Research Article

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Low Folate Levels Are Associated with Reduced Risk of Colorectal Cancer in a Population with Low Folate Status

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Abstract

Background: A diet rich in folate is associated with a reduced colorectal cancer risk, whereas the role of circulating levels is less clear. The aim of this study was to relate prediagnostic plasma folate, vitamin B12, and homocysteine concentrations to the risk of colorectal cancer.

Methods: This was a prospective case–control study of 331 cases and 662 matched controls nested within the population-based Northern Sweden Health and Disease Study. Median follow-up time from recruitment to diagnosis was 10.8 years.

Results: Plasma folate concentrations were positively related to colorectal cancer risk; multivariate odds ratios were 1.62 [95% confidence intervals (CI), 1.08–2.42] and 1.42 (95% CI, 0.94–2.21) for the middle and highest versus lowest tertile, respectively. In subjects with follow-up <10.8 years, a statistically significant doubled risk was observed for the middle and highest versus lowest tertile, whereas findings for longer follow-up times were null. A positive risk relationship was also observed for tumor stage III–IV but not I–II. Plasma vitamin B12 concentrations were inversely associated with rectal cancer risk. Homocysteine was not significantly related to colorectal cancer risk.

Conclusions: In this population-based, nested case–control study, low plasma folate concentrations were associated with a reduced colorectal cancer risk. This protective role was mainly observed in subjects with higher tumor stage or shorter follow-up time between recruitment and diagnosis. Low circulating folate status may protect against colorectal cancer or suppress progression of preneoplastic or neoplastic lesions.

Impact: These findings may have relevance for the ongoing debate about mandatory folic acid fortification of flour. *Cancer Epidemiol Biomarkers Prev; 23(10); 2136–44. ©2014 AACR.*

Introduction

Dietary supplementation with 0.4 mg/d of folic acid, the synthetic form of the water-soluble vitamin folate (vitamin B9), is recommended before and during early pregnancy to reduce the incidence of neural tube defects (NTD; ref. 1). To ensure adequate folate status in women at gestation, mandatory folic acid fortification of flour has been introduced in more than 50 countries worldwide, whereas efforts toward fortification in European countries have been obstructed by concerns of a possible role for folic acid in cancer development including, but not limited to, colorectal cancer development (2). However, a recent meta-analysis of cancer incidence in all available large randomized trials on folic acid supplementation with doses up to 40 mg/d did not indicate a significant increase of site-specific cancer incidence during the first 5 years of treatment (3), and prospective studies of natural food folates, primarily found in liver, legumes, and certain vegetables, and folic acid consumption suggest that a high intake may reduce the risk of colorectal cancer (4–6). Results of prospective studies of circulating folate levels have been inconsistent, and have not confirmed a potential protective role of folate on colorectal cancer risk (7–17). In a previous population-based study from northern Sweden, a bell-shaped relationship between plasma folate concentrations and colorectal cancer risk was observed, with a doubling of risk for subjects in the middle versus lowest quintile (9). The results from that study may have had an impact on the decision of Swedish authorities to abstain from mandatory folic acid fortification in 2007. Many new colorectal cancer cases have been registered in the same northern Swedish cohort after the cutoff date for the previous study, and a new, larger, nonoverlapping study is, therefore, now possible.

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Different forms of folate are thought to influence colorectal cancer development through their role as essential cofactors and cosubstrates in one-carbon metabolism (18). Intracellular folates act as carriers of one-carbon moieties for methylation of proteins, DNA and RNA, histone regulation, and also for synthesis of nucleotide precursors necessary for DNA synthesis. These pathways involve the transfer of methyl groups. The methylenetetrahydrofolate reductase (MTHFR) reaction is responsible for the conversion of 5,10-methylenetetrahydrofolate (THF) into 5-methylTHF, an important cosubstrate in the remethylation cycle. A common polymorphism in this gene, *MTHFR 677*TT, impairs MTHFR activity and effectively shifts the balance toward 5,10-methyleneteTHF, which is a substrate for nucleotide synthesis. For the transfer of the methyl group from 5-methylTHF to homocysteine and the formation of methionine in the methionine synthase reaction, cobalamin (vitamin B12) is an essential cofactor. Functional folate status is, thus, also dependent on sufficient vitamin B12 supply. Methionine, on the other hand, is essential for the formation of *S*-Adenosyl methionine, which functions as a universal methyl donor (19).

The aim of this study was to investigate the role of prediagnostic plasma concentrations of folate, vitamin B12, and total homocysteine for future colorectal cancer risk in the same population-based cohort as our previous report, but including only new cases and encompassing a longer follow-up time between recruitment and diagnosis.

Materials and Methods

Study design and cohorts

This was a nested case–control study within the Northern Sweden Health and Disease Study (NSHDS). Subjects from two population-based subcohorts were included, the Västerbotten Intervention Programme (VIP; 85% of the study subjects) and the Mammography Screening Project in Västerbotten (MSP; 15% of the study subjects). These cohorts have been described in detail previously (20). In VIP, established in 1985, residents of the Västerbotten County are invited to participate in a health survey upon turning 30 (years, 1990–1996), 40, 50, and 60 years of age, comprising a medical examination and laboratory tests, donation of a fasting blood sample for future research, and completion of an extensive participant-administered lifestyle questionnaire. As of December 31, 2009, the cutoff date for case identification for the present study, the VIP included 115,147 blood samples from 85,877 individuals. Previous drop-out analyses showed only small differences between participants and nonparticipants about health and social factors, and these differences have also been observed to decrease over time (21, 22), thus indicating that selection bias is minimal. Studies of cancer incidence rates also support the population-based nature of the VIP cohort (23). The age-standardized colorectal cancer incidence per 100,000 individuals in Sweden is similar to that of the United States, the United Kingdom, Germany, and Finland, but lower than Canada and Norway (24). In the MSP, established in 1995 and concluded in 2006, women residing in the Västerbotten County, roughly 50 to 70 years of age, were invited to complete a lifestyle questionnaire and donate a blood sample for future research while attending mammography screening (54,401 blood samples from 28,802 women).

Study subjects

Colorectal cancer cases diagnosed between February 14, 2003 and March 31, 2009 were identified by linkage with the essentially complete Cancer Registry of Northern Sweden (ICD-10, 18.0 and 18.2–18.9 for colon, 19.9 and 20.9 for rectum). Four cases were excluded because of the location of the primary tumor being in the appendix, and seven cases were excluded because the location of the primary tumor could not be verified. Cases with a previous cancer diagnosis, other than nonmelanoma skin cancer, insufficient plasma sample volume available, prioritized to other studies, or with no matching control were also excluded. Five cases and their 10 controls were excluded because of suspected falsely low plasma folate concentration. Folate degradation is related to oxidation processes (25). It has been demonstrated that plasma folate concentrations are sensitive to storage conditions and degrade at room temperature and even during medium-term (up to 1 year), and long-term storage at temperatures −20°C and −25°C, respectively (25, 26). In contrast, folate and vitamin B12 seem to be stable at storage temperatures below −70°C for at least 1 year (25). Blood samples in NSHDS are stored at −80°C and data on folate stability for longer storage times at temperatures below −25°C are not available. Methionine in serum is prone to oxidation during long-term storage, which results on the formation of methionine sulfoxide (26). Of the subjects in our present study, 98% had a low ratio of methionine sulfoxide to methionine (less than 10% of total plasma concentrations of methionine), whereas four cases and their controls had markedly increased plasma concentrations of methionine sulfoxide (more than a third of the plasma concentration of methionine, indicating improper sample storage), and one case had a physiologically improbable plasma concentration of methionine (3.59 μmol/L; normal range, 18–33 μmol/L) and had missing data on plasma methionine sulfoxide. Tumor characteristics (stage and site) were extracted from pathology reports. There was no overlap between the cases in our previous report and those in the current study (9).

For each case, two controls were selected, matched by age (±1 year), sex, cohort, and year of blood sampling and data collection. Controls were all alive and free from diagnosed cancer other than nonmelanoma skin cancer at the time of diagnosis of their index cases. The study protocol and data handling procedures were approved by

the Research Ethics Committee of Umeå University, Umeå, Sweden (dnr 03-186). All participants gave a written informed consent at the time of recruitment to the VIP or MSP cohort. All samples were anonymized.

Blood sampling and analyses

Samples in the VIP were collected in the morning, and only 12 of 825 study subjects (1.5%) had fasted <4 hours, and 146 (18%) <8 hours. In the MSP, blood samples were collected throughout the day, and 163 of 168 study subjects (97%) had fasted for less than 4 hours. The blood samples used in the present study were collected in EDTA sample tubes, separated into plasma, buffy coat, and erythrocyte fractions, aliquoted, and cryopreserved at −80°C. Samples are frozen within 1 hour of collection, either at −80°C or at −20°C for up to 1 week before transfer to a −80°C freezer at the Northern Sweden Medical Biobank for long-term storage. All biochemical analyses were performed at Bevital AS, Bergen, Norway (27). Folate and cobalamin concentrations were determined with a microbiologic method, using *Lactobacillus casei* for folate and *Lactobacillus leichmannii* for cobalamin, adapted to a microtiter plate format, and carried out by a robotic workstation. Concentrations of total homocysteine were measured with an isotope dilution gas chromatography–mass spectrometry method (28). Concentration of methionine sulfoxide was determined using a liquid chromatography-tandem mass spectrometry method. The coefficients of variation for serum folate and cobalamin are 5%, whereas the coefficients of variations for homocysteine and methionine/methionine sulfoxide are <5% (29). The investigators and laboratory staff were blinded to case and control status.

Statistical analyses

Mann–Whitney and χ^2 tests were used to compare baseline characteristics and study variables in cases and controls. Odds ratios (OR) for disease and 95% confidence intervals (CI) were calculated by conditional logistic regression. In this report, plasma metabolite tertiles were used instead of quintiles, used in our previous report, to preserve power in subgroup analyses. Results for quintiles are available in Supplementary Table S1 (9). Sex-specific tertile cutoffs were based on the variable distributions in the control subjects. Because cases and controls were matched by cohort, tertile cutoffs were not stratified for cohort. Tests for trend were performed by including tertiles (labeled with sex-specific tertile medians) as continuous variables in regression analyses. Covariates for the multivariate analysis were selected to reproduce the procedure of our previous study (9). Variables included were body mass index (BMI; <25, 25–30, and >30 kg/m^{-2}), smoking (current, not current smoker), intake of alcohol (sex-specific quartiles), and recreational and occupational physical activity (self-reported on a scale from 1 to 5). The selected covariates are all established risk factors for colorectal cancer (30). Stratified analyses were also performed for sex, age at recruitment (divided at 59 years to approximate the median but to avoid dividing the large sampling cluster at 60 years), time from recruitment to diagnosis (divided at the median of 10.8 years), tumor site (right and left colon, and rectum), tumor stage (stage I–II and III–IV), and cohort (VIP and MSP). Missing values in covariates were treated as dummy variables in multivariate analyses. Heterogeneity between results of the subgroup analysis was investigated by a χ^2-based test. Statistical tests and corresponding P values were two sided and P values <0.05 were considered statistically significant. IBM SPSS Statistics version 21.0 (IBM Corporation) was used for all statistical analysis.

Results

A total of 331 cases and 662 controls were included in the study. One control lacked data for plasma concentrations of vitamin B12. Baseline characteristics of cases and controls and tumor characteristics are shown in Table 1. Median age at recruitment was 59.7 for both cases and controls. Because of the MSP cohort, women were slightly overrepresented. Subjects in the MSP were older at recruitment [median age at recruitment, 64.6 years (25th–75th percentile, 58.5–67.8 years) compared with 59.5 years (25th–75th percentile, 50.0–60.0 years) for the VIP]. Right-sided colon cancer was also more common in the MSP (48.2% of tumors, as opposed to 33.5% in the VIP). Overall, no significant differences in baseline characteristics were observed between cases and controls, although plasma vitamin B12 concentrations were slightly higher in controls ($P = 0.055$). Median time from blood draw to diagnosis of cases was 10.8 years, with a range of 0.3 to 19.8 years.

The OR for colorectal cancer according to tertiles of plasma folate, vitamin B12, and total homocysteine concentrations are shown in Table 2. Plasma folate concentrations were positively related to colorectal cancer risk, with multivariate OR of 1.62 (95% CI, 1.08–2.42) and 1.42 (95% CI, 0.94–2.21) for the middle and highest versus lowest tertile, respectively. Plasma vitamin B12 and homocysteine concentrations were not significantly related to colorectal cancer risk.

In Table 3, heterogeneity was investigated in subgroups based on sex, age, follow-up time between baseline and colorectal cancer diagnosis, tumor site, tumor stage, and cohort. For plasma folate concentrations, the same distribution of OR for colorectal cancer was observed in most subgroups as in the full study group, with the exception of follow-up time above the median, rectal tumor localization, and stage I–II, for which associations were essentially null. Low plasma folate concentrations were associated with a reduced colorectal cancer risk in subjects with follow-up times below the median of 10.8 years, OR 2.49 (95% CI, 1.33–4.64) for the middle versus lowest tertile and OR 2.43 (95% CI, 1.27–4.65) for the highest versus lowest tertile. A positive risk relationship was observed for tumor stage III–IV but not I–II [multivariate OR for stage III–IV, middle versus lowest tertile of plasma folate concentrations, 2.51 (95% CI, 1.37–4.63)]. Because of the

Table 1. Baseline characteristics of colorectal cancer cases and their matched controls, and tumor characteristics of cases

	Cases		Controls		
	N	Median/%	*N*	Median/%	*P*[a]
Sex, men/women	135/196	40.8%/59.2%	270/392	40.8%/59.2%	0.406
Age[b], y	331	59.7 (50.1–60.1)	662	59.7 (50.1–60.1)	0.823
Follow-up time[b], y	331	10.8 (7.9–14.0)			NA
BMI[b], kg/m^2	320	25.8 (23.6–28.1)	646	25.6 (23.2–28.1)	0.610
P-folate[b], nmol/L	331	7.9 (5.5–11.6)	662	8.0 (5.1–11.7)	0.411
P-vitamin B12[b], pmol/L	331	402.9 (328.1–488.5)	661	415.7 (340.0–505.8)	0.055
P-homocysteine[b], μmol/L	331	10.1 (8.3–12.1)	662	10.0 (8.3–11.7)	0.487
Current smoking	318	65 (20.5%)	650	133 (20.5%)	0.994
Tumor site					
Right colon	119	36.0%			
Left colon	104	31.4%			
Rectum	108	32.6%			
Stage[c]					
I–II	165	52.5%			
III–IV	149	47.5%			

[a]The Mann-Whitney test for continuous variables, the χ^2 test for categorical variables.
[b]Median and 25th–75th percentile.
[c]Stage could not be determined for 17 cases.

low number of stage IV cases ($N = 82$), stage-specific results are not presented, but ORs for stage III and IV demonstrated no significant heterogeneity (data not shown). Statistically significant risk estimates for the middle versus lowest tertile were also found for right-sided colon cancer [OR, 2.53 (95% CI, 1.12–5.70)], female sex [OR, 1.83 (95% CI, 1.05–3.17)] and age at recruitment ≥59 years [OR, 2.12 (95% CI, 1.15–3.92)]. Plasma vitamin B12 concentrations were inversely associated with rectal cancer risk (P-trend = 0.019); multivariate OR 0.46 (95% CI, 0.25–0.86) for the middle versus lowest tertile and 0.46 (95% CI, 0.24–0.86) for highest versus lowest tertile (Supplementary Table S2). No statistically significant results were found in the tests of heterogeneity comparing the risk estimates for folate tertiles between subgroups with the exception of the OR for the highest versus lowest tertile in subjects with follow-up times above and below the median of 10.82 years ($P_{\text{Heterogeneity}} = 0.045$). For

Table 2. ORs for colorectal cancer by plasma folate, vitamin B12, and total homocysteine concentrations

	Lowest	Middle	Highest	P_{trend}
Folate tertiles[a]				
Cases/controls	94/221	129/221	108/220	
Unadjusted OR (95% CI)	1.00	1.55 (1.05–2.28)	1.31 (0.87–1.98)	0.503
Adjusted OR[b] (95% CI)	1.00	1.62 (1.08–2.42)	1.42 (0.94–2.21)	0.322
Vitamin B12 tertiles[c]				
Cases/controls	117/220	120/221	94/220	
Unadjusted OR (95% CI)	1.00	1.00 (0.73–1.38)	0.77 (0.55–1.11)	0.154
Adjusted OR[b] (95% CI)	1.00	0.98 (0.78–1.36)	0.74 (0.52–1.07)	0.116
Homocysteine tertiles[d]				
Cases/controls	112/221	100/221	119/220	
Unadjusted OR (95% CI)	1.00	0.90 (0.65–1.25)	1.07 (0.77–1.48)	0.654
Adjusted OR[b] (95% CI)	1.00	0.87 (0.62–1.24)	1.01 (0.72–1.43)	0.764

[a]Tertile cutoffs for plasma folate concentrations were for men, 5.8 and 9.5 nmol/L; and for women, 6.1 and 10.7 nmol/L.
[b]Adjusted for BMI, current smoking, recreational and occupational physical activity, and alcohol intake.
[c]Tertile cutoffs for plasma vitamin B12 concentrations were for men, 352 and 456 pmol/L; and for women, 370 and 480 pmol/L.
[d]Tertile cutoffs for plasma total homocysteine concentrations were for men, 9.3 and 11.4 μmol/L; and for women, 8.5 and 10.6 μmol/L.

Table 3. Multivariate ORs for colorectal cancer risk according to plasma folate after stratification by baseline and tumor characteristics

	Tertiles of plasma folate concentrations[a]			
	Lowest	Middle	Highest	P_{trend}
Sex				
Men OR (95% CI)[b]	1.00	1.30 (0.70–2.41)	1.17 (0.61–2.25)	0.621
Cases/controls	39/90	51/90	45/90	
Women OR (95% CI)[b]	1.00	1.83 (1.05–3.17)	1.56 (0.87–2.80)	0.411
Cases/controls	55/130	78/131	63/131	
Age at screening[c]				
<59 y OR (95% CI)[b]	1.00	1.45 (0.81–2.63)	1.41 (0.73–2.73)	0.429
Cases/controls	65/146	62/98	61/131	
≥59 y OR (95% CI)[b]	1.00	2.12 (1.15–3.92)	1.61 (0.88–2.95)	0.446
Cases/controls	29/75	67/123	47/89	
Follow-up[d]				
≤10.82 y OR (95% CI)[b]	1.00	2.49 (1.33–4.64)	2.43 (1.27–4.65)	0.057
Cases/controls[e]	26/82	69/117	70/131	
>10.82 y OR (95% CI)[b]	1.00	1.15 (0.65–2.03)	0.96 (0.51–1.81)	0.812
Cases/controls[e]	68/139	60/104	38/89	
Tumor site				
Right colon OR (95% CI)[b]	1.00	2.53 (1.12–5.70)	2.14 (0.94–4.88)	0.275
Cases/controls[e]	34/83	48/82	37/73	
Left colon OR (95% CI)[b]	1.00	1.56 (0.76–3.20)	1.57 (0.74–3.30)	0.328
Cases/controls[e]	28/71	38/67	38/70	
Rectum OR (95% CI)[b]	1.00	1.29 (0.65–2.59)	0.89 (0.42–1.86)	0.590
Cases/controls[e]	32/67	43/72	33/77	
Tumor stage				
I–II OR (95% CI)[b]	1.00	1.24 (0.68–2.27)	1.00 (0.50–1.99)	0.828
Cases/controls[e]	47/101	66/118	51/109	
III–IV OR (95% CI)[b]	1.00	2.51 (1.37–4.63)	1.72 (0.95–3.12)	0.387
Cases/controls[e]	42/110	61/91	50/105	
Cohort				
VIP OR (95% CI)[b]	1.00	1.53 (0.98–2.38)	1.30 (0.82–2.07)	0.574
Cases/controls	78/179	109/187	88/184	
MSP OR (95% CI)[b]	1.00	2.31 (0.81–6.54)	2.31 (0.74–7.23)	0. 277
Cases/controls	16/42	20/34	20/36	

[a]Tertile cutoffs for plasma folate concentrations were for men, 5.8 and 9.5 nmol/L; and for women, 6.1 and 10.7 nmol/L.
[b]Adjusted for BMI, current smoking, recreational and occupational physical activity, and alcohol intake.
[c]Age 59 years selected to approximate the median of 59.7 years without dividing the large sampling cluster at 59 to 61 years.
[d]Follow-up time between screening and diagnosis stratified at the median.
[e]Controls were given the corresponding value of their respective case.

vitamin B12, statistically significant heterogeneity was found between the OR for both the middle and highest versus lowest tertiles in rectal cancer and right-sided colon cancer ($P_{\text{Heterogeneity}} = 0.004$ and $P_{\text{Heterogeneity}} = 0.028$, respectively), as well as for the OR for the middle versus lowest tertile in rectal cancer and left-sided colon cancer ($P_{\text{Heterogeneity}} = 0.019$).

Discussion

The main finding of this population-based, nested case–control study, of a reduced colorectal cancer risk in subjects with the lowest circulating folate concentrations, essentially confirms the results of our previous report from the same population. In the present investigation, which had a much longer follow-up, the positive association between plasma folate concentrations and colorectal cancer risk was essentially attributable to subjects with follow-up times below the median of 10.8 years. Also in line with our previous studies, plasma vitamin B12 concentrations were inversely associated with rectal cancer risk, whereas plasma total homocysteine concentrations were not related to colorectal cancer risk (9, 31).

The associations between plasma folate concentrations and colorectal cancer risk in the present study are strikingly similar to the findings of our previous report (9), and correspond well to the findings for colon, but not rectal, cancer in a nested case–control study from the Finnish Alpha-Tocopherol, Beta-Carotene Cancer Prevention cohort (13). A reduced colorectal cancer risk in subjects with lower plasma folate concentrations was also found in a large American prospective study on plasma folate and colorectal cancer risk (16). In all these reports, the study populations had relatively low folate status and were not exposed to food folic acid fortification. Concerns over the safety of folic acid fortification have been raised by ecological studies demonstrating temporal increases in colorectal cancer after folic acid supplementation of cereals in Chile (32) and United States and Canada (33), the conclusions of which have been questioned in another recent analysis (34). A possible role for folate metabolism in modulating colorectal cancer risk has been demonstrated by folates' influence in methylation patterns in genes responsible for DNA integrity. However, the largest meta-analysis to date addressing cancer risk in randomized trials of folic acid supplementation found no statistically significant increase in cancer risk in the folic acid supplemented groups (3). Except for the prospective studies already mentioned (9, 13, 16) and one study reporting an inverse association between plasma folate concentrations and colorectal cancer risk (8), other prospective studies of circulating folate concentrations and colorectal cancer risk have tended to report null associations (7, 10–12, 15, 17). Conversely, prospective studies of dietary folate intake and colorectal cancer risk have generally indicated an inverse association (4–6). The disparity in the findings for colorectal cancer risk in relation to plasma folate concentrations, dietary intake of folate-rich food stuffs, and exposure to folic acid has been demonstrated in the Women's Health Initiative Observational Study, in which a high total intake of natural folates and folic acid was associated with a decreased risk of colorectal cancer, but in which a transient increase in colorectal cancer risk was observed in conjunction with the start of mandatory folic acid fortification of flour in the United States (35).

Folate has been proposed to have a dual role in colorectal cancer, protecting the normal mucosa but promoting growth in established preneoplastic lesions or cancer (19). Low folate status might, thereby, delay or prevent progression to clinical significance and diagnosis depending on the timing of folate exposure. In concordance with this hypothesis, a doubling of risk was observed for the middle and highest versus the lowest plasma folate tertile in the subgroup with follow-up times below the median of 10.8 years, whereas the association in the subgroup with longer follow-up times was essentially null. Given the median follow-up time of 10 years, undiagnosed colorectal cancer or preneoplastic lesions were likely to be confined to the subgroup with shorter follow-up times. A doubling of risk of stage III–IV tumors was also observed for the middle versus lowest tertile of plasma folate concentrations, whereas for stages I–II the results were essentially null. A similar association between advanced tumor stage and plasma folate was observed in a recent prospective study on colorectal cancer risk in Chinese men in which the highest tertile of plasma folate was associated with an increased risk [OR, 2.66 (95% CI 1.03–6.86); ref. 17]. These results suggest that lower folate status may hinder the progression and aggressiveness of established preneoplastic lesions and colorectal cancer.

The association between plasma folate concentrations and risk (higher risk for the middle versus lowest tertile) was also statistically significant for women, right-sided, i.e., proximal, colon cancer, and subjects older than 59 years at baseline (Table 3). These traits are all more common in the subtype of colorectal cancer with frequent CpG island methylation (CpG Island Methylator Phenotype, CIMP-high/CIMP-2). Given the central role of folate in DNA methylation, further characterization of the tumors in this cohort according to CIMP status would be interesting (36, 37). A general proximal to distal gradient along the colorectum has also recently been proposed for certain tumor characteristics, including CIMP (38, 39). However, the limited sample size of our study did not allow for subgroup analyses according to more specific tumor sites than right and left colon and rectum.

In the general population, a common polymorphism in a gene related to folate metabolism, *MTHFR* 677TT, decreases the risk of colorectal cancer (40), and it has been suggested that the protective role of low concentrations of plasma folate seen in some studies is due to a reduction in circulating 5-methylTHF, the dominating form of folate in plasma, caused by the 677TT polymorphism. The 677TT polymorphism shifts the balance of intracellular folates by decreasing the conversion of 5,10-methyleneTHF into 5-methylTHF. Circulating 5-methylTHF may, therefore, not reflect intracellular 5,10-methyleneTHF, and the association between circulating folates and cancer risk might be less predictable if 5,10-methyleneTHF has a different influence on cancer development than other folates (16, 41). The MTHFR enzyme may also be impaired by vitamin B2 deficiency (42), and further studies on the interactions between folate, vitamin B2, *MTHFR* polymorphisms, and the risk of developing colorectal cancer may be warranted. Investigation of a number of other factors such as choline and betaine (an alternative source of methyl groups), vitamin B6, and other polymorphisms than *MTHFR* could also provide further insight into the role of one-carbon metabolism in colorectal tumorigenesis.

Plasma concentrations of vitamin B12 and total homocysteine were not significantly associated with colorectal cancer risk, whereas plasma vitamin B12 concentrations were inversely associated with rectal cancer risk. These results are similar to the findings of our previous report from the same population (9, 31). Two prospective studies of circulating vitamin B12 have been published since,

none of which found a statistically significant association between vitamin B12 and colorectal cancer risk (11, 43).

The main strengths of this study were the population-based, prospective design with two individually matched controls per case and, given that colorectal cancer is a slowly developing disease, the very long follow-up time (median, 10.8 years). The latter allowed analysis of subgroups in which (pre-) neoplastic lesions at baseline were possibly present (follow-up <10.8 years) or unlikely to have been present (follow-up >10.8 years). The main weakness of our study was the single baseline blood sample from each participant, preventing assessment of changes in folate status over time. We were not able to control for a number of factors, including nonsteroidal anti-inflammatory drugs, hereditary colorectal cancer and vitamin D status. Vitamin D status, which has been demonstrated to modulate colorectal cancer risk (44), is likely lower in the NSHDS cohort compared with populations closer to the equator. However, despite the inevitable risk of residual confounding, we were able to adjust for a number of important potential confounders, all established risk factors of colorectal cancer. Because controls were matched for cohort, and the cohorts in turn consisted either of almost exclusively fasting blood samples (VIP) or nonfasting blood samples (MSP), controls can be considered to be matched for fasting status. Factors such as degradation of folate during storage were accounted for by matching for year of recruitment. Erythrocyte folate concentrations might provide a more robust measure of folate status over time than plasma folate but are even more prone to storage instability, and in large-scale epidemiologic studies, plasma folate seems to be a more suitable marker of folate status (45).

Our cohort is characterized by low natural food folate intake and no mandatory folic acid fortification and, concordantly, considerably lower folate status compared with other European countries (46) and the United States both before and after the introduction of folic acid fortification (47). Although this provides an opportunity to study the impact of the lower spectrum of plasma folate concentrations on colorectal cancer risk, these population differences must also be taken into account when extrapolating our findings to countries with higher nutritional folate supply or with mandatory folic acid fortification. Although the present study may raise concerns about folic acid supplementation and food fortification, the findings of the recent meta-analysis of randomized studies on high-dose folic acid interventions (3), which did not show a statistically significant increase in overall cancer incidence over a period of 5 years, suggest that short-term exposure to folic acid supplementation is likely to be safe. Considering the crucial role of folate in embryogenesis, folic acid supplementation should still be encouraged before and during early pregnancy.

In conclusion, in this case–control study nested within the population-based NSHDS, low plasma folate concentrations were associated with a reduced risk of colorectal cancer. This protective role was mainly observed in subjects with higher tumor stage or shorter follow-up time between recruitment and diagnosis. On the basis of our findings, and those of previous prospective studies of populations with similar low nutritional folate intake and no exposure to mandatory food folic acid fortification (9, 13, 16), it seems that low circulating folate status may protect against colorectal cancer or suppress tumor progression. These findings should be taken into consideration in the ongoing debate about mandatory folic acid fortification of flour.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: B. Van Guelpen, J. Schneede, J. Hultdin, G. Hallmans, I. Johansson, R. Palmqvist
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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): B. Gylling, J. Hultdin, G. Hallmans, I. Johansson, R. Palmqvist
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): B. Gylling, B. Van Guelpen, J. Schneede, J. Hultdin, I. Johansson
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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): B. Gylling, J. Schneede, P.M. Ueland, G. Hallmans, R. Palmqvist
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