Plasma Methionine, Choline, Betaine, and Dimethylglycine, in relation to Colorectal Cancer Risk in the European Prospective Investigation into Cancer and Nutrition (EPIC)

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ABSTRACT

Background: Disturbances in one carbon metabolism may contribute to carcinogenesis by affecting methylation and synthesis of DNA. Choline and its oxidation product betaine are involved in this metabolism and can serve as alternative methyl group donors when folate status is low.

Methods: We conducted a case-control study nested within the European Prospective Investigation into Cancer and Nutrition (EPIC), to investigate plasma concentrations of the methyl donors methionine, choline, betaine, and dimethylglycine (DMG) in relation to colorectal cancer (CRC) risk. Our study included 1,367 incident CRC cases (965 colon; 402 rectum) and 2,323 controls matched by gender, age group, and study center. Multivariate-adjusted odds ratios (OR) and 95% confidence intervals (CI) for CRC risk were estimated by conditional logistic regression comparing the fifth to the first quintile of plasma concentrations.

Results: Overall, methionine (OR: 0.79, 95%CI: 0.63-0.99, *P*-trend=0.05), choline (OR: 0.77, 95%CI: 0.60-0.99, *P*-trend=0.07), and betaine (OR: 0.85, 95%CI: 0.66-1.09, *P*-trend=0.06) concentrations were inversely associated with CRC risk of borderline significance. Among women, but not men, high choline concentration was associated with decreased CRC risk (OR: 0.62, 95%CI: 0.43-0.88, *P*-trend=0.01). In participants with folate concentration below 11.3 nmol/L, high betaine concentration was associated with reduced CRC risk (OR: 0.71, 95%CI: 0.50-1.00, *P*-trend=0.02), which was not observed for those having a higher folate status. Plasma DMG was not associated with CRC risk.

Conclusions: Individuals with high plasma concentrations of methionine, choline, and betaine may be at reduced risk of colorectal cancer.

INTRODUCTION

Colorectal cancer (CRC) is third most common cancer world-wide [1] and the second most commonly diagnosed cancer in Europe with 447,000 estimated new cases in the year 2012 [2]. In 2011, the World Cancer Research Fund (WCRF) concluded that there is convincing evidence of physical activity and dietary fibre intake to protect against colorectal cancer, whereas red meat, processed meat, intake of ethanol from alcoholic drinks, as well as body fatness and abdominal fatness, are associated with increased colorectal cancer risk [3].

One-carbon metabolism includes donors of methyl groups for DNA methylation and DNA synthesis, both of which are involved in carcinogenesis. The B-vitamin folate as well as related methyl group donors involved in one-carbon metabolism, are hypothesized to potentially affect DNA methylation status and thereby have the potential to prevent carcinogenesis [4]. Choline and its oxidation product betaine can not only serve as alternative methyl group donors for the remethylation of homocysteine to methionine during folate deficiency, but, unlike folate, also provide additional methyl-groups for the synthesis of formate in the mitochondria, which subsequently can be used for one-carbon transmethylation reactions in the cytosol, including those involved in the production of purines and thymidalate. Conversely, during choline deprivation, methyl groups from the methyl carrier folate are used [5-7].

Only a few studies with inconclusive results have reported on choline and betaine status in relation to CRC. One cross-sectional study suggested an inverse association between plasma concentrations of methionine and betaine and high-risk colorectal adenomas [8]. Dietary methionine intake was associated with decreased proximal CRC risk among men [9]. Another study supported a positive association between choline intake and colorectal adenoma in women [10]. However, null associations for dietary choline have been reported [11], and the majority of prospective cohort studies and population-based case-control studies on dietary methionine and CRC do not suggest an association [9, 12-19]. The

association between plasma folate and CRC risk has been inconsistent in several studies [20-25]. This relation has previously been investigated in the European Prospective Investigation into Cancer and Nutrition (EPIC), but no association of plasma folate with CRC risk was observed [26].

We conducted a large population-based case-control study nested within the European Prospective Investigation into Cancer and Nutrition (EPIC) cohort. We investigated associations between plasma concentrations of methionine, choline, betaine, and dimethylglycine (DMG; the product of the enzymatic conversion from betaine), in relation to overall CRC risk, and risk of colon and rectum cancer separately. In view of the hypothesis that these alternative methyl group donors become particularly important when folate status is low, we evaluated whether the associations were different among individuals with high or low folate status.

SUBJECTS AND METHODS

Study population

The methods and design of the EPIC study have previously been described [27]. EPIC is a large-scale population based prospective cohort study designed to investigate the relation between diet, nutritional and metabolic characteristics, various lifestyle factors and the risk of cancer [27]. In brief, the EPIC cohort is based on participants recruited from 23 collaborating centers in 10 European countries (Denmark, France, Germany, Greece, Italy, the Netherlands, Norway, Spain, Sweden and the United Kingdom).

Data were collected between 1992 and 1998 and included baseline dietary questionnaires, standardized questionnaires on various lifestyle factors and personal history, and anthropometric data according to a standard protocol. Anthropometric data were measured on almost all subjects, except in the French and Oxford cohorts where these data were measured only for a restricted number of participants, but additional self-reports were obtained from all individuals. In Norway only self-reports were available [28].

Collection of blood samples

Blood samples were collected from 80% of the participants at baseline, and at least 30 mL was drawn from each of the participants, either non-fasting or fasting. The samples were then transported to local laboratories for processing and aliquoting. During transport they were stored at temperatures from 5°C to 10°C and protected from light exposure [29, 30]. Exceptions from this procedure were the EPIC-Oxford and EPIC-Norway centers, where whole blood samples were transported to a central laboratory via mail. The whole blood samples were protected from light, but were exposed to ambient temperatures for up to 48 hours. As some B-vitamins and related metabolites are unstable under such conditions [31],

all EPIC-Oxford (55 cases, 107 controls) and EPIC-Norway (5 cases, 9 controls) samples were excluded from the present analyses [29].

Storage of blood samples

Separation of blood into fractions of 0.5 mL (serum, plasma, erythrocytes and buffy coat for DNA extraction) were done in all countries except Denmark and Sweden (constituting 38.4% of all participants) because the collection in these countries was initiated many years before the common EPIC protocol [32]. The fractions were placed into heat sealed straws and stored in liquid nitrogen at a temperature of - 196°C. Half of the samples were stored at local study centers and the other half at the EPIC biorepository at the International Agency for Research on Cancer (IARC; Lyon, France). Storage conditions in Denmark and Sweden are described elsewhere [28, 29].

Follow up for cancer incidence

Follow up in EPIC is mainly based on national population-based cancer registries (Denmark, Italy, Netherlands, Norway, Spain, Sweden and the United Kingdom). Other sources of CRC diagnosis were health insurance records, pathology registries or through self-reporting (France, Germany and Greece). Self-reported cancer cases were verified through pathology reports and physicians, available for at least 95% of the cases. In our study, time between inclusion and diagnosis of CRC varied from 3 days to 11.5 years (mean 3.7 years).

Study design and selection of study subjects

Case definition and selection

We conducted a nested case-control study within the EPIC cohort. Colon cancer was defined as the ICD-10 (The 10th Revision of the International Statistical Classification of Diseases, Injury and Causes of Death) diagnosis C18.0-C18.7, as well as tumors that were overlapping

or unspecified; C18.8 and C18.9. Overlapping tumors are defined, as malignant neoplasm of overlapping sites of colon, were the primary site of the tumor is impossible to define. Cancers of the rectum were defined as the diagnosis C19 or C20. CRC is defined as a combination of the colon and rectal cancer cases. The present study included 1,367 CRC cases (colon *n* = 965; rectum *n* = 402).

Control selection

For each identified cancer case, 1 to 2 controls were randomly selected from all cohort members with available blood samples who were alive and free of cancer (except non-melanoma skin cancer) at the time of diagnosis of the index case. The controls were matched by gender, age group (±2.5 years), and study center, except for the Danish cases, which were *post hoc* matched [29].

Laboratory measurements

Plasma methionine, choline, betaine, and DMG, were determined by a method based on normal-phase liquid chromatography and tandem mass spectrometry [33]. 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, [34]). In addition, SNPs of genes related to one-carbon metabolism were determined by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry [35]. These included *methylene-tetrahydrofolate reductase (MTHFR) 677C→T*, *MTHFR 1298A→C* and *betaine-homocysteine methyltransferase (BHMT) 742G→A*. All laboratory analyses were performed at BEVITAL AS, Bergen, Norway.

Statistical methods

Because distributions of plasma concentrations were right-skewed, differences between cases and controls of the measured one-carbon biomarkers were assessed non-parametrically by Kruskal Wallis test. Categorical variables were evaluated by χ^2 test and the remaining continuous variables (age (y), body mass index (BMI, kg/m²), total energy intake (kJ) and total meat intake (g/day)) by ANOVA.

Multivariate-adjusted odds ratios (OR) and 95% confidence intervals (CI) were estimated by conditional logistic regression for CRC risk in relation to quintiles of methionine, choline, betaine, and DMG concentrations, taking the lowest quintiles as reference categories. Quintile cut-off values were based on the distributions among controls. Tests for linear trend over quintiles were performed by fitting the ordinal exposure variables as continuous variables. These analyses were conducted for overall CRC and for colon and rectum cancer separately. To decrease the possibility of reverse causality we also conducted an analysis where we excluded the cases (n=163; 11.2%) diagnosed within the first year of follow up. We adjusted for potential confounders; Body Mass Index (BMI; kg/m^2), smoking status (never, former and current), physical activity (inactive, moderately inactive, moderately active, and active), alcohol consumption (abstainers, >0 –<30g/day and ≥30g/day), and dietary intake of fibre, red meat, processed meat, and total energy.

We also estimated ORs in analyses stratified for median time from blood donation to cancer diagnosis (below and above median follow up time of 3.6 years), sex, and age at recruitment (<60 years versus ≥ 60 years). In addition, unconditional logistic regression analyses were conducted, in which the matching criteria were included as covariates, in order to estimate interactions with sex and age category, and to estimate CRC risk for subgroups of median folate concentration (above and below median) among controls (<11.3 nmol/L and ≥11.3 nmol/L).

It could be expected that serum concentrations are differentially associated with cancer risk across the genotypes due to the influence on enzymatic activity. Therefore, in addition to previously reported associations of one-carbon genetic variants with CRC risk [26, 36], we analysed the associations between *BHMT* (742G→A) genotypes and CRC risk, and between serum concentrations with CRC risk across *BHMT* (742G→A) as well as *MTHFR* (677C→T and 1298A→C) genotypes with CRC risk.

All statistical analyses were conducted using STATA, version 11.

RESULTS

Characteristics of the study population

Altogether, 1,367 cases and 2,323 matched controls were included in the analyses. Mean age at blood donation was 59.0 years and mean age at diagnosis was 62.7 years. Baseline characteristics of cases and controls are summarized in Table 1. BMI, current smoking, alcohol consumption, and meat intake were significantly higher among cases than controls, whereas fibre intake and the level of physical activity were lower among cases. Plasma concentrations of methionine, choline, betaine, and folate were lower among cases than among controls. However, genotype frequencies did not differ between cases and controls.

All four methyl group donors, methionine, choline, betaine, and DMG, showed higher median concentrations in men compared to women among controls (P<0.01). Furthermore, median concentrations of methionine were lower, and those of betaine, choline and DMG were higher in controls over the age of 60 (P<0.01) (data not shown).

Associations between plasma concentrations of methyl group donors and CRC risk

Analyses adjusted for BMI, smoking status, physical activity, alcohol consumption, and intake of fibre, red meat, processed meat, and energy, revealed that high methionine (OR: 0.79, 95%CI: 0.63-0.99, P-trend=0.05), high choline (OR: 0.77, 95%CI: 0.60-0.99, P-trend=0.07), and high betaine (OR: 0.85, 95%CI: 0.66-1.09, P-trend=0.06) concentrations were associated with lower CRC risk of borderline significance (Table 2). Exclusion of the cases diagnosed within the first year of follow-up modestly attenuated the associations of methionine and betaine, whereas choline remained associated with reduced CRC risk (P-trend=0.05) (data not shown).

Choline was inversely associated with colon cancer risk, while we did not observe significant associations between the remaining plasma concentrations and risk of colon cancer or rectum cancer separately (Table 3). Plasma methionine was associated with

reduced CRC risk exclusively in those cases who were diagnosed with CRC within 3.6 years after blood collection, and not in those diagnosed at a later time. Choline, betaine, and DMG were not significantly associated with CRC risk in either of these groups (data not shown).

Subgroup analysis

Subgroup analysis (Table 4) revealed an inverse association between choline and CRC risk in women (OR: 0.62, 95%CI: 0.43-0.88, *P*-trend=0.01), but not in men (OR: 1.03, 95%CI: 0.71-1.50, *P*-trend=0.87). The inverse associations of methionine and betaine were observed among individuals <60 years of age, but not among those ≥60 years. However, the tests for interaction, based on unconditional logistic regression models, were not significant for these associations. Further, an increased CRC risk for higher levels of DMG in the age group <60 years was also present, whereas among those ≥60 years no association was observed.

In the analyses stratified by folate concentration, plasma betaine was inversely associated with CRC risk in the group with folate concentration below the median of 11.3 nmol/L, but not among those with folate concentration above the median (Table 5). A similar, though borderline significant inverse association was observed for high choline concentrations among individuals with lower folate status. Neither methionine or choline, nor DMG were differentially associated with CRC risk across the categories of folate status.

The polymorphisms and their association with CRC risk

No associations between the genotypes and CRC risk or of serum concentrations across genotypes were observed (data not shown).

DISCUSSION

In this large-scale population-based European nested case control study, we investigated plasma concentrations of methionine, choline, betaine, and DMG in relation to CRC risk. Overall, plasma methionine, choline, and betaine status were modestly inversely associated with CRC risk. Plasma choline was associated with reduced CRC risk among women, but not among men. The inverse assocations of methionine and betaine were confined to individuals **<**60 years at recruitment. Finally, we observed that higher betaine concentration was associated with a reduced CRC risk among individuals with folate concentration below the median of 11.3 nmol/L, but not among those with higher folate status.

This study is the largest prospective study on plasma methionine and the first on plasma DMG, choline and betaine concentrations in relation to CRC risk to date. The large sample size and extensive data collection on modifiable risk factors for CRC allowed subgroup analyses. Strength of this study is the nested case-control design, where blood samples were taken prior to cancer diagnosis. The mean time between inclusion and cancer diagnosis was relatively short (median 3.6 years), which may have led to reverse causality if undiagnosed (pre-clinical) cancer has affected exposure status. Although possibly resulting from reduced power to demonstrate an underlying true association, the inverse associations of methionine and betaine with CRC risk tended to attenuate after exclusion of cases diagnosed within the first year of follow up. Nevertheless, the possibility cannot be excluded that reverse causation has biased the estimated associations to some extent. Another advantage of our study was that the main exposure variables were measured in blood rather than obtained from dietary questionnaires [9-11, 17], which rely on subjects´ memory or ability in recording dietary intake [27].

In the EPIC study, extensive lifestyle factors and other relevant information have been collected for each cohort member, which allowed us to address potential confounders and assessment of potential effect modification. Potential confounders such as BMI,

smoking status, physical activity, alcohol consumption, and dietary intake of red meat, processed meat, fibre, and energy were adjusted for, but the results from this adjusted analysis were essentially the same as from the crude analyses.

The blood samples were collected according to a standardized protocol [27] at each study center and all the biochemical analysis were conducted at one laboratory, thereby eliminating variability in sampling procedures and assay methods. However, a possible drawback of a single blood sample is that it may not have captured long term plasma concentrations of each individual, and may therefore not represent lifetime exposure. Variations in plasma concentrations over time may occur due to life-style changes and diet variation. Further, the measured blood levels may not directly reflect the dietary intake or body stores of nutrients.

Inverse associations between plasma levels or dietary intake of methionine and betaine with risk of colorectal adenomas (CRA) or CRC, were previously reported in a cross-sectional analysis of the Norwegian Colorectal Cancer Prevention (NORCCAP) screening study [8] and the prospective Netherlands Cohort Study on diet and Cancer (NLCS) [9]. Conversely, a positive association between choline intake and CRA risk was previously observed in the Nurses Health Study [10]. However, one would expect high choline to be protective against neoplasia, as choline deficiency has the potential to induce DNA damage by uracil misincorporation and to alter DNA methylation patterns [37]. For instance, in an intervention study, subjects fed a choline deficient diet for 10 days had more subsequent DNA damage in lymphocytes compared to when taking the recommended daily intake of choline [38]. Furthermore, a recent population-based case-control study in China reported that, especially among former and current smokers, dietary choline and betaine intake was associated with reduced lung cancer risk [39].

Although not associated with overall CRC risk, we observed that high plasma choline may protect against CRC in women. This may be partly due to differences in the metabolism

of choline between men and women. Choline is not only obtained from the diet, but is also synthesized endogenously from phosphatidylethanolamine by the enzyme phosphatidylethanolamine-N-methyltransferase (PEMT), the activity of which is increased by estrogen [40]. This may explain why postmenopausal women tend to be less resistant against choline deficiency compared to premenopausal women [41]. Moreover, results from the Long Island Breast Cancer Study suggest that high dietary betaine and choline intakes were associated with decreased all-cause and breast cancer-specific mortality [42], and that rare variants of the *PEMT* rs12325817 SNP, which is associated with decreased choline biosynthesis, was associated with a 30% increased risk of breast cancer [42]. In our study, the majority of the women were postmenopausal, as mean age at inclusion in the EPIC cohort was 58.8 years and 58.6 years in cases and controls, respectively. We also observed that mean choline concentration was lower among women than among men in our study (data not shown), and women may therefore have benefited more from a high choline status.

When stratifying according to age at recruitment, the inverse associations of methionine and betaine were observed exclusively among participants **<**60 years at baseline whereas DMG was associated with increased CRC risk in this age group. These differences could not be explained by an underlying difference between the two age categories, with respect to the time between cohort inclusion and cancer diagnosis or large differences of the main exposure variables (data not shown). However, given that there was no statistically significant interaction observed for methionine and betaine, it may be questioned whether there has been a true age effect. Nevertheless, although speculative, individuals ≥60 years at baseline may have benefited less from higher plasma concentrations of the studied methyl group donors possibly partly because they had undiagnosed colorectal adenomas more often compared to the younger age group. In this respect, a large screening study of individuals 50-64 years of the general Norwegian population revealed that 17.1% of the

screened participants had at least one distal CRA [8], and this proportion is likely to increase with increasing age.

Although in the EPIC cohort plasma folate and vitamin B12 were not associated with CRC risk [26, 29], inverse associations were observed of plasma concentrations of vitamins B2 and B6 with CRC risk [29]. In addition, dietary intake of vitamins B2 and B6 were associated with reduced CRC risk in the Women's Health Initiative Observational Study [43]. B-vitamins are components of a network with major effects on the transfer of one-carbon units, as B-vitamins were more strongly related to plasma tHcy when concentrations of other B vitamins were low [44]. Similarly, choline and betaine may serve as alternative methyl group donors when folate status is low [7]. Our observation of a possible protective role of choline and betaine among individuals with lower folate status may support this possibility.

Finally, the number of CRC cases identified in the EPIC cohort so far may have been insufficient to demonstrate an association, if any, of *BHMT* genotypes in the current study, and of other related one-carbon genetic variants with CRC risk [26, 36].

This study suggests that methionine, choline, and betaine may play a protective role in colorectal carcinogenesis and that these methyl group donors should be investigated further with respect to CRC risk. Repeated blood samples in order to more accurately reflect lifetime exposure could be an important focus for future research. A longer follow-up period is also recommendable to exclude the potential problem of reverse causality.

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Table 1. Baseline characteristics of colorectal cancer cases and matched controls in the European Prospective Investigation into Cancer and Nutrition (EPIC)			
	Cases	**Controls**	***P*-difference**
No. of individuals	1,367	2,323	
Sex, female, n(%)	700 (51.2)	1,213 (52.2)	0.55*
Age at recruitment (years; mean (SD))	58.9 (7.1)	58.7 (7.5)	0.38§
Body Mass Index (kg/m^2; mean(SD))	26.8 (4.3)	26.4 (3.9)	0.005§
Smoking status, n (%)			0.05*
Never	561 (41.0)	1,025 (44.1)	
Former	451 (33.0)	775 (33.4)	
Current	346 (25.3)	510 (22.0)	
Unknown	9 (0.7)	13 (0.6)	
Physical activity, n (%)			0.05*
Active	123 (9.0)	242 (10.4)	
Moderately active	574 (42.0)	1,019 (43.9)	
Moderately inactive	423 (30.9)	697 (30.0)	
Inactive	219 (16.0)	304 (13.1)	
Unknown	28 (2.1)	61 (2.6)	
Alcohol consumption, n(%)			0.001*
Abstainers	172 (12.6)	347 (14.9)	
>0g/day and <30 g/day	908 (66.5)	1,597 (68.8)	
≥ 30 g/day	285 (20.9)	379 (16.3)	
Dietary intakes (mean (SD))			
Energy (kcal/day)	2176 (710)	2136 (643)	0.08§
Total meat (g/day)	118.3 (69.8)	109.7 (56.4)	<0.001§
Red meat (g/day)	53.9 (39.5)	47.5 (35.5)	<0.001§
Processed meat (g/day)	38.2 (47.8)	35.3 (32.2)	0.03§
Fibre, mean (g/day)	22.2 (8.2)	22.9 (7.9)	0.003§
Plasma concentrations (median (5th-95th percentile))			
Methionine (µmol/L)	23.7 (16.6-37.0)	24.2 (17.0-37.4)	0.009¶
Choline (µmol/L)	9.3 (6.2-14.2)	9.4 (6.3-14.4)	0.02¶
Betaine (µmol/L)	31.5 (18.4-52.6)	33.0 (18.5-53.8)	0.005¶
Dimethylglycine (µmol/L)	3.6 (2.4-5.9)	3.6 (2.3-6.1)	0.86¶
Folate (nmol/L)	10.9 (5.1-32.1)	11.3 (4.9-34.0)	0.03¶
MTHFR 677C→T, %			0.86*
CC	41.8	42.7	
CT	46.1	45.8	
TT	12.1	11.6	
MTHFR 1298A→C, %			0.96*
AA	45.9	45.8	
AC	43.0	43.4	
CC	11.1	10.8	
BHMT 742G→A, %			0.45*
GG	50.8	48.6	
GA	41.0	42.5	
AA	8.2	8.8	
* Chi2-test, unknown category not included § ANOVA ¶ Kruskal Wallis test			

Table 2. Conditional logistic regression analyses with corresponding odds (OR) ratios and 95% confidence intervals for colorectal cancer, according to quintiles of methionine, betaine, choline and dimethylglycine concentrations

			OR **	
Plasma concentration	**Quintiles (range) ***	**cases/controls**	**Crude analyses**	**Adjusted analyses *****
Methionine	1 (< 20.2)	335/465	Reference	Reference
(µmol/L)	2 (20.2 –< 22.9)	264/465	0.79 (0.63-0.97)	0.79 (0.63-0.98)
	3 (22.9 –< 25.6)	260/464	0.81 (0.65-1.00)	0.83 (0.66-1.03)
	4 (25.6 –< 29.6)	249/465	0.77 (0.61-0.96)	0.77 (0.61-0.97)
	5 (≥ 29.6)	259/464	0.78 (0.62-0.98)	0.79 (0.63-0.99)
			p-trend=0.04	p-trend=0.05
Choline	1 (< 7.7)	317/460	Reference	Reference
(µmol/L)	2 (7.7 –< 8.9)	268/472	0.84 (0.68-1.04)	0.83 (0.66-1.03)
	3 (8.9 –< 10.1)	276/462	0.92 (0.73-1.15)	0.91 (0.73-1.15)
	4 (10.1 –< 11.7)	254/457	0.86 (0.68-1.09)	0.82 (0.64-1.04)
	5 (≥ 11.7)	250/472	0.84 (0.66-1.07)	0.77 (0.60-0.99)
			p-trend=0.26	p-trend=0.07
Betaine	1 (< 24.8)	297/461	Reference	Reference
(µmol/L)	2 (24.8 –< 30.4)	304/469	1.01 (0.81-1.25)	1.03 (0.82-1.28)
	3 (30.4 –< 35.3)	282/459	0.94 (0.76-1.17)	0.98 (0.79-1.22)
	4 (35.3 –< 42.1)	246/468	0.79 (0.63-1.00)	0.84 (0.66-1.06)
	5 (≥ 42.1)	236/466	0.78 (0.62-1.00)	0.85 (0.66-1.09)
			p-trend=0.01	p-trend=0.06
DMG	1 (< 2.9)	271/464	Reference	Reference
(µmol/L)	2 (2.9 –< 3.3)	267/452	1.09 (0.87-1.36)	1.05 (0.84-1.31)
	3 (3.3 –< 3.9)	288/473	1.15 (0.92-1.44)	1.12 (0.89-1.41)
	4 (3.9 –< 4.6)	277/467	1.21 (0.96-1.51)	1.13 (0.89-1.42)
	5 (≥ 4.6)	262/467	1.18 (0.93-1.50)	1.10 (0.86-1.40)
			p-trend=0.12	p-trend=0.33

* Quintiles are based on the distribution of serum concentrations among controls
** Case-control matching factors included sex, age, and study center
*** Adjusted for BMI, smoking status, physical activity and alcohol consumption

Table 3. Conditional logistic regression analyses with corresponding odds (OR) ratios and 95% confidence intervals for colon and rectum cancer, according to quintiles of methionine, betaine, choline and dimethylglycine concentrations

		Colon cancer		Rectum cancer	
Plasma concentration	**Quintiles (range) ***	**cases/ controls**	**OR ****	**cases/ controls**	**OR ****
Methionine	1 (< 20.2)	215/294	Reference	97/132	Reference
(µmol/L)	2 (20.2 –< 22.9)	159/308	0.73 (0.56-0.94)	81/121	0.97 (0.64-1.47)
	3 (22.9 –< 25.6)	172/305	0.85 (0.66-1.10)	68/121	0.77 (0.50-1.17)
	4 (25.6 –< 29.6)	149/289	0.76 (0.58-1.00)	75/140	0.77 (0.50-1.19)
	5 (≥ 29.6)	159/278	0.80 (0.61-1.06)	82/146	0.79 (0.52-1.19)
			p-trend= 0.18		p-trend= 0.16
Choline	1 (< 7.7)	207/281	Reference	92/140	Reference
(µmol/L)	2 (7.7 –< 8.9)	158/298	0.79 (0.61-1.03)	82/139	0.95 (0.64-1.42)
	3 (8.9 –< 10.1)	177/297	0.85 (0.65-1.11)	82/126	1.11 (0.73-1.71)
	4 (10.1 –< 11.7)	155/299	0.75 (0.56-0.99)	79/124	1.04 (0.66-1.64)
	5 (≥ 11.7)	156/299	0.74 (0.55-0.99)	67/131	0.87 (0.54-1.40)
			p-trend= 0.05		p-trend= 0.76
Betaine	1 (< 24.8)	206/291	Reference	73/118	Reference
(µmol/L)	2 (24.8 –< 30.4)	184/299	0.97 (0.75-1.26)	100/144	1.22 (0.80-1.86)
	3 (30.4 –< 35.3)	163/305	0.88 (0.68-1.14)	92/124	1.30 (0.85-1.98)
	4 (35.3 –< 42.1)	152/285	0.86 (0.66-1.14)	69/140	0.81 (0.51-1.28)
	5 (≥ 42.1)	148/294	0.84 (0.63-1.12)	68/134	0.89 (0.55-1.43)
			p-trend= 0.16		p-trend=0.18
DMG	1 (< 2.9)	182/292	Reference	74/128	Reference
(µmol/L)	2 (2.9 –< 3.3)	170/306	1.01 (0.77-1.32)	76/108	1.14 (0.75-1.73)
	3 (3.3 –< 3.9)	176/286	1.16 (0.89-1.51)	83/152	1.01 (0.66-1.56)
	4 (3.9 –< 4.6)	178/300	1.09 (0.83-1.42)	77/126	1.23 (0.78-1.93)
	5 (≥ 4.6)	147/290	1.02 (0.76-1.37)	92/146	1.28 (0.84-1.99)
			p-trend= 0.67		p-trend= 0.25

* Quintiles are based on the distribution of serum concentrations among controls

** Case-control matching factors were sex, age, and study center. Adjusted for BMI, smoking status, physical activity, alcohol consumption, and intakes of energy, fibre, red meat, and processed meat

Table 4. Conditional logistic regression analyses with corresponding odds (OR) ratios and 95% confidence intervals for colorectal cancer, according to quintiles of methionine, betaine, choline and dimethylglycine concentrations, by sex and age

		Sex				Age at recruitment			
		Men		Women		<60 years		≥60 years	
Plasma concentration	**Quintiles (range) ***	**cases/ controls**	**OR ****	**cases/ controls**	**OR ****	**cases/ controls**	**OR ****	**cases/ controls**	**OR ****
Methionine	1 (< 20.2)	130/170	Reference	205/295	Reference	165/230	Reference	170/235	Reference
(µmol/L)	2 (20.2 –< 22.9)	106/190	0.73 (0.52-1.03)	158/275	0.84 (0.63-1.11)	139/257	0.75 (0.55-1.03)	125/208	0.86 (0.61-1.19)
	3 (22.9 –< 25.6)	127/210	0.86 (0.61-1.21)	133/254	0.80 (0.59-1.07)	146/268	0.76 (0.56-1.04)	114/196	0.87 (0.62-1.22)
	4 (25.6 –< 29.6)	145/260	0.78 (0.56-1.09)	104/205	0.75 (0.54-1.04)	148/255	0.80 (0.58-1.11)	101/201	0.68 (0.48-0.98)
	5 (≥ 29.6)	159/280	0.75 (0.53-1.05)	100/184	0.85 (0.62-1.18)	131/267	0.64 (0.46-0.90)	128/197	1.03 (0.74-1.45)
			P-trend=0.17		*P*-trend=0.17		*P*-trend=0.03		*P*-trend=0.69
		P-interaction=0.91 ***				*P*-interaction=0.32 ***			
Choline	1 (< 7.7)	110/185	Reference	207/275	Reference	200/312	Reference	117/148	Reference
(µmol/L)	2 (7.7 –< 8.9)	131/194	1.18 (0.83-1.67)	137/278	0.65 (0.49-0.87)	152/269	0.85 (0.64-1.14)	116/203	0.76 (0.52-1.10)
	3 (8.9 –< 10.1)	140/228	1.15 (0.81-1.65)	136/234	0.77 (0.57-1.04)	150/255	0.89 (0.66-1.22)	126/207	0.92 (0.64-1.33)
	4 (10.1 –< 11.7)	138/241	1.06 (0.74-1.52)	116/216	0.67 (0.48-0.94)	116/239	0.73 (0.52-1.02)	138/218	0.93 (0.64-1.34)
	5 (≥ 11.7)	148/262	1.03 (0.71-1.50)	102/210	0.62 (0.43-0.88)	109/202	0.77 (0.54-1.10)	141/270	0.75 (0.52-1.10)
			P-trend=0.87		*P*-trend=0.01		*P*-trend=0.10		*P*-trend=0.40
		P-interaction=0.11 ***				*P*-interaction=0.58 ***			
Betaine	1 (< 24.8)	80/105	Reference	217/356	Reference	174/297	Reference	123/164	Reference
(µmol/L)	2 (24.8 –< 30.4)	125/180	0.89 (0.60-1.32)	179/289	1.09 (0.83-1.42)	171/249	1.16 (0.86-1.56)	133/220	0.92 (0.65-1.31)
	3 (30.4 –< 35.3)	148/229	0.90 (0.62-1.30)	134/230	1.01 (0.76-1.34)	155/247	1.01 (0.75-1.37)	127/212	0.92 (0.65-1.30)
	4 (35.3 –< 42.1)	146/279	0.72 (0.49-1.06)	100/189	0.91 (0.66-1.26)	110/241	0.72 (0.51-1.01)	136/227	0.94 (0.66-1.34)
	5 (≥ 42.1)	168/317	0.79 (0.54-1.15)	68/149	0.81 (0.56-1.19)	117/243	0.82 (0.58-1.17)	119/223	0.85 (0.58-1.25)
			P-trend=0.11		*P*-trend=0.25		*P*-trend=0.03		*P*-trend=0.52
		P-interaction=0.91 ***				*P*-interaction=0.28 ***			
DMG	1 (< 2.9)	90/134	Reference	181/330	Reference	152/308	Reference	119/156	Reference
(µmol/L)	2 (2.9 –< 3.3)	115/187	0.99 (0.68-1.46)	152/265	1.06 (0.80-1.41)	137/266	1.09 (0.80-1.47)	130/186	0.97 (0.67-1.41)
	3 (3.3 –< 3.9)	142/232	1.05 (0.72-1.51)	146/241	1.17 (0.87-1.57)	151/268	1.19 (0.88-1.62)	137/205	1.08 (0.74-1.56)
	4 (3.9 –< 4.6)	156/261	0.99 (0.69-1.44)	121/206	1.24 (0.91-1.69)	149/226	1.51 (1.10-2.07)	128/241	0.81 (0.56-1.19)
	5 (≥ 4.6)	164/296	0.98 (0.67-1.42)	98/171	1.23 (0.87-1.74)	138/209	1.60 (1.14-2.24)	124/258	0.78 (0.53-1.15)
			P-trend=0.89		*P*-trend=0.12		*P*-trend=0.001		*P*-trend=0.11
		P-interaction=0.93 ***				*P*-interaction=0.001 ***			

* Quintiles are based on the distribution of serum concentrations among controls

** Case-control matching factors included sex, age, and study center. Adjusted for BMI, smoking status, physical activit, alcohol consumption, and intakes of energy, fibre, red meat, and processed meat

****P*-values for interaction are based on unconditional logistic regression with case-control matching factors modeled as co-variates, adjusted for BMI, smoking status, physical activity, alcohol consumption, and intakes of energy, fibre, red meat, and processed meat

Table 5. Logistic regression analyses with corresponding odds (OR) ratios and 95% confidence intervals for colorectal cancer, according to quintiles of methionine, betaine, choline and dimethylglycine concentrations, for low (under median of 11.3 nmol/L) and high (above median) folate concentrations

		Folate <11.3 nmol/L		Folate ≥11.3 nmol/L	
Plasma concentration	**Quintiles (range) ***	**cases/ controls**	**OR ***	**cases/ controls**	**OR ***
Methionine	1 (< 20.2)	170/218	Reference	165/245	Reference
(µmol/L)	2 (20.2 –< 22.9)	139/228	0.76 (0.57-1.03)	125/235	0.81 (0.60-1.09)
	3 (22.9 –< 25.6)	132/239	0.79 (0.55-1.01)	128/225	0.87 (0.64-1.17)
	4 (25.6 –< 29.6)	133/239	0.73 (0.54-0.98)	116/225	0.79 (0.58-1.08)
	5 (≥ 29.6)	147/232	0.76 (0.56-1.02)	112/232	0.76 (0.56-1.04)
			P-trend=0.07		*P*-trend=0.11
		P-interaction=0.85			
Choline	1 (< 7.7)	195/245	Reference	122/213	Reference
(µmol/L)	2 (7.7 –< 8.9)	129/250	0.64 (0.48-0.86)	139/221	1.08 (0.79-1.48)
	3 (8.9 –< 10.1)	148/227	0.80 (0.60-1.08)	128/234	0.95 (0.69-1.31)
	4 (10.1 –< 11.7)	125/219	0.71 (0.52-0.96)	129/237	0.91 (0.66-1.26)
	5 (≥ 11.7)	124/215	0.71 (0.52-0.96)	126/257	0.81 (0.58-1.13)
			P-trend=0.07		*P*-trend=0.12
		P-interaction=0.13			
Betaine	1 (< 24.8)	182/266	Reference	115/193	Reference
(µmol/L)	2 (24.8 –< 30.4)	176/256	0.98 (0.75-1.30)	128/213	1.03 (0.74-1.42)
	3 (30.4 –< 35.3)	147/232	0.89 (0.66-1.20)	135/225	1.01 (0.73-1.40)
	4 (35.3 –< 42.1)	123/214	0.80 (0.59-1.09)	123/254	0.83 (0.60-1.16)
	5 (≥ 42.1)	93/188	0.71 (0.50-1.00)	143/277	0.99 (0.67-1.30)
			P-trend=0.02		*P*-trend=0.37
		P-interaction=0.86			
DMG	1 (< 2.9)	141/243	Reference	130/219	Reference
(µmol/L)	2 (2.9 –< 3.3)	133/213	1.06 (0.78-1.44)	134/238	0.93 (0.68-1.27)
	3 (3.3 –< 3.9)	155/241	1.10 (0.81-1.48)	133/231	0.98 (0.71-1.34)
	4 (3.9 –< 4.6)	151/208	1.25 (0.92-1.71)	126/259	0.78 (0.57-1.07)
	5 (≥ 4.6)	141/251	0.93 (0.68-1.28)	121/215	0.90 (0.65-1.25)
			P-trend=0.94		*P*-trend=0.29
		P-interaction=0.28			

* Quintiles are based on the distribution of serum concentrations among controls

**Unconditional logistic regression with case-control matching factors sex, age, and study center modeled as co-variates, adjusted for BMI, smoking status, physical activity, alcohol consumption, and intakes of energy, fibre, red meat, and processed meat