Research Article

Cancer Epidemiology, Biomarkers & Prevention

Plasma Folate, Related Genetic Variants, and Colorectal Cancer Risk in EPIC

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Abstract

Background: A potential dual role of folate in colorectal cancer (CRC) is currently subject to debate. We investigate the associations between plasma folate, several relevant folate-related polymorphisms, and CRC risk within the large European Prospective Investigation into Cancer and Nutrition cohort.

Methods: In this nested case-control study, 1,367 incident CRC cases were matched to 2,325 controls for study center, age, and sex. Risk ratios (RR) were estimated with conditional logistic regression and adjusted for smoking, education, physical activity, and intake of alcohol and fiber.

Results: Overall analyses did not reveal associations of plasma folate with CRC. The RR (95% confidence interval; P_{trend}) for the fifth versus the first quintile of folate status was 0.94 (0.74-1.20; 0.44). The polymorphisms *MTHFR677C→T, MTHFR1298A→C, MTR2756A→G, MTRR66A→G*, and *MTHFD11958G→A* were not associated with CRC risk. However, in individuals with the lowest plasma folate concentrations, the *MTHFR 677TT* genotype showed a statistically nonsignificant increased CRC risk [RR (95% CI; P_{trend}) *TT* versus *CC* = 1.39 (0.87-2.21); 0.12], whereas those with the highest folate concentrations showed a nonsignificant decreased CRC risk [RR *TT* versus *CC* = 0.74 (0.39-1.37); 0.34]. The *SLC19A180G→A* showed a positive association with CRC risk [RR *AA* versus *GG* 1.30 (1.06-1.59); <0.01].

Conclusions: This large European prospective multicenter study did not show an association of CRC risk with plasma folate status nor with *MTHFR* polymorphisms.

Impact: Findings of the present study tend to weaken the evidence that folate plays an important role in CRC carcinogenesis. However, larger sample sizes are needed to adequately address potential gene-environment interactions. *Cancer Epidemiol Biomarkers Prev; 19(5); 1328–40. ©2010 AACR.*

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Introduction

It has been proposed that folate status may affect colorectal cancer (CRC) risk because of the role of folate in the synthesis of nucleic acid and in DNA methylation (1). Although not consistent, the majority of studies on folate intake, as summarized in two meta-analyses (2, 3), indicate a 20% to 40% CRC risk reduction in individuals with the highest folate intake compared with those with the lowest folate intake. The Nurses Health Study showed no relation between dietary folate and CRC risk, but a 75% CRC risk reduction was observed in women using multivitamin supplements with ≥400 μg folic acid for 15 years or longer compared with those taking them <15 years or never (4). However, a temporally increased CRC incidence has been reported to coincide with mandatory folic acid supplementation in the United States and Canada (5). In addition, explorative analyses of the first randomized controlled intervention trial of folic acid for the secondary prevention of colorectal polyps revealed that supplementation was associated with a 67% increased risk in advanced lesions during the last part of a follow-up period of 6 to 8 years (6). As a result of these contradictory findings, a potential dual role of folate has been hypothesized (1, 7, 8), that is, that folate deficiency in normal colorectal tissues may promote carcinogenesis, whereas folate deficiency could have an inhibitory effect on the progression of established neoplasms (1).

Folate metabolism involves multiple enzymes (9) that provide methyl groups to various reactions. The trifunctional enzyme methylenetetrahydrofolate dehydrogenase/methenyltetrahydrofolate cyclohydrolase/formyltetrahydrofolate synthetase (MTHFD1) catalyses the conversion from tetrahydrofolate to the corresponding 10-formyl, 5,10-methenyl, and 5,10-methylene forms, which are cofactors for *de novo* purine and pyrimidine and subsequent DNA synthesis (10).The reduced folate carrier 1 (SLC19A1) plays a role in cellular uptake of 5-methyltetrahydrofolate (11). Methionine synthase reductase (MTRR) is required for activation of methionine synthase (MTR; refs. 12-15), which requires 5-methyltetrahydrofolate as a methyl donor for the remethylation of homocysteine to methionine to provides methylgroups for, e.g., DNA methylation. Regeneration of 5-methyltetrahydrofolate from tetrahydrofolate is catalyzed by the enzyme methylenetetrahydrofolate reductase (MTHFR; Fig. 1; ref. 16). Among the one carbon–related single nucleotide polymorphisms (SNP) mentioned above, only *MTHFR 677C→T* has been shown to significantly and consistently affect life-long folate and homocysteine status, whereas data for the other SNPs are sparse and inconsistent (17-19). The *677C→T* and *1298A→C* polymorphisms in the *methylenetetrahydrofolate* (*MTHFR*) gene have been studied in greatest detail in relation to CRC risk. Two meta-analyses indicate inverse associations between *677TT* and *1298CC* genotypes, and CRC risk (20, 21). In contrast, the effects of genetic variants of *MTR* (22-24), *MTRR* (18, 19, 25), *MTHFD1* (26, 27) and *SLC19A1* (9, 28) on CRC risk have been less studied and show inconsistent associations. Furthermore, the *TT* variant of the *MTHFR 677C→T* SNP, compared with the *CC* or *CT* variants, is either a risk factor for CRC (25, 29-31) or colorectal adenomas (32, 33) at low folate concentrations and protective at high folate concentrations, whereas other studies did not show such a dual effect (22, 34-37). In addition to an interaction of *MTHFR* genotype with folate status, there is also some evidence for an interaction with alcohol consumption (29, 38).

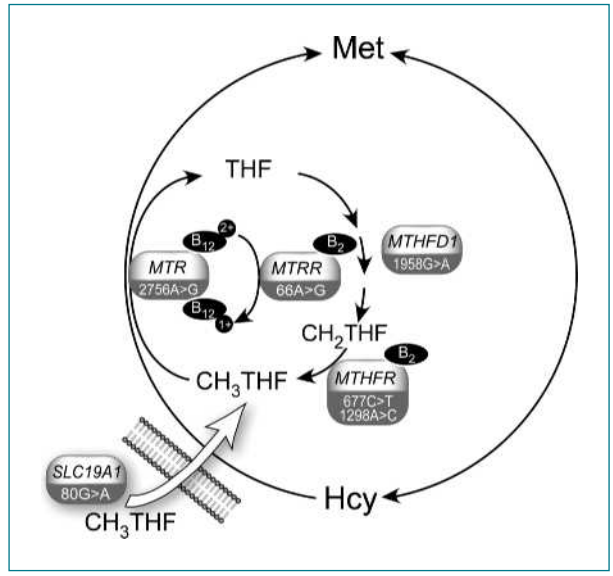

Figure 1. Key pathways of the folate metabolism. CH_2THF, methylenetetrahydrofolate; CH_3THF, methyltetrahydrofolate; Hcy, homocysteine; Met, methionine; MTHFD1, provision of one-carbon units for purine and pyrimidine synthesis; MTHFR, provision of 5-methylfolate for homocysteine remethylation; MTR, remethylation of homocysteine to methionine; MTRR, activation of MTR; SLC19A1, reduced folate carrier-1 (transport and binding of folate); THF, tetrahydrofolate.

The associations between plasma folate and CRC risk has been investigated in several previous studies (30, 37, 39-42). However, the associations were inconsistent. Therefore, we investigated the association between plasma folate status and CRC in a very large-scale prospective cohort study within the European Prospective Investigation into Cancer and Nutrition (EPIC) study. We also

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assessed the potential interaction between plasma folate status, SNPs related to one-carbon metabolism, and alcohol consumption.

Materials and Methods

Study population and collection of blood samples

The design and methods of the EPIC study have been previously described in detail (43). Briefly, the EPIC cohort recruited participants from 23 centers in 10 European countries (Denmark, France, Greece, Germany, Italy, the Netherlands, Norway, Spain, Sweden, and United Kingdom). Between 1992 and 1998, country-specific dietary questionnaires, standardized life-style and personal history questionnaires, and anthropometric data were collected from all the cohort members, and a blood sample was taken from 80% of the participants.

In each of the recruitment centers, both fasting or nonfasting blood samples of at least 30 mL were drawn from those participants who provided a blood sample and stored at 5°C to 10°C, protected from light, and transported to local laboratories for processing and aliquoting as previously described (43, 44). The only exceptions are the EPIC-Oxford and EPIC-Norway centers where blood samples were collected from a network of general practitioners (Oxford and Norway) and health conscious people (Oxford), and transported in the mail to a central laboratory in Norfolk (Oxford) and Tromsø (Norway), respectively. The whole blood samples were protected from light, but exposed to ambient temperatures for up to 48 hours. As it is possible that some B vitamins might be partly degraded by such handling (45), all EPIC-Oxford (55 cases and 107 controls) and EPIC-Norway (5 cases and 9 controls) samples were excluded from the present analyses.

In all countries, except Denmark and Sweden, blood was separated into 0.5-mL fractions (serum, plasma, red cells, and buffy coat for DNA extraction). Each fraction was placed into plastic CBS straws, which were heat sealed and stored in liquid nitrogen (−196°C). One half of all aliquots were stored at the local study center and the other half in the central EPIC biorepository at the IARC (Lyon, France). In Denmark, blood fraction aliquots of 1.0 mL were stored locally at −150°C under nitrogen vapor. In Sweden, samples were stored in −80°C freezers.

This study was approved by the Ethical Review Board of the IARC and those of all EPIC centers. All EPIC participants have provided written consent for the use of their blood samples and all data.

Follow-up for cancer incidence

In EPIC, follow-up is based on population cancer registries (Denmark, Italy, the Netherlands, Norway, Spain, Sweden, and the United Kingdom) or through a combination of methods, including linkage with health insurance records, contact with cancer and pathology registries, and active contact of study subjects or next of kin (France, Germany, and Greece). Follow-up began at the date of enrollment and ended at the date of CRC diagnosis. Follow-up was closed between 1999 and 2004. Depending on study center, complete follow-up for the centers using record linkage with cancer registries, data were available until December 31, 1999 (Turin), June 30, 2000 (Bilthoven), December 31, 2000 (Asturias, Murcia), December 31, 2001 (Florence, Varese, Ragusa, Naples, Granada, Navarra, San Sebastian, Malmö, and United Kingdom), December 31, 2002 (Denmark and Umeå), and June 30, 2003 (Utrecht). For the centers using active follow-up, the last contact dates were June 30, 2002 (France), September 11, 2002 (Greece), December 6, 2003 (Heidelberg), and March 11, 2004 (Potsdam).

Indicators of data quality of all cancer registries in the study are considered to be of good quality (46). In France, Germany, and Greece, self-reported cancer cases through active follow-up were verified by physicians and pathology reports. In these countries, pathology reports are available for at least 95% of cases and are confirmed at least 95% of self-reported cancers for other cancer sites, indicating a well-functioning case identification system (47-50). In the present study, histologic confirmation of CRC was available for 93%, whereas 6% was diagnosed through clinical observation, cytology, or autopsy; information on 1% was missing. Current information about the efficacy of follow-up until 2007 shows that 1.57% of the total population is lost to follow-up, from which 0.18% was reluctant to continue to participate, 0.80% emigrated to another region or country, and the remaining 0.57% are lost to follow-up for unknown reasons (51), implying even lower rates for follow-up until 2004 in the present study.

Nested case-control study design and selection of study subjects

Case ascertainment and selection. For the present study, colon cancers were defined as tumors in the cecum, appendix, ascending colon, hepatic flexure, transverse colon, splenic flexure, and descending and sigmoid colon (C18.0-C18.7 as per the 10th Revision of the International Statistical Classification of Diseases, Injury and Causes of Death), as well as tumors that were overlapping or unspecified (C18.8 and C18.9). Cancers of the rectum were defined as tumors occurring at the recto-sigmoid junction (C19) or rectum (C20). CRC is defined as a combination of the colon and rectal cancer cases. The present study includes a total of 1,367 incident CRC cases (colon $n = 850$; rectal $n = 517$). The distribution of cases (colon/rectal) by country was as follows: 206/172 for Denmark, 100/84 for Sweden, 30/3 for France, 14/13 for Greece, 98/61 for Germany, 106/43 for Italy, 102/49 for the Netherlands, 80/43 for Spain, and 114/49 for United Kingdom. Ninety-six percent ($n = 1,299$) of cancer cases were classified as adenocarcinomas, whereas the remaining 4% of the tumors were classified as carcinomas or unspecified forms.

Control selection. For each identified cancer case, control cohort members with available blood samples were

randomly selected from all cohort members with available blood samples who were alive and free of cancer (except nonmelanoma skin cancer) at the time of diagnosis of the index case. Controls were matched to cases by gender, age group (±2.5 y), and study center (to account of center-specific differences in questionnaire design, blood collection procedures, etc.). An exception were Danish cases (n = 378) and controls (n = 373) who were *post hoc* matched using the "greedy" algorithm, a macro (gmatch) provided by the Mayo Clinic College of Medicine (52, 53) to be run in SAS. Cases and controls were exactly matched for sex and study center, and with a maximum age difference of 5 years. The mean (range) difference in age between cases and controls within each caseset was −1.03 (−5.0 to 4.9) years.

Laboratory measurements

Plasma folate was determined by a *Lactobacillus casei* microbiological assay, adapted to a microtiter plate format and carried out by a robotic workstation (Micro-lab AT plus 2; Hamilton Bonaduz AG; ref. 54), and plasma total homocysteine (tHcy) was determined by a method based on methylchloroformate derivatization and gas chromatography-mass spectrometry (55). In total, 1.89% of the samples could not be analyzed due to, e.g., insufficient sample volumes. Within- and between-day coefficients of variation for folate and tHcy were 3% to 20% (54) and 6% to 22% (55), respectively.

The SNPs of genes related to one-carbon metabolism were determined by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (56). These included *MTHFR* (*MTHFR 677C→T*; rs#1801133 and *MTHFR 1298A→C*; rs#1801131), *MTR* (*MTR 2756A→G*; rs#1805087), *MTR reductase* (*MTRR*; *MTRR 66A→G*; rs#1801394), *MTHFD1* (*MTHFD1 1958G→A*; rs#2236225), and *reduced folate carrier-1* (*SLC19A1 80G→A*; rs#1051266).

Statistical methods

Differences between cases and controls in median baseline covariates and differences in median folate and tHcy concentrations for selected characteristics were assessed by Mann-Whitney *U* tests, whereas categorical variable differences were assessed by χ^2 tests.

Relative risks (RR) and 95% confidence intervals (95% CI) for CRC in relation to plasma folate and tHcy were calculated by conditional logistic regression using the SAS LOGISTIC procedure (SAS statistical software, version 9.1, SAS Institute), stratified by the case-control set. In our study, the RR indicated the incidence rate ratio, which is reflected by the odds ratio calculated from conditional logistic regression (57). The RRs for CRC risk were examined by quintile categories with cutoff points based on the distribution of folate and homocysteine in all 2,325 controls combined. Because excluding the 4% (n = 61) of cases with other histologies than adenocarcinoma did not modify any associations, we included them in the statistical analyses. Potential confounders were investigated, including smoking status (never, former, current, and missing), alcohol consumption (continuous), dietary fiber (continuous), intake of red and processed meat (continuous), physical activity (inactive, moderately inactive, moderately active, and active), educational level (none, primary school completed, technical/professional school, secondary school, university degree, and not specified), and body mass index (kg/m^2). Although none of these variables altered the risk estimates as obtained by the matched analyses, we present matched analyses that were further adjusted for these covariates to allow comparisons to previous publications. Likelihood ratio tests were used to assess linear trends in RRs across the categories using values for quintile categories as the quantitative score of exposure. All models were run separately and tested for effect modification for the anatomical site of cancer (colon versus rectal), European region [North (Denmark, Sweden) versus Central (France, United Kingdom, the Netherlands, and Germany) versus South (Italy, Spain, and Greece)], time from blood donation to cancer diagnosis [≤3.6 (median follow-up time) y versus >3.6 y)], age (≤60 y versus >60 y), sex, and alcohol intake (0-30 g/d versus ≥30 g/d). Effect modification was tested by adding the product term of folate and potential effect modifiers in the model. All statistical tests were two tailed and $P < 0.05$ was considered statistically significant.

The associations between the polymorphisms and CRC risk were studied with conditional logistic regression. The risk estimates were calculated with the wild-type as the reference category. A trend test with equally spaced integer weights for the genotypes were used to summarize the effect of each polymorphism. Effect modification of the SNP-CRC associations by folate concentrations and alcohol consumption were studied with conditional logistic regression, but by stratifying on country instead of the matched sets and with age and sex as covariates.

Results

Characteristics of cases and controls. The present study investigates 1,367 cancer cases and 2,325 matched controls. The sex distribution and mean age at recruitment were comparable among cases and controls. The median (range) follow up time between blood donation and CRC diagnosis was 3.6 (0.0-10.3) years. Median folate concentrations in cases were slightly lower compared with their matched controls ($P_{difference}$ = 0.08), whereas no differences in tHcy concentrations were observed between cases and controls (Table 1).

Data integrity. Table 2 shows folate and tHcy concentrations among the 2,325 control cohort members by sex; age; smoking status; European region; alcohol intake; and the SNPs *MTHFR 677C→T*, *MTHFR 1298A→C*, *MTR 2756A→G*, *MTRR 66A→G*, *MTHFD1 1958G→A*, and *SLC19A1 80G→A*. Folate concentrations were lower in males, individuals younger than 60 years, smokers, those living in Sweden and Denmark, and in those with the homozygote variant of the *MTHFR*

Table 1. Characteristics of cohort members

	Cases	Controls	$P_{difference}$
No. of individuals	1,367	2,325	
Sex, female, *n* (%)	700 (51)	1,215 (53)	0.54
Mean age (y; min-max)			
At recruitment	58.9 (30.1-76.9)	58.7 (30.0-76.6)	0.37
At blood donation	59.1 (36.8-77.0)	58.9 (36.6-76.6)	0.42
At diagnosis	62.8 (37.7-81.2)	N.a.	
Lag time	3.6 (0.01-10.3)	N.a.	
Smoking status, *n* (%)			0.02
Never	561 (41)	1,026 (43)	
Former	451 (33)	775 (33)	
Smoker	346 (25)	511 (22)	
Mean/median (P5-P95)			
Folate (nmol/L)	13.6/10.9 (5.1-32.1)	14.2/11.3 (4.9-34.0)	0.08
tHcy (μmol/L)	10.2/9.5 (6.2-16.2)	10.3/9.6 (6.3-16.4)	0.33

NOTE: Differences in plasma concentrations were assessed by Mann-Whitney *U* tests, whereas categorical variable differences were assessed by χ^2 tests.
Abbreviation: N.a., not applicable

677C→T polymorphism. tHcy concentrations were higher in males, individuals older than 60 years, smokers, and in those with the homozygote variant of the *MTHFR 677C→T* and the homozygote wild-type of the *MTHFR 1298A→C* polymorphisms ($P_{all\ differences} < 0.01$; Table 2). Plasma folate and tHcy concentrations correlated strongly within each European region after adjustment for age, sex, and study center, with Spearman correlation coefficients of −0.43 in Northern, −0.35 in Central, and −0.32 in Southern European countries ($P_{all\ correlations} < 0.0001$).

Associations of folate with CRC. Overall analyses do not show an association of plasma folate with CRC risk, nor after stratifying according to age, sex, alcohol intake, and time between blood donation and cancer diagnoses (Table 3). Cancer risk for tumors in the colon or the rectum was similar compared with overall CRC risk (data not shown). However, individuals living in Northern European countries showed an inverse association between plasma folate and rectal cancer risk, with an RR (95% CI; P_{trend}) of the highest versus the lowest folate concentrations of 0.56 (0.29-1.09; 0.04; data not shown). tHcy concentrations were not associated with cancer risk in the colorectum, colon, or rectum for any of the subgroup analyses (data not shown).

We further explored whether the association between CRC risk and plasma folate in those with low and high alcohol consumption differed among males and females. Among individuals drinking <30 g alcohol/day, the RR (95% CI; P_{trend}) for the fifth versus the first quintile of folate status for males was 0.79 (0.52-1.23; 0.19) and 0.96 (0.67-1.37; 0.73) for females. Among those drinking >30 g alcohol/day, the RR (95% CI; P_{trend}) for the fifth versus the first quintile of folate status for males was 0.91 (0.47-1.75; 0.87) and 2.59 (0.53-12.75; 0.47) for females. The association between folate and CRC risk within different categories of alcohol intake did not differ by gender ($P_{interaction\ <30\ g\ alcohol/d} = 0.56$; $P_{interaction\ \geq 30\ g\ alcohol/d} = 0.15$) and within each gender not by alcohol intake ($P_{interaction\ males} = 0.96$; $P_{interaction\ females} = 0.15$).

The folate related SNPs and their associations with CRC risk. All the SNPs were in Hardy-Weinberg equilibrium (χ^2 test $P > 0.05$ for all SNPs) and the genotype distributions did not differ among cases and their matched controls (Table 4). We observed a borderline significant inverse association between the *MTR 2756A→G* polymorphism and CRC risk ($P_{trend} = 0.06$). Compared with the *SLC19A1 GG* homozygotes, both the *GA* and *AA* genotypes were associated with significantly increased CRC risk ($P_{trend} < 0.01$). Neither the *MTHFR, MTRR*, nor the *MTHFD1* polymorphisms were associated with altered CRC cancer risk (Table 4). In addition, we investigated the associations between CRC risk and *MTHFR 677C→T* in *1298AA* individuals, and *MTHFR 1298A→C* association in *677CC* individuals to take potential effects of linkage disequilibrium between these two SNPs into account. These restrictions did not alter the associations between the MTHFR SNPs and CRC risk as shown in Table 4.

We further stratified the analyses by European region. The homozygote rare variant genotypes of the *MTHFR 677C→T* and *SLC19A1 80G→A* polymorphisms were more prevalent in Southern European countries (15% for *MTHFR 677TT* and 22% for *SLC19A1 80AA*) compared with Northern European countries (8% for *MTHFR 677TT* and 17% for *SLC19A1 80AA*; $P < 0.01$). In contrast, the homozygote rare variant genotypes of the *MTRR*

Table 2. Folate and homocysteine concentrations [mean/median (P5-P95)] by selected characteristics in control cohort members (n = 2,320)

		n	Folate (nmol/L)	tHcy (μmol/L)
Sex	Male	1,108	13.4/11.0 (4.7-31.6)	10.9/10.1 (6.7-17.3)
	Female	1,212	14.9/11.7 (5.3-37.0)	9.8/9.1 (6.0-15.2)
	$P_{difference}$*	<0.01	<0.01	
Age	<60 y	1,277	13.3/11.1 (4.8-30.0)	9.7/8.9 (6.1-15.5)
	≥60 y	1,043	15.2/11.7 (5.2-38.6)	11.0/10.4 (6.8-17.2)
	$P_{difference}$*		<0.01	<0.01
Smoking status	Never	1,024	15.2/12.3 (5.5-36.6)	9.9/9.4 (6.2-15.5)
	Former	774	14.6/11.6 (5.4-34.6)	10.3/9.8 (6.4-15.8)
	Current	509	11.5/9.3 (3.9-27.8)	11.1/9.8 (6.3-19.7)
	Unknown†	13	13.6/12.2 (8.9-23.1)	9.3/9.6 (6.4-13.6)
	P_{trend}‡		<0.01	<0.01
European region	North§	736	12.0/9.6 (4.0-27.9)	10.4/9.6 (6.3-17.1)
	Central	997	15.8/12.5 (5.7-38.1)	10.4/9.8 (6.5-16.1)
	South	587	14.2/12.4 (5.2-29.2)	10.0/8.9 (5.9-15.8)
	$P_{difference}$†		<0.01	0.11
Alcohol intake (g/d)	Abstainers intake	166	15.1/12.7 (4.5-36.8)	9.9/8.9 (5.9-16.1)
	1-30	1,783	14.2/11.3 (4.9-34.0)	10.3/9.6 (6.3-16.3)
	≥30	371	13.7/11.3 (5.2-32.7)	10.8/9.6 (6.4-17.0)
	$P_{difference}$*		0.33	0.03
MTHFR 677	*CC*	1,131	14.4/11.5 (5.2-33.4)	10.1/9.5 (6.3-15.6)
	CT	933	13.9/11.3 (4.9-32.8)	10.2/9.5 (6.4-15.7)
	TT	254	13.2/9.8 (4.2-37.2)	11.9/10.4 (6.5-22.9)
	P_{trend}‖		0.01	<0.01
MTHFR 1298	*AA*	1,002	13.6/10.9 (4.8-34.5)	10.5/9.7 (6.4-17.1)
	AC	950	14.2/11.5 (5.1-32.6)	10.3/9.6 (6.3-15.7)
	CC	236	14.9/11.2 (5.1-34.5)	10.1/9.3 (6.4-16.4)
	P_{trend}‖		0.08	0.01
MTR 2756	*AA*	1,395	14.2/11.5 (5.0-34.6)	10.4/9.7 (6.3-16.5)
	AG	710	13.6/10.8 (4.9-31.6)	10.4/9.5 (6.3-16.6)
	GG	82	13.5/10.3 (4.7-30.6)	9.9/9.2 (6.4-14.2)
	P_{trend}‖		0.24	0.48
MTRR 66	*AA*	404	14.5/11.3 (4.8-36.6)	10.0/9.3 (6.4-16.4)
	AG	1,025	13.6/11.1 (4.9-30.6)	10.3/9.6 (6.3-15.8)
	GG	603	13.9/11.3 (5.0-32.3)	10.5/9.7 (6.3-17.0)
	P_{trend}‖		0.92	0.06
MTHFD1 1958	*GG*	679	13.7/11.3 (4.7-31.0)	10.4/9.6 (6.3-16.9)
	GA	1,061	14.4/11.1 (5.0-35.6)	10.3/9.6 (6.3-15.9)
	AA	448	13.4/11.1 (5.0-30.1)	10.6/9.8 (6.6-16.6)
	P_{trend}‖		0.41	0.71
SLC19A1 80	*GG*	743	13.8/11.3 (5.1-32.0)	10.4/9.5 (6.3-16.4)
	GA	1,066	14.4/11.3 (4.8-35.5)	10.4/9.8 (6.4-16.3)
	AA	378	13.1/10.8 (4.8-28.7)	10.2/9.5 (6.1-17.7)
	P_{trend}‖		0.23	0.12

*$P_{difference}$ (two sided) calculated by Mann-Whitney U test; abstainers were excluded from statistical analyses on alcohol consumption.

†$P_{difference}$ (two sided) calculated by Kruskal-Wallis test; category unknown was excluded from statistical analyses smoking status.

‡Category unknown not included in the test for trend.

§North: Sweden and Denmark; Central: France, United Kingdom, the Netherlands, and Germany; South: Italy, Spain, and Greece.

‖P_{trend} (two sided) calculated by regression models.

Table 3. Adjusted RRs (95% CI) for CRC by quintiles of plasma folate concentrations

		N case/control	Q1	Q2: 7.6-9.9	Q3: 9.9-13.0	Q4: 13.0-18.3	Q5: >18.3	P_{trend}	$P_{interaction}$
Matched analyses	Overall	1,367/2,318	1	0.97 (0.78-1.20)	1.06 (0.86-1.31)	0.87 (0.69-1.09)	0.91 (0.73-1.14)	0.29	
Matched plus covariate-adjusted analyses	Overall	1,327/2,239	1	1.02 (0.82-1.27)	1.08 (0.87-1.35)	0.92 (0.72-1.16)	0.94 (0.74-1.20)	0.44	
Sex	Males	644/1,060	1	0.99 (0.72-1.37)	1.07 (0.77-1.47)	0.83 (0.58-1.18)	0.86 (0.60-1.23)	0.26	
	Females	683/1,179	1	1.06 (0.78-1.44)	1.10 (0.81-1.50)	1.00 (0.73-1.39)	1.00 (0.72-1.39)	0.90	0.50
Age*	≤60 y	706/1,185	1	0.97 (0.72-1.31)	0.97 (0.72-1.30)	0.76 (0.55-1.03)	0.91 (0.65-1.27)	0.22	
	>60 y	621/1,054	1	1.02 (0.74-1.40)	1.09 (0.78-1.52)	1.01 (0.72-1.43)	0.94 (0.67-1.32)	0.72	0.96
European Region	North	540/695	1	1.09 (0.79-1.51)	0.88 (0.61-1.26)	0.76 (0.51-1.13)	0.73 (0.48-1.13)	0.06	
	Central	495/971	1	1.05 (0.70-1.58)	1.34 (0.91-1.99)	1.09 (0.72-1.63)	1.34 (0.90-1.99)	0.18	
	South	292/573	1	1.04 (0.65-1.68)	1.21 (0.76-1.93)	1.02 (0.63-1.67)	0.85 (0.51-1.41)	0.56	0.17
Alcohol intake*	Abstainer	88/155	1	0.87 (0.31-2.45)	2.66 (0.93-7.61)	0.93 (0.33-2.64)	0.84 (0.29-2.42)	0.65	
	1-30 g/d	963/1,719	1	0.92 (0.71-1.18)	0.95 (0.74-1.23)	0.81 (0.61-1.05)	0.89 (0.68-1.17)	0.26	
	≥30 g/d	276/365	1	1.18 (0.70-1.99)	0.92 (0.54-1.56)	1.07 (0.61-1.87)	1.09 (0.61-1.96)	0.96	0.82
Median follow-up	<3.6 y	651/1,145	1	1.16 (0.84-1.59)	1.17 (0.85-1.61)	1.05 (0.75-1.46)	0.96 (0.68-1.35)	0.70	
	≥3.6 y	676/1,094	1	0.88 (0.65-1.21)	1.01 (0.73-1.40)	0.83 (0.59-1.17)	0.97 (0.69-1.37)	0.80	0.73

NOTE: Lower quintile is reference category. All analyses are matched for age, sex, study center, and date of blood collection. The matched + covariate adjusted analyses are further adjusted for smoking status, education level, physical activity, fiber intake, intake of red and processed meat, alcohol consumption, and body mass index.

*Subgroup analyses by age and alcohol consumption are matched for sex and study center, and further adjusted for age, date of blood collection, smoking status, education level, physical activity, fiber intake, intake of red and processed meat, alcohol consumption, and body mass index.

Table 4. Associations [RR (95% CI)] of SNPs with CRC risk

SNP		N case/control	Odds ratio (95% CI)
MTHFR 677	*CC*	567/1,019	1
	CT	608/1,076	1.02 (0.88-1.18)
	*TT**	154/271	1.02 (0.81-1.28)
	P_{trend}		0.79
MTHFR 1298	*AA*	605/1,099	1
	AC	574/1,007	1.05 (0.91-1.21)
	*CC**	151/259	1.09 (0.87-1.37)
	P_{trend}		0.45
MTR 2756	*AA*	890/1,498	1
	AG	385/774	0.83 (0.72-0.97)
	GG	54/92	0.98 (0.69-1.39)
	P_{trend}		0.06
MTRR 66	*AA*	264/435	1
	AG	597/1,111	0.88 (0.73-1.06)
	*GG**	377/661	0.92 (0.76-1.13)
	P_{trend}		0.54
MTHFDI1958	*GG*	414/721	1
	GA	626/1,117	0.98 (0.84-1.15)
	*AA**	269/489	0.98 (0.80-1.19)
	P_{trend}		0.86
SLC19A1 80	*GG*	390/792	1
	GA	660/1,126	1.21 (1.03-1.41)
	*AA**	258/408	1.30 (1.06-1.59)
	P_{trend}		<0.01

*Prevalence in control cohort members significantly different among European regions (P_{all} < 0.01).

66A→G polymorphism was more prevalent in Northern European countries (34%) compared with Southern European countries (25%; $P < 0.01$), whereas the *AA* genotype of the *MTHFD1 1958G→A* SNP was most prevalent in Central European countries (24%) compared with Northern (19%) and Southern (20%) countries ($P < 0.01$). In spite of this genetic variation among European regions, stratification did not reveal significantly different associations between the SNPs and CRC by European regions (data not shown).

Interactions between SNPs, plasma folate, and alcohol consumption in CRC risk. We studied each polymorphism separately in combination with quintiles of folate status (Table 5). Although not statistically significant, we observed that the rare *MTHFR 677TT* genotype conferred an increased CRC risk in individuals with the lowest folate concentrations [RR (95% CI) *TT* versus *CC* = 1.39 (0.87-2.21)], whereas the *TT* genotype was associated with a decreased CRC risk in individuals with the highest folate concentrations [RR (95% CI) *TT* versus *CC* = 0.74 (0.39-1.37)]. Overall, the association between *MTHFR 677C→T* was not significantly modified by folate status ($P_{interaction}$ = 0.11). The associations between the other SNPs and CRC were not modified by folate status (Table 5).

Table 6 shows that individuals with the *MTHFR 677TT* genotype who consumed ≥30 g of alcohol/day had a slight increased CRC risk (P_{trend} 0.09) compared with those drinking less alcohol/day (P_{trend} 0.46; $P_{interaction}$ = 0.11). These associations were not modified by medians of folate status ($P_{interaction}$ = 0.38). Examination of combinations of other SNPs under investigation, alcohol intake, and folate status did not modify the associations between those SNPs and CRC risk ($P_{interactions} > 0.31$; data not shown).

Discussion

In this large EPIC study, we investigated the associations of plasma folate status and genetic variability in five folate-related genes with CRC risk. Overall analysis did not reveal significant associations between plasma folate concentrations or the *MTHFR* genotypes and CRC risk. However, CRC risk increased with the number of T alleles of the *MTHFR 677C→T* polymorphism in individuals with low folate status and decreased in individuals with high folate status. Such an association has been reported by others (25, 29-31), but associations in our study did not reach statistical significance. For the first time, a positive association of the variant genotypes of the *SLC19A1 80G→A* SNP with CRC risk is observed.

The present study is the largest prospective investigation with data on plasma folate and CRC risk published thus far and its large sample size allowed us to perform subgroup analyses. Extensive information on life-style factors has been collected for each cohort member, which allowed the adjustment for potential confounders and the assessment of possible effect modification. Another important strength of this study is the collection of blood samples before cancer diagnosis. All study centers collected and stored blood samples according to a standardized protocol (43) and all biochemical analyses were done in one laboratory, thereby optimizing sample treatment and avoiding between-laboratory method variability. The observed associations between folate status and age, sex, smoking status, and the folate-related polymorphisms in the present study are in line with previous findings (17, 58, 59), confirming the integrity of biochemical data in this large multicenter study.

Studies on plasma folate and CRC risk are inconclusive, showing either an inverse association (40, 42), a positive association (37), or no association in the present and other (30, 39, 41) studies. It is unknown whether any of the Japanese (39), European (37, 41), and American (30, 40, 42) studies collected data under policies of voluntary folic acid fortification. Therefore, it is unclear whether folic acid fortification may explain contrasting study findings; however, relatively small sample sizes, ethnicity, and different ranges of plasma folate concentrations may do so. Conceivably, an association between CRC risk and plasma folate status may be more pronounced in a population that includes a higher proportion of individuals with low folate status. Different dietary habits

Table 5. RRs (95% CI) for CRC in folate related SNPs by quintiles of plasma folate status

SNP		Quintiles of folate status										OR (95% CI)
		1		2		3		4		5		
		N case/ control	OR (95% CI)	N case/ control	OR (95% CI)	N case/ control	OR (95% CI)	N case/ control	OR (95% CI)	N case/ control		
MTHFR 677	*CC*	103/182	1	101/177	1	116/196	1	99/190	1	105/188		1
	CT	137/201	1.25 (0.90-1.75)	122/200	1.11 (0.79-1.56)	125/203	1.02 (0.74-1.42)	98/207	0.92 (0.65-1.30)	96/191		0.92 (0.65-1.30)
	TT	46/64	1.39 (0.87-2.21)	37/66	1.06 (0.65-1.73)	26/44	0.96 (0.55-1.67)	25/41	1.16 (0.66-2.03)	17/39		0.74 (0.39-1.37)
	P_{trend}		0.12		0.70		0.96		0.89			0.34
MTHFR 1298	*AA*	148/221	1	121/210	1	118/200	1	97/204	1	92/167		1
	AC	105/183	0.87 (0.63-1.20)	110/182	1.08 (0.77-1.51)6	123/194	1.10 (0.79-1.53)	98/193	1.06 (0.75-1.50)	103/198		0.96 (0.67-1.36)
	CC	33/42	1.25 (0.75-2.08)	29/51	0.92 (0.54-1.55)	27/49	0.98 (0.58-1.67)	27/41	1.28 (0.74-2.24)	23/53		0.81 (0.47-1.42)
	P_{trend}		0.83		0.97		0.82		0.42			0.50
SLC19A1	*AA*	84/148	1	81/149	1	76/149	1	67/159	1	62/138		1
	AG	143/217	1.20 (0.85-1.70)	132/215	1.06 (0.74-1.52)	126/218	1.18 (0.83-1.70)	114/198	1.44 (0.99-2.09)	120/218		1.27 (0.87-1.85)
	GG	59/81	1.27 (0.82-1.97)	46/79	1.00 (0.62-1.59)	66/76	1.68 (1.08-2.61)	41/81	1.26 (0.78-2.03)	36/61		1.43 (0.85-2.39)
	P_{trend}		0.25		0.95		0.03		0.21			0.14
MTRR 66	*AA*	72/77	1	46/86	1	53/83	1	38/71	1	47/87		1
	AG	121/214	0.61 (0.41-0.91)	111/201	0.98 (0.63-1.52)	132/213	0.90 (0.59-1.36)	104/211	0.96 (0.60-1.53)	96/186		0.93 (0.60-1.45)
	GG	69/124	0.57 (0.37-0.90)	70/120	0.98 (0.60-1.0)	71/122	0.91 (0.57-1.44)	70/120	1.10 (0.67-1.82)	63/117		0.96 (0.60-1.54)
	P_{trend}		0.02		0.95		0.70		0.62			0.89
MTR 2756	*AA*	186/264	1	184/279	1	183/282	1	149/286	1	147/284		1
	AG	86/162	0.78 (0.56-1.09)	69/149	0.71 (0.50-1.01)	69/141	0.74 (0.52-1.05)	66/139	0.87 (0.60-1.24)	63/119		1.02 (0.71-1.47)
	GG	14/20	1.05 (0.51-2.16)	7/16	0.66 (0.26-1.66)	15/18	1.22 (0.59-2.50)	7/13	0.94 (0.36-2.44)	8/15		1.09 (0.45-2.67)
	P_{trend}		0.34		0.05		0.40		0.49			0.85
MTHFDi	*GG*	96/134	1	75/137	1	90/134	1	70/147	1	64/127		1
	GA	129/225	0.88 (0.68-1.15)	133/210	0.74 (0.55-1.00)	129/212	0.89 (0.68-1.17)	107/200	0.90 (0.66-1.22)	105/214		1.03 (0.76-1.39)
	AA	61/88	1.02 (1.00-1.04)	52/96	1.01 (0.99-1.04)	49/97	1.00 (0.98-1.03)	45/91	1.02 (0.99-1.04)	49/76		1.01 (0.98-1.03)
	P_{trend}		0.94		0.72		0.13		0.99			0.36

NOTE: All analyses are stratified on country and sex and adjusted for age. $P_{interaction}$ *MTHFR677**folate = 0.11; *MTHFR1298**folate = 0.81; *SLC19A1**folate = 0.67; *MTRR66**folate = 0.12; *MTR2756**folate = 0,26; *MTHFDI**folate = 0.59.

Table 6. RRs (95% CI) for CRC with *MTHFR 677C→T* in subgroups of alcohol consumption and folate status

		Alcohol consumption				$P_{interaction}$	$P_{interaction}$
		1-29 g/d		≥30 g/d			
Plasma folate	*MTHFR 677*	N ca/co	OR (95% CI)	N ca/co	OR (95% CI)	MTHFR 677 alcohol	MTHFR 677 alcohol folate
Overall	*CC*	431/797	1	101/159	1		
	CT	431/839	0.95 (0.80-1.12)	127/148	1.41 (1.00-2.00)		
	TT	107/211	0.92 (0.71-1.20)	36/45	1.38 (0.82-2.32)		
	P_{trend}		0.46		0.09	0.11	
<11.3 nmol/L	*CC*	301/549	1	68/107	1		
	CT	318/591	0.99 (0.81-1.20)	92/110	1.33 (0.87-2.03)		
	TT	84/164	0.93 (0.69-1.26)	27/34	1.42 (0.76-2.62)		
	P_{trend}		0.71		0.15	0.35	
>11.3 nmol/L	*CC*	130/248	1	33/52	1		
	CT	113/248	0.86 (0.63-1.17)	35/38	1.30 (0.67-2.51)		
	TT	23/47	0.90 (0.52-1.56)	9/11	1.36 (0.49-3.74)		
	P_{trend}		0.42		0.41	0.28	0.38

and a large variation in folate status (30, 37, 39, 40) in this large multicountry study is therefore one of its strengths. In the present study, 9% of all cohort members were folate deficient, as defined by folate concentrations <6.0 nmol/L. In Sweden and Denmark, the prevalence was 15%, compared with 5% and 8% in Central and Southern Europe, respectively. Moreover, mean plasma folate concentrations were lower in Northern European countries compared with other European regions. The lower folate concentrations, and consequently higher prevalence of folate deficiency in Northern Europe, may explain the inverse association for rectal cancer. In contrast, although overall folate status was reported to be low, a Swedish study observed low plasma folate concentrations to be associated with a decreased CRC risk in a smaller subgroup with a follow up time of >4.2 years (37).

Regarding dietary intake of folate and CRC, the majority of studies reported an inverse association between folate and CRC risk (2, 3, 60), which was predominantly present for dietary folate and not for total folate intake (3), suggesting that the use of folic acid supplements may attenuate the inverse association with CRC risk. It is unclear whether this is the case in the present study because data on supplement use, and specifically use of B vitamins, in the EPIC cohort are sparse. However, in a subsample of the EPIC population, single 24-hour recalls revealed a clear North-South gradient in supplement use, with higher consumption in Northern European countries than in Southern European countries and higher consumption for women than for men (61). Although many countries apply mandatory folic acid fortification of flour (62), no such policies have been implemented in Western European countries and national voluntary fortification policies vary considerably throughout the European Union (63).

The discrepant results of studies on the association of folate intake and plasma folate with CRC risk may be explained by the fact that plasma folate does not necessarily reflect folate intake due to disturbances in folate bioavailability, or measurement errors that are inevitable when using food frequency questionnaires. In some studies with a short follow-up period, preclinical cancer might have been present in controls when their blood sample was taken, which could have influenced the plasma folate concentrations. However, exclusion of cases and their matched controls diagnosed within 1 to 3.6 years from blood donation did not materially alter the associations in the present study.

In addition to some small prospective studies that observed inverse associations between plasma folate and CRC risk (30, 40, 42), several small randomized placebo-controlled intervention studies on surrogate end points of CRC, as summarized by Kim (1), suggest that folic acid supplementation may have beneficial effects on DNA stability, DNA repair, and DNA methylation, which are considered important in carcinogenesis. However, these trials have a short intervention period and do not, therefore, provide strong evidence for a role of folate in colorectal carcinogenesis. Nevertheless, these observations are not in agreement with the observations of a temporal increased CRC incidence concurrent with mandatory folic acid supplementation in the United States and Canada (5), and results from the Aspirin/Folate Polyp Prevention Study suggested increased risk of advanced lesions after folic acid supplementation (6). The apparent contradictory findings from epidemiologic (2, 3, 23, 37, 39-42) and intervention studies (1, 6, 64) may reflect differences in study designs, as well as a dual role of folate whereby the timing of low and high folate status in relation to the progress of colorectal carcinogenesis is thought to be

of crucial importance. The present study is not designed to resolve this hypothesized dual effect of folate status.

The present study investigated SNPs that occurred in genes related to folate absorption, transport, and use (65). Two meta-analyses observed a CRC risk reduction of 7% to 17% in individuals with the *MTHFR 677TT* genotype compared with individuals with the *677CC* genotype in worldwide populations (20, 21). However, stratification for geographic regions revealed moderate significant risk reductions in U.S. and Asian studies, but a null-finding for European studies (20), which agrees with the present study but not with a large Norwegian study (24). In addition, previous studies have shown that the *MTHFR 677TT* genotype might be a risk factor for CRC (25, 29-31) and adenoma (32, 33) at low folate status and protective at high folate status, which is confirmed to some extend by results from the present study. The variant genotype of the *MTR 2756A→G* polymorphism is rare (17) and less studied, but there is some evidence that the homozygote genotype is also associated with decreased CRC risk (23, 24), although this was not confirmed in the present and other studies (23, 25). Results from studies on *MTRR 66A→G* (18, 19, 25), *MTHFD1 1958G→A* (26, 27), and the *SLC19A1 80G→A* (9, 28) polymorphisms are sparse and inconclusive. We did not observe an association of *MTRR 66A→G* and *MTHFD1 1958G→A* SNPs with CRC risk. The positive association between the *SLC19A1 80G→A* polymorphism is a novel finding that requires confirmation in future studies.

In line with alcohol as a risk factor for CRC (66) and an antagonistic effect of alcohol on folate status (67), many studies on folate intake (66, 68) and some on plasma folate (30, 40, 41) have shown an increased CRC risk in individuals with low folate status and high alcohol consumption compared with those with high folate status and low alcohol consumption (30, 40, 41). This was not observed in the present and another study (39). Small subgroups, ethnicity, and different measures and ranges of folate status and alcohol intake may explain contrasting findings. It has been suggested that the alcohol effect is mediated by impaired one-carbon metabolism because previous studies showed an interaction between plasma folate status, *MTHFR* polymorphisms, and alcohol intake for CRC risk (22, 34-36). Furthermore, individuals with the *MTHFR 677TT* genotype showed a decreased risk when combined with high folate and low alcohol intake, and increased risk in individuals with low folate and high alcohol intake (25, 30, 31). Although the present study observed a borderline significant increased CRC risk in individuals with the *CT* and *TT* genotype in individuals who consumed ≥30 g alcohol/day compared with those consuming less alcohol, stratification by folate status attenuated these results. However, a large sample size is critical to investigate gene-environment interactions with moderate RRs and sample size requirement may even exceed the number of cases and controls included in the present study

In conclusion, the overall results of the current study did not reveal significant associations between the *MTHFR 677C→T* polymorphism and plasma folate with CRC risk, which is in contrast to results from meta-analyses (2, 3, 20, 21). However, a much longer follow-up period and repeated measures of plasma folate are required to elucidate potential time-dependent associations between folate and CRC risk. In addition, larger sample sizes are needed to adequately investigate gene-environment interactions.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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