ (rs2235515, RR = 1.49, $p_{trend} = 0.03$). However, interactions between these dietary factors and genotypes were not statistically significant.

We also investigated the associations between methyl donor intake and CRC risk according to the number of rare alleles within each functional group (i.e. folate metabolizing enzymes, *DNMT3B* and histone methyltransferases). It appeared that methionine was inversely associated with CRC if subjects were homozygous to both of the common variants of the *DNMT3B* rs2424913 and rs406193 $C > T$ SNPs (RR = 0.44, $p_{trend} = 0.05$, $P_{interaction} = 0.07$, Table 3). Moreover, relatively high vitamin B2 intake was associated with reduced CRC risk in subjects carrying less than one rare variant of folate metabolizing enzymes (RR = 0.30, $p_{trend} = 0.005$, $P_{interaction} = 0.36$, Table 4). No dietary associations were observed according to the number of rare alleles in the studied histone methyltransferases (Table 5).

With respect to CpG island promoter hypermethylation, we observed no overall associations between folate, methionine, vitamins B2 or B6 with CIMP (Table 6). Moreover, there were no clear associations between methyl donor intake and CIMP, *MLH1* hypermethylation or MSI when accounting for genetic status of individuals (data not shown).

Discussion

In the current prospective case-cohort study, we observed no clear associations between dietary folate and vitamin B6 with CRC risk when accounting for genetic variants of folate metabolizing enzymes, DNA methyltransferases, or histone methyltransferases. However, relatively high methionine intake may protect against CRC if enzymatic activity of DNMT3B is not affected by two $C > T$ SNPs in its encoding gene. In addition, subjects with high vitamin B2 intake may be at reduced CRC risk in combination with

Table 2 Intake of vitamins B2 and B6 and CRC risk stratified by genetic status

Gene and SNP (rs number, MAF)*	Tertile of intake	Vitamin B2†					Vitamin B6‡				
		Common homozygotes		Heterozygotes and rare homozygotes			Common homozygotes		Heterozygotes and rare homozygotes		
		n§	RR (95% CI)	n	RR (95% CI)	$p_{interaction}$	n	RR (95% CI)	n	RR (95% CI)	$p_{interaction}$
MTHFR c.665C > T (rs1801133, 0.30)	1	103	Ref.	117	Ref.	*0.73*	81	Ref.	105	Ref.	*0.32*
	2	92	0.80 (0.54–1.17)	112	1.02 (0.70–1.49)		96	1.24 (0.85–1.83)	111	1.11 (0.76–1.61)	
	3	78	0.66 (0.42–1.04)	105	0.89 (0.58–1.35)		96	1.50 (0.98–2.28)	118	1.05 (0.69–1.61)	
	p_{trend}**		*0.08*		*0.54*			*0.12*		*0.63*	
MTHFR c.1286A > C (rs1801131, 0.37)	1	100	Ref.	118	Ref.	*0.46*	90	Ref.	98	Ref.	*0.89*
	2	87	0.72 (0.47–1.09)	116	1.06 (0.75–1.49)		87	1.11 (0.74–1.66)	116	1.16 (0.81–1.68)	
	3	86	0.76 (0.48–1.19)	95	0.85 (0.57–1.27)		96	1.19 (0.77–1.85)	115	1.23 (0.82–1.85)	
	p_{trend}		*0.29*		*0.42*			*0.53*		*0.35*	
MTR c.2756A > G (rs1805087, 0.19)	1	133	Ref.	88	Ref.	*0.34*	115	Ref.	74	Ref.	*0.62*
	2	137	0.95 (0.68–1.31)	68	0.85 (0.54–1.34)		145	1.33 (0.95–1.86)	64	0.91 (0.58–1.44)	
	3	127	0.81 (0.56–1.17)	61	0.87 (0.53–1.44)		137	1.17 (0.80–1.72)	79	1.31 (0.84–2.05)	
	p_{trend}		*0.30*		*0.52*			*0.49*		*0.22*	
MTRR c.66A > G (rs1801394, 0.56)	1	49	Ref.	174	Ref.	*0.26*	39	Ref.	153	Ref.	*0.69*
	2	44	0.78 (0.39–1.56)	162	0.93 (0.70–1.25)		43	1.48 (0.80–2.74)	165	1.03 (0.76–1.38)	
	3	36	0.59 (0.27–1.32)	153	0.89 (0.65–1.23)		47	1.49 (0.74–3.00)	171	1.11 (0.80–1.53)	
	p_{trend}		*0.12*		*0.56*			*0.19*		*0.55*	
DNMT3B C > T (rs2424913, 0.42)	1	79	Ref.	146	Ref.	*0.39*	68	Ref.	125	Ref.	*0.54*
	2	72	1.03 (0.64–1.69)	135	0.84 (0.61–1.15)		71	1.60 (0.96–2.67)	138	1.04 (0.76–1.42)	
	3	66	1.07 (0.63–1.82)	123	0.69 (0.48–0.99)		78	1.42 (0.85–2.37)	141	1.18 (0.82–1.70)	
	p_{trend}		*0.81*		*0.05*			*0.21*		*0.44*	
DNMT3B C > T (rs406193, 0.14)	1	171	Ref.	52	Ref.	*0.71*	145	Ref.	45	Ref.	*0.39*
	2	151	0.86 (0.63–1.17)	53	0.86 (0.50–1.48)		163	1.12 (0.83–1.52)	45	1.28 (0.70–1.36)	
	3	146	0.84 (0.59–1.19)	43	0.66 (0.37–1.19)		160	1.12 (0.80–1.58)	58	1.90 (1.00–3.60)	
	p_{trend}		*0.33*		*0.20*			*0.60*		*0.04*	
EHMT1 G > A (rs4634736, 0.10)	1	180	Ref.	42	Ref.	*0.74*	161	Ref.	30	Ref.	*0.70*
	2	165	0.93 (0.69–1.25)	40	0.94 (0.49–1.82)		169	1.07 (0.80–1.44)	38	1.33 (0.68–2.58)	
	3	157	0.85 (0.62–1.18)	30	0.69 (0.32–1.50)		172	1.15 (0.84–1.58)	44	1.52 (0.71–3.26)	
	p_{trend}		*0.38*		*0.38*			*0.40*		*0.29*	
EHMT2 G > A (rs535586, 0.35)	1	98	Ref.	125	Ref.	*0.66*	81	Ref.	108	Ref.	*0.66*
	2	88	0.93 (0.62–2.38)	116	0.85 (0.59–1.21)		85	0.98 (0.65–1.48)	123	1.25 (0.88–1.78)	
	3	79	0.85 (0.54–1.34)	105	0.75 (0.50–1.12)		99	1.17 (0.75–1.83)	115	1.25 (0.85–1.86)	
	p_{trend}		*0.53*		*0.21*			*0.45*		*0.30*	

Table 2 continued

Gene and SNP (rs number, MAF)*	Tertile of intake	Vitamin B2†					Vitamin B6‡				
		Common homozygotes		Heterozygotes and rare homozygotes			Common homozygotes		Heterozygotes and rare homozygotes		
		n§	RR (95% CI)	n	RR (95% CI)	$p_{interaction}$	n	RR (95% CI)	n	RR (95% CI)	$p_{interaction}$
PRDM2 G > A	1	123	Ref.	95	Ref.	*0.18*	101	Ref.	83	Ref.	*0.24*
(rs2235515, 0.23)	2	113	0.84 (0.59–1.19)	84	0.94 (0.62–1.44)		105	1.31 (0.92–1.88)	99	0.95 (0.63–1.42)	
	3	94	0.67 (0.44–1.00)	87	1.02 (0.65–1.60)		124	1.49 (1.00–2.22)	84	0.89 (0.56–1.41)	
	p_{trend}		*0.05*		*0.88*			*0.03*		*0.41*	

* SNP: Single Nucleotide Polymorphism, MAF: Minor Allele Frequency among subcohort members

† Among subcohort members within tertiles: median vitamin B2 intake: 1.19, 1.50 and 1.84 mg/day; accumulated time at risk: 4,116, 4,109 and 4,090 person years

‡ Among subcohort members within tertiles: median vitamin B6 intake: 1.20, 1.44 and 1.70 mg/day; accumulated time at risk: 4,125, 4,118 and 4,073 person years

§ Number of colorectal cancer cases

¶ RRs based on a model containing the variables folate, methionine, vitamin B2, vitamin B6, and further adjusted for age, sex, family history of colorectal cancer, body mass index, smoking status, alcohol consumption and total energy intake

** p-value for linear trend

optimal MTHFR activity in individuals homozygous for the common *c.665CC* (rs1801133) variant, with common *PRDM2 GG* (rs2235515) genotype and among those with the variant allele of *DNMT3B C > T* (rs2424913). We observed a strong inverse association between vitamin B2 intake and CRC risk among individuals carrying ≤ 1 rare allele in the combination of any of the folate metabolizing enzymes *MTHFR*, *MTR*, or *MTRR*. There were no associations with the CIMP phenotype overall, or within strata of the studied genotypes.

The *MTHFR c.665C > T* (rs1801133) polymorphism reduces binding of the MTHFR enzyme to its cofactor flavin adenine dinucleotide (FAD), a metabolite of vitamin B2, resulting in loss of enzymatic activity [15]. The potentially resulting reduced bioavailability of methyl groups may induce DNA hypomethylation in for example blood cells [35, 36] or CpG island promoter hypermethylation in CRC [19, 37]. We observed an inverse association between vitamin B2 and CRC risk, predominantly among subjects homozygous for the *MTHFR c.665CC* (rs1801133) variant, suggesting that vitamin B2 may maximize the catalytic activity of MTHFR when binding to FAD is optimal. Similarly, it was recently observed that high vitamin B2 plasma concentrations, in combination with *MTHFR c.665CC or CT* genotypes, may reduce risk of CRA recurrence, whereas such an inverse association was not observed among individuals with the *MTHFR c.665TT* variant [38].

The rare variant of another *MTHFR* polymorphism, *MTHFR c.1286A > C* (rs1801131), may also reduce enzymatic MTHFR activity [16], and was associated with CIMP in colorectal cancer [18], possibly in combination with low folate and methionine intakes and high alcohol consumption [17]. However, we previously observed that this polymorphism was neither associated with overall CRC or with the CIMP phenotype [39], nor when methyl donor intake was accounted for in the current study. Possibly, the use of different panels to identify CIMP-high (a "classic" panel [17] or a new panel [18, 39] which may be more robust [2]) may have contributed to this inconsistency. Moreover, different essays to measure DNA methylation were used, i.e. MSP [17, 39] or a quantitative method [18]. However, in addition to this variety of approaches, it is also important to realize that the one-carbon metabolism is involved in both DNA synthesis as well as DNA methylation, both of which may have an effect on colorectal carcinogenesis [40]. The relative contribution of each of these biological processes in carcinogenesis remains to be established and may not have been similar in the investigated study populations. Furthermore, global DNA hypomethylation and CIMP are possibly inversely associated in CRC [41], and methyl group donors may have an effect on both of these potentially distinct methylation-associated pathways in colorectal carcinogenesis. In this respect, low

Table 3 Dietary folate, methionine, vitamins B2 and B6 and CRC risk for combinations of genotypes DNA methyltransferase 3B

	Tertile of intake	DNA methyltransferase 3B						*p*-value for interaction
		0*		1		2		
		n	RR (95% CI)	*n*	RR (95% CI)	*n*	RR (95% CI)	
Folate	1	53	Ref.	151	Ref.	22	Ref.	*0.48*
	2	37	0.72 (0.39–1.35)	107	0.63 (0.45–0.89)	20	0.67 (0.27–1.67)	
	3	39	0.76 (0.39–1.48)	163	1.10 (0.78–1.55)	23	0.78 (0.31–1.93)	
	p_{trend}		*0.37*		*0.54*		*0.79*	
Methionine	1	53	Ref.	137	Ref.	20	Ref.	*0.07*
	2	37	0.74 (0.37–1.47)	142	1.18 (0.85–1.63)	24	1.71 (0.77–3.79)	
	3	39	0.44 (0.21–0.94)	142	1.12 (0.79–1.60)	21	1.48 (0.56–3.90)	
	p_{trend}		*0.05*		*0.66*		*0.47*	
Vitamin B2	1	47	Ref.	154	Ref.	22	Ref.	*0.87*
	2	41	1.09 (0.56–2.12)	137	0.78 (0.57–1.08)	25	1.30 (0.57–2.98)	
	3	41	1.52 (0.71–3.22)	130	0.72 (0.50–1.05)	18	0.79 (0.29–1.74)	
	p_{trend}		*0.36*		*0.10*		*0.41*	
Vitamin B6	1	42	Ref.	127	Ref.	21	Ref.	*0.29*
	2	42	1.80 (0.88–3.70)	147	1.13 (0.83–1.54)	18	0.98 (0.37–2.57)	
	3	45	1.37 (0.68–2.75)	147	1.15 (0.81–1.63)	26	2.12 (0.79–5.72)	
	p_{trend}		*0.44*		*0.46*		*0.18*	

* Number of variant alleles (i.e. heterozygotes or homozygotes for the rare allele)

Table 4 Dietary folate, methionine, vitamins B2 and B6 and CRC risk for combinations of genotypes in folate metabolizing enzymes

	Tertile of intake	Folate metabolizing enzymes						*p*-value for interaction
		≤1*		2		≥3		
		n	RR (95% CI)	*n*	RR (95% CI)	*n*	RR (95% CI)	
Folate	1	41	Ref.	95	Ref.	83	Ref.	*0.99*
	2	32	0.52 (0.24–1.14)	66	0.56 (0.37–0.86)	61	0.76 (0.44–1.31)	
	3	42	1.23 (0.58–2.63)	102	0.91 (0.60–1.40)	75	0.89 (0.51–1.54)	
	p_{trend}		*0.51*		*0.97*		*0.66*	
Methionine	1	40	Ref.	85	Ref.	81	Ref.	*0.38*
	2	41	1.50 (0.70–3.21)	89	1.24 (0.82–1.90)	70	0.73 (0.44–1.21)	
	3	34	0.99 (0.38–2.53)	89	1.06 (0.68–1.65)	68	0.70 (0.40–1.24)	
	p_{trend}		*0.90*		*0.90*		*0.18*	
Vitamin B2	1	43	Ref.	91	Ref.	83	Ref.	*0.36*
	2	40	0.76 (0.37–1.54)	90	0.95 (0.63–1.43)	71	0.94 (0.58–1.53)	
	3	32	0.30 (0.11–0.81)	82	0.88 (0.55–1.39)	65	1.05 (0.61–1.80)	
	p_{trend}		*0.005*		*0.96*		*0.87*	
Vitamin B6	1	31	Ref.	83	Ref.	71	Ref.	*0.82*
	2	46	0.94 (0.94–4.03)	88	1.05 (0.70–1.56)	69	1.07 (0.63–1.80)	
	3	38	2.32 (1.00–5.36)	92	1.06 (0.68–1.65)	79	1.39 (0.80–2.42)	
	p_{trend}		*0.07*		*1.00*		*0.21*	

* Number of variant alleles (i.e. heterozygotes or homozygotes for the rare allele)

folate and high alcohol intakes were associated with LINE-1 hypomethylation as an indicator for global DNA hypomethylation [8], which is in agreement with in vivo experimental data [7].

We did not observe associations between methyl donor intake and the CIMP phenotype in CRC, either overall or after stratifying the analyses for the genetic variants of folate metabolizing enzymes or methyltransferases.

Table 5 Dietary folate, methionine, vitamins B2 and B6 and CRC risk for combinations of genotypes in histone methyltransferases

	Tertile of intake	Histone methyltransferases						*p*-value for interaction
		0*		1		≥2		
		n	RR (95% CI)	*n*	RR (95% CI)	*n*	RR (95% CI)	
Folate	1	43	Ref.	103	Ref.	72	Ref.	0.76
	2	30	0.47 (0.24–0.90)	76	0.72 (0.47–1.10)	51	0.71 (0.43–1.17)	
	3	44	0.91 (0.46–1.80)	90	0.92 (0.61–1.39)	78	1.11 (0.67–1.85)	
	p_{trend}		*0.64*		*0.69*		*0.65*	
Methionine	1	41	Ref.	93	Ref.	68	Ref.	0.61
	2	35	1.03 (0.51–2.06)	89	1.08 (0.71–1.64)	70	1.12 (0.68–1.85)	
	3	41	1.14 (0.58–2.23)	87	1.12 (0.71–1.76)	63	0.77 (0.44–1.35)	
	p_{trend}		*0.80*		*0.76*		*0.34*	
Vitamin B2	1	45	Ref.	96	Ref.	75	Ref.	0.99
	2	37	0.71 (0.37–1.37)	94	0.94 (0.63–1.40)	64	0.81 (0.50–1.30)	
	3	35	0.55 (0.26–1.13)	79	0.86 (0.54–1.37)	62	0.78 (0.47–1.31)	
	p_{trend}		*0.22*		*0.45*		*0.49*	
Vitamin B6	1	36	Ref.	85	Ref.	60	Ref.	0.29
	2	29	0.85 (0.44–1.64)	100	1.38 (0.94–2.04)	73	1.11 (0.68–1.79)	
	3	52	1.69 (0.79–3.60)	84	1.01 (0.66–1.55)	68	1.39 (0.81–2.39)	
	p_{trend}		*0.10*		*0.93*		*0.34*	

* Number of variant alleles (i.e. heterozygotes or homozygotes for the rare allele)

Moreover, overall associations between methyl donor intake and CIMP in CRC were not observed in another population-based study [11], while in the same cohort, a diet-gene association with CIMP in CRC was observed for only one out of thirteen one-carbon metabolism genes [17]. However, these studies, as well as our study, may have lacked adequate power to demonstrate such associations. The effect of methyl donor intake on gene promoter hypermethylation may indeed be weak though, and to demonstrate whether such an effect is modified by genetic variability of the methyl metabolism requires studies with large numbers of cases. Nonetheless, we did observe an inverse association between vitamin B2 and CRC risk in individuals carrying ≤1 variant allele out of the four studied SNPs of folate metabolizing enzymes, suggesting that the combination of common wild-type genotypes, which possibly results in higher bioavailability of methyl groups, protects against CRC in these people.

The findings of our study may indicate that relatively high methionine intake protects against CRC if enzymatic DNMT3B activity is not affected by two polymorphisms. DNMT3B activity, which may be increased by the *DNMT3B* $C > T$ (rs2424913) polymorphism [42], was associated with CIMP-high in CRC [43], and with increased risk of various other types of cancer [42, 44, 45]. In addition, experimental research suggested that DNMT3B overexpression induced formation of tumors with promoter hypermethylation [46]. *DNMT3B* $C > T$ (rs2424913) was also associated with increased colorectal adenoma risk in individuals with low folate and methionine intakes [47], suggesting a nutrient–gene interaction in colorectal carcinogenesis. In view of the function of the DNMT3B enzyme of incorporating methyl groups into DNA, an interaction between methionine intake, *DNMT3B* polymorphisms and CpG island hypermethylation may be expected, but we did not observe clear associations between methyl donor intake and CIMP, *MLH1* hypermethylation or MSI when accounting for *DNMT3B* genotypes.

The potential protective effects of vitamin B2 or methionine may only be present among individuals with ≤1 polymorphism in folate metabolizing enzymes or among those with common wild-type genotypes of *DNMT3B*, respectively. This suggests that the occurrence of only one rare variant may be compensated for, but that the combination of several polymorphic genes may lead to disruption of a particular metabolic or regulatory function and to the abolishment of beneficial effects of nutrients. However, we should be careful in drawing definite conclusions because the sample size of our study may have been insufficient to conduct stratified analyses with adequate precision. Moreover, the *P*-values for interaction were not statistically significant, suggesting the absence of heterogeneity of diet associations with CRC across genotypes. In addition, we conducted several stratified analyses, and these multiple comparisons do not exclude the possibility of reporting chance findings. Nonetheless, although these observations

Table 6 Associations of folate, methionine, vitamins B2 and B6 with CIMP in CRC

Tertile (median within tertile)	PY†	CIMP+¶		CIMP−*	
		n‡	RR (95% CI)§	n	RR (95% CI)
Folate (μg/day)					
1 (151.4)	6,502	54	Ref.	128	Ref.
2 (200.1)	6,631	57	1.05 (0.71–1.57)	124	0.92 (0.69–1.23)
3 (264.6)	6,618	42	0.83 (0.52–1.35)	134	1.05 (0.75–1.47)
p-value for linear trend			*0.54*		*0.73*
Methionine (mg/day)					
1 (1323)	6,613	55	Ref.	134	Ref.
2 (1587)	6,621	48	0.80 (0.51–1.26)	128	0.88 (0.67–1.17)
3 (1880)	6,518	50	0.80 (0.49–1.31)	124	0.81 (0.59–1.10)
p-value for linear trend			*0.42*		*0.18*
Vitamin B2 (mg/day)					
1 (1.19)	6,607	53	Ref.	131	Ref.
2 (1.48)	6,617	48	0.96 (0.63–1.46)	134	1.06 (0.81–1.39)
3 (1.83)	6,528	52	1.16 (0.72–1.87)	121	0.97 (0.72–1.31)
p-value for linear trend			*0.62*		*0.82*
Vitamin B6 (mg/day)					
1 (1.18)	6,573	48	Ref.	115	Ref.
2 (1.43)	6,675	56	1.21 (0.79–1.85)	129	1.15 (0.87–1.54)
3 (1.70)	6,504	49	1.13 (0.71–1.80)	142	1.33 (0.97–1.83)
p-value for linear trend			*0.72*		*0.11*

Associations are irrespective of genetic status and are therefore based on a larger number of subcohort members and CRC cases

† Number of accumulated Person Years (PY) within categories of dietary intake

‡ Number of cases within tertiles of dietary intake

§ Incidence Rate Ratio (RR) from a Cox regression model including the variables folate, methionine, vitamins B2 and B6. Adjusted for age, sex, family history of colorectal cancer, body mass index, smoking behavior, alcohol consumption and energy intake

¶ CpG Island Methylator Phenotype (CIMP); ≥3 out of 5 CIMP markers methylated

* 0–2 out of 5 CIMP markers methylated

are based on subgroup analyses, and thus have to be interpreted with some caution, this study may indicate that combining genotypes is important to reveal associations of dietary factors with cancer risk. Such an approach has not been followed in previous studies investigating associations between genetic factors and cancer risk, and we recommend that combinations of genotypes should be considered in addition to overall analyses in future studies.

Subgroup analyses in the present study indicated that vitamin B2 and methionine may protect against CRC among individuals who do not carry rare variants of folate metabolizing enzymes and a DNA methyltransferase. However, larger studies are needed to investigate a potential interaction between dietary methyl donor intake, genetic variation of folate metabolizing enzymes and epigenetic regulators, and methylation endpoints in CRC with more precision. Because multiple genes may collectively affect the folate metabolism, combining genotypes of related genes is a useful approach of investigating associations of dietary methyl donors and CRC.

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