Systematic Reviews and Meta- and Pooled Analyses

Quantifying the Dose-Response Relationship Between Circulating Folate Concentrations and Colorectal Cancer in Cohort Studies: A Meta-Analysis Based on a Flexible Meta-Regression Model

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Most epidemiologic studies on folate intake suggest that folate may be protective against colorectal cancer, but the results on circulating (plasma or serum) folate are mostly inconclusive. We conducted a meta-analysis of case-control studies nested within prospective studies on circulating folate and colorectal cancer risk by using flexible meta-regression models to test the linear and nonlinear dose-response relationships. A total of 8 publications (10 cohorts, representing 3,477 cases and 7,039 controls) were included in the meta-analysis. The linear and nonlinear models corresponded to relative risks of 0.96 (95% confidence interval (CI): 0.91, 1.02) and 0.99 (95% CI: 0.96, 1.02), respectively, per 10 nmol/L of circulating folate in contrast to the reference value. The pooled relative risks when comparing the highest with the lowest category were 0.80 (95% CI: 0.61, 0.99) for radioimmunoassay and 1.03 (95% CI: 0.83, 1.22) for microbiological assay. Overall, our analyses suggest a null association between circulating folate and colorectal cancer risk. The stronger association for the radioimmunoassay-based studies could reflect differences in cohorts and study designs rather than assay performance. Further investigations need to integrate more accurate measurements and flexible modeling to explore the effects of folate in the presence of genetic, lifestyle, dietary, and hormone-related factors.

Abbreviations: CI, confidence interval; CRC, colorectal cancer; PLP, pyridoxal 5’-phosphate; RR, relative risk; THF, tetrahydrofolic acid.

Worldwide, colorectal cancer (CRC) is the third most common cancer in men and the second most common in women, and in the developed countries it is the second most common cause of death from cancer (1). However, the etiology of CRC is poorly understood. Observational studies have shown that modifiable lifestyle factors, such as smoking, alcohol drinking, physical activity, obesity, and diet, are associated with CRC risk, but each seems to explain only a small proportion of the total CRC incidence (2).

Most epidemiological studies (prospective and retrospective) report an inverse linear association between folate intake and CRC (3). In 2007, the World Cancer Research Fund (London, United Kingdom) released a report stating that “there is limited evidence suggesting that foods containing folate protect against CRC” (4, p. 284). Folate plays a pivotal role in the 1-carbon metabolism pathway and has been hypothesized to be associated with CRC risk through at least 2 distinct mechanisms, including DNA hypomethylation and subsequent proto-oncogene activation and uracil misincorporation during DNA synthesis, leading to DNA instability (5).

However, the associations between folate and CRC have been inconsistent in prospective studies that use circulating (serum or plasma) folate (6–12) and in clinical trials of colorectal adenoma (13–17). For example, in the Aspirin/Folate Polyp Prevention Study (13), participants with recent histories of colorectal adenoma were randomized into groups that received 1 mg/day of folic acid or placebo. During 6 years of follow-up, the relative risks for any adenoma and advanced
adenoma were 1.13 (95% confidence interval (CI): 0.93, 1.37) and 1.67 (95% CI: 1.00, 2.80), respectively. Such associations may imply that folate is involved in CRC progression. Nevertheless, the other 3 large-scale trials showed null associations in general (14, 15, 17).

Whereas folate deficiency could increase cancer risk, folate supplementation could promote the progression of existing cancerous or precancerous lesions (18). Indirect evidence relating folate to CRC includes a reversal of the downward trend in CRC rates in the United States, Canada, and Chile after the introduction of mandatory folic acid fortification of flour (19, 20). On this basis, it was suggested that the cancer prevention effect of folate is time- and dose dependent.

Although there have been several review articles on folate intake and CRC risk, there has been no systematic evaluation of studies that have investigated the association of circulating folate with CRC development. Circulating folate is likely to be a valid marker that reflects folate intake and folate available to tissues (21); hence, the circulating form of folate may be a better indicator than intake for evaluating the association between folate and CRC; however, results from studies of circulating folate have been inconclusive.

There have been discussions on the interchangeability between radioimmunoassay and microbiological assay for folate. The radioimmunoassay is precise, but there are accuracy problems. Although the microbiological assay detects most biologically active folate species with comparable sensitivity, the radioimmunoassay binds different folate species with different affinities (22). The differences in measurements could also contribute to the inconclusive observations.

Given that folate has been postulated to both reduce and increase the risk of CRC, the identification of U-shaped curves may be necessary. Because cancer development and treatment may influence the blood levels of biomarkers, we sought to address the question by using the results from case-control studies nested within prospective cohort studies of circulating folate.

**MATERIALS AND METHODS**

**Search strategy and selection criteria**

We conducted a literature search of the PubMed database until February 2012. The search was restricted to English-language articles and human studies and used the following search terms: “folate” and “colorectal cancer” in the abstract and title. We also checked the reference lists of articles retrieved from the PubMed search. Studies were included in the meta-analysis if they met the following criteria: case-control studies nested within a prospective cohort study; the exposure of interest was circulating (plasma or serum) levels of folate; and the outcome of interest was colorectal, colon, or rectal cancer. When several publications were available from the same study, the most recent publication, or the one including the largest number of subjects, was included. Figure 1 illustrates the study search and selection process.

**Figure 1.** Flow chart of the study search and selection.

<table>
<thead>
<tr>
<th>First Author, Year (Reference No.)</th>
<th>Cohort</th>
<th>Sex</th>
<th>Age, Years at Recruitment</th>
<th>Size</th>
<th>No. of Cases</th>
<th>No. of Controls</th>
<th>Biological Sample Assay Type</th>
<th>Circulating Folate Range in Controls, nmol/L</th>
<th>Percentile Range</th>
<th>Matching Variables</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eussen, 2010 (11)</td>
<td>EPIC</td>
<td></td>
<td>Female, male</td>
<td>520,000</td>
<td>1,367</td>
<td>2,325</td>
<td>Plasma MA</td>
<td>4.9 – 34.0</td>
<td>5%, 95%</td>
<td>Age, sex, smoking status, educational level, fiber intake, red meat and processed meat intakes, BMI, alcohol consumption, family history of CRC, history of endoscopy, and history of CRC screening.</td>
</tr>
<tr>
<td>Kato, 1999 (6)</td>
<td>NYUWHS</td>
<td>Female</td>
<td>35 – 65</td>
<td>15,785</td>
<td>105</td>
<td>523</td>
<td>Serum RIA</td>
<td>12.2 – 43.7</td>
<td>25%, 75%</td>
<td>Age, menopausal status at enrollment, date of enrollment, and date of subsequent blood donation.</td>
</tr>
<tr>
<td>Marchand, 2009 (31)</td>
<td>MEC</td>
<td>Male</td>
<td>45 – 75</td>
<td>67,594</td>
<td>224</td>
<td>411</td>
<td>Plasma RIA</td>
<td>23.8 – 63.7</td>
<td>25%, 75%</td>
<td>Age, sex, study year, race/ethnicity, location, date of blood draw, and fasting status.</td>
</tr>
<tr>
<td>Lee, 2012</td>
<td>HPFS</td>
<td>Male</td>
<td>40 – 75</td>
<td>18,225</td>
<td>173</td>
<td>345</td>
<td>Plasma RIA</td>
<td>5.4 – 32.0</td>
<td>6%, 94%</td>
<td>Age, month and year of blood collection.</td>
</tr>
<tr>
<td>Lee, 2012</td>
<td>NHS</td>
<td>Female</td>
<td>30 – 45</td>
<td>32,626</td>
<td>189</td>
<td>377</td>
<td>Plasma RI A</td>
<td>7.2 – 49.5</td>
<td>6%, 94%</td>
<td>Year of blood collection and fasting status.</td>
</tr>
<tr>
<td>Lee, 2012</td>
<td>HPFS</td>
<td>Male</td>
<td>40 – 75</td>
<td>18,225</td>
<td>173</td>
<td>345</td>
<td>Plasma RIA</td>
<td>5.4 – 32.0</td>
<td>6%, 94%</td>
<td>Year of blood collection and fasting status.</td>
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<tr>
<td>Chuang et al., 2013;178(7):1028–1037</td>
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<tr>
<td>First Author, Year (Reference No.)</td>
<td>Cohort</td>
<td>Years at Recruitment</td>
<td>Sex</td>
<td>Age, years</td>
<td>Cohort Size</td>
<td>No. of Cases</td>
<td>No. of Controls</td>
<td>Biological Sample</td>
<td>Assay Type</td>
<td>Circulating Folate Range in Controls, nmol/L</td>
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</tr>
<tr>
<td>Lee, 2012 (12)</td>
<td>PHS</td>
<td>1982–1984</td>
<td>Male</td>
<td>40–84</td>
<td>14,916</td>
<td>240</td>
<td>408</td>
<td>Plasma</td>
<td>MA</td>
<td>4.2–30.1³</td>
</tr>
<tr>
<td>Otani, 2008 (8)</td>
<td>JPHC²</td>
<td>1990–1995</td>
<td>Female, male</td>
<td>40–69</td>
<td>133,323</td>
<td>375</td>
<td>750</td>
<td>Plasma</td>
<td>RIA</td>
<td>13.1–20.1 (men); 15.4–24.7 (women)</td>
</tr>
<tr>
<td>Shrubsole, 2009 (10)</td>
<td>SWHS</td>
<td>1996–2000</td>
<td>Female</td>
<td>40–70</td>
<td>74,942</td>
<td>303</td>
<td>1,188</td>
<td>Plasma</td>
<td>MA</td>
<td>4.8–709.3</td>
</tr>
<tr>
<td>Van Guelpen, 2006 (7)</td>
<td>NSHDC⁹</td>
<td>1985–2002</td>
<td>Female, male</td>
<td>25–74</td>
<td>85,000</td>
<td>226</td>
<td>437</td>
<td>Plasma</td>
<td>RIA</td>
<td>5.8–11.1</td>
</tr>
</tbody>
</table>

Table continues
Data extraction

The following data were extracted from each study: the first author’s last name; publication year; name of the cohort; years of recruitment and blood collection; participants’ sex and age; sample sizes of cases, controls, and cohort sizes, both overall and in each exposure stratum (contacting corresponding authors if this information was missing); matching criteria; biological samples; circulating folate assay; range of circulating folate in the controls; variables adjusted for in the analyses; and the relative risk estimates with the corresponding 95% confidence intervals for each category of circulating folate levels.

Statistical analysis

We used a flexible meta-regression model, which provides the best fitting 2-term fractional-polynomial model, to test a linear or a nonlinear dose-response relationship between circulating folate concentrations and CRC risk. The statistical methods used for this analysis are described in detail elsewhere (23, 24). In brief, this approach takes into account the correlation within the same study among reported dose-specific log (relative risk) estimates due to the common reference group, the heterogeneity among studies, and the nonlinear trend component of the dose-response relationship. For each study, the midpoint/median level of circulating folate for each category, except the highest, and a level 1.2 times the lower cut point of the highest category were assigned to each corresponding relative risk estimate (25). The best fitting model is defined as the one with the smallest Akaike’s Information Criterion. Because folate levels never reach null values, we investigated the relationship between folate levels and CRC risk on the basis of the contrast of each folate level with the reference category (26). All circulating folate values were converted to nmol/L (27). The heterogeneity among the studies was tested with the Q statistic (28) by using the linear trend estimates. The statistical analyses were repeated by converting the exposure levels using the equation provided by Fazili et al. (29) to improve the comparability of the measurements among studies. We also combined the study-specific relative risks, comparing the highest with the lowest category, with an assumption that the measurement error due to interlab variation in absolute concentrations would be less likely to influence a comparison based on study-specific quantiles. The degree of heterogeneity was estimated by using the $I^2$ statistic, which represents the percentage of total variation contributed by between-study variance (28).

RESULTS

A total of 399 publications were retrieved from the PubMed search. After careful filtering and checking, we identified 9 potentially relevant articles concerning circulating folate in relationship to the risk of CRC from case-control studies nested within prospective cohort studies. One publication (30) was excluded because of a later report with longer follow-up (9) from the same study population. One publication included results for 3 distinct cohorts (the Nurses’ Health Study, the Health Professionals Follow-up Study, and the Physicians’ Health Study). Therefore, the meta-analysis on the dose-response
The relationship between circulating folate and CRC includes results for 10 cohorts from 8 publications (Table 1). Almost all publications appeared after 2006, but only 1 cohort in the United States, the Multiethnic Cohort Study, collected blood after 1998 (i.e., after the folic acid fortification policy was implemented). The 10 cohorts contributed 3,477 cases and 7,039 controls, and the European Prospective Investigation into Cancer and Nutrition cohort alone contributed more than one-third (35.8%) of the cases and controls.

Figure 2 presents the cohort-specific dose-risk functions. Because no heterogeneity among studies was detected ($Q = 8.53$, $P = 0.58$), a fixed-effect model was used in further analyses. Under a linear fixed-effect model, we observed a non-statistically significant weak inverse dose-response relationship between circulating folate concentrations and CRC risk (relative risk (RR) = exp(−0.00375 · $x$), Akaike’s Information Criterion = −58.4 (Figure 3A), where $x$ = circulating folate in contrast to the reference value). A stronger, but still non-statistically significant inverse association was observed for the studies that used radioimmunoassay (RR = exp(−0.00599 · $x$), Akaike’s Information Criterion = −39.0 (Figure 3B), which corresponds to a relative risk of 0.94 (95% CI: 0.88, 1.01) per 10 nmol/L of circulating folate in contrast to the reference value).

Because of the potential reversal in the risk estimates at high folate levels, a nonlinear fixed-effect model was also fitted. The fixed-effect model with power terms $p_1 = 3$ and $p_2 = 2$ presented the lowest Akaike’s Information Criterion value (−253.4) among the estimable second-order fractional polynomial models tested. The equation of the best fitting model was a relative risk of exp(−1.44E−7 · $x^3$−0.00011 · $x^2$). The estimated summary relative risk for CRC obtained with the best-fitting fractional polynomial model was 0.99 (95% CI: 0.96, 1.02) per 10 nmol/L of circulating folate levels in contrast to the reference value. Interestingly, there seemed to be a reversal of the weak inverse association noted at lower concentrations at approximately 14 nmol/L (lowest RR = 0.92, 95% CI: 0.75, 1.13) if we restrict the analysis to the 3 studies that used the microbiological assay. However, the estimation was based on only 3 studies, and each of them had a limited range of circulating folate (<20 nmol/L), thus leading to imprecise estimates at high values.

In sensitivity analyses based on the linear models, the exclusion of the Multiethnic Cohort Study, which was conducted in the post–folic acid fortification era (RR = 0.98, 95% CI: 0.92, 1.05 per 10 nmol/L in contrast to the reference value), or the European Prospective Investigation into Cancer and Nutrition Study, which contributed the largest proportion of cases and controls (RR = 0.97, 95% CI: 0.91, 1.03 per 10 nmol/L), did not change the estimates.

After the correction of measurements from different assays by using the equation provided by Fazli et al. (29), we obtained results similar to those without the correction (per 10 nmol/L in contrast to the reference value, RR = 0.97, 95% CI: 0.93, 1.01 (Figure 3A); and RR = 0.99, 95% CI: 0.97, 1.02 (Figure 3C)). The combined relative risk comparing the highest with the lowest category was 0.91 (95% CI: 0.77, 1.05) (Figure 4). There was a stronger and statistically significant inverse association between circulating folate and CRC risk in studies that used the radioimmunoassay (RR = 0.80, 95% CI: 0.61, 0.99).

**DISCUSSION**

The findings from our meta-analysis of nested case-control studies with prospectively collected blood specimens indicate a null association between circulating folate and CRC risk. Furthermore, there is no evidence that a very high circulating folate concentration is associated with increased CRC risk in the nonlinear model. Nevertheless, when analysis is restricted to studies that used microbiological assay, there seems to be an increased risk at high levels of circulating folate. However, this observation was based on few studies, and the ranges used for the estimation were usually limited. Further studies are warranted.

In most of the studies included in this meta-analysis, the highest values of circulating folate were lower than 40 nmol/L, except in the Multiethnic Cohort Study (31), which was the only study in our analyses that collected blood samples after folic acid fortification was initiated in the United States in 1998. In the nationally representative National Health and Nutrition Examination Survey of the US population, conducted during 1988–1994, only 7% of participants had serum folate concentrations higher than 45.3 nmol/L; however, the percentage had increased to 38% in the 1999–2000 survey (32).

If folate does play a role in CRC prevention, the observed null association of circulating folate levels with CRC could be caused by the relatively narrow range of folate concentrations within the single studies, as well as the relatively low highest absolute concentrations, to provide a meaningful risk estimate in the study populations that were not exposed to mandatory folic acid fortification policy. Further, the impact of the narrow range in our study is the imprecise risk estimates at high concentrations (e.g., what we observed in the studies that used microbiological assay). As a result, the interpretation at >20 nmol/L must be cautious. Inclusion of postfortification blood samples may be helpful to expand the range of circulating folate.

Moreover, folate is not stable in serum and plasma and is therefore easily affected by storage and processing factors. For example, circulating folate could have been degraded ~60% after being stored at −25°C for ~30 years (33). Further, microbiologically active folate was degraded in room temperature at a rate of 0.16% per hour in serum, 1.61% in ethylenediaminetetraacetic acid, 0.08% in heparin, and 0.11% in citrate plasma in the first 24 hours after collection (34). Such instability can lead to artificially low absolute levels, making accurate quantification of exposure difficult and substantially attenuating the association between folate status and study outcomes. We are not able to address the effect of degradation in the current meta-analysis because the folate degradation kinetics during blood processing and storage vary by study, possibly for individual samples within the same study, and by folate forms in the individual samples. Interestingly, the 2 studies (6, 31) that showed the strongest inverse association had relative shorter storage times (i.e., years since recruitment to publication). However, another 2 studies with similar storage times, the Northern Sweden Health and Disease Cohort (7), for which the mean follow-up was 4.2 years for the cases but the recruitment covered a long period (1985–2002), and the Shanghai Women’s Health Study (10), showed no association between circulating folate and CRC risk.

Theoretically, the absolute folate concentrations also vary by assay method, with the microbiological assay producing
higher readings than the radioimmunoassay. A major reason for this is that the radioimmunoassay only partially recovers 5-methyltetrahydrofolate (5CH\textsubscript{3}THF), the main circulating form of folate (29, 35). Because radioimmunoassay suffers from more severe measurement error, we expected stronger inverse association after correction for measurement error or

Figure 2. Black squares indicate relative risk estimates, and dotted lines are 95% confidence intervals. Lines indicate the predicted risk with the linear fixed-effects model. The vertical axis is on a log scale. The absolute concentrations (in nmol/L) used for the modeling at each category were as follows: A) 6, 16, 25, and 37 for Kato (6); B) 2, 6, 10, 13, and 18 for Van Guelpen (7); C) 11, 14, 17, and 24 for Otani (men) (8); D) 13, 17, 21, and 30 for Otani (women) (8); E) 6, 7, 9, 10, and 13 for Weinstein (9); F) 12, 29, 48, and 73 for Le Marchand (31); G) 11, 22, and 31 for Shrubsole (10); H) 4, 9, 12, 16, and 22 for Eussen (11); I) 9, 14, 22, and 41 for Lee (Nurses' Health Study) (12); J) 7, 11, 17, and 27 for Lee (Health Professionals Follow-up Study) (12); and K) 6, 10, 13, and 25 for Lee (Physicians' Health Study) (12).

Figure 3. The dose-response relationships between circulating folate and colorectal cancer risk. A) Linear fixed-effect model, B) linear fixed-effect model by assay, C) nonlinear fixed-effect model, and D) nonlinear fixed-effect model by assay. The dashed lines represent 95% confidence intervals.
in studies that used the microbiological assay. We partially corrected the measurement error caused by assay method by adopting the equation provided by Fazili et al. (29) for calibrating the specific radioimmunoassay kit used in the National Health and Nutrition Examination Survey and repeated the meta-analyses. However, the correction did not change the results noticeably. A comparison of the summary relative risk associated with the highest category of folate status relative to the lowest category with a Forest plot provided the same conclusion (i.e., a null association) (Figure 4). Interestingly, we observed a statistically significant inverse association between circulating folate and CRC risk in the studies that measured folate by radioimmunoassay. Whereas the laboratory measurement error is more likely to be nondifferential, the grouping of continuous exposure data into categories may result in differential misclassification (36). Because the degradation rate can be different by folate species (33, 34), the selective underrecovery of 5CH3THF by radioimmunoassay complicated the prediction of the direction of misclassification by assay.

It has been suggested that the methylenetetrahydrofolate reductase gene 677T allele is associated with a reduced CRC risk (37). Methylenetetrahydrofolate reductase mediates the conversion of 5,10-methylenetetrahydrofolate (5,10-CH = THF), which is required for DNA synthesis and repair, into 5CH3THF, which is involved in DNA methylation, and the enzyme activity decreases with the increase of the T allele of the polymorphism (38). Therefore, some studies have suggested that the inverse association of the methylenetetrahydrofolate reductase gene 677T allele and CRC risk could be caused by an increase of 5,10-CH = THF levels for DNA synthesis and repair (39, 40). The recently developed liquid chromatography–tandem mass spectrometry detects a spectrum of folate species (29) and may help to partition the effects from DNA synthesis/repair or methylation. In contrast to the null associations reported in studies that used circulating folate as the main exposure, a pooled analysis from 13 cohorts reported a statistically significant inverse association between folate intake and CRC risk with a linear dose-response relationship (41). Because previous dose-response controlled trials have shown increasing serum folate levels with folic acid dose ($P_{\text{trend}} < 0.001$) (21, 42), we expected a similar association for similar ranges of exposure. Interestingly, similar contradictory results were also reported for vitamin B6 intake and circulating levels of pyridoxal 5′-phosphate (PLP), which is the coenzyme form of vitamin B6 (43). That is, circulating PLP was inversely associated with CRC risk, but vitamin B6 intake was not associated (or weakly associated after excluding the influential study) with CRC. The arguments to explain these discrepancies include the fact that PLP is not identical to dietary vitamin B6 (44), and that low PLP may reflect low-grade inflammation instead of vitamin B6 deficiency (45). It is also possible that PLP and circulating folate confound their associations with...
CRC because both of them play important roles in 1-carbon metabolism. It is unclear whether the effects are from folate alone, from vitamin B6/PLP, from their combination, or from other B vitamins in the 1-carbon metabolism.

Recently, a randomized, placebo-controlled trial of a combination supplement of folic acid, vitamin B6, and vitamin B12 showed neither beneficial effects nor increased risk for colorectal adenoma over 9 years of follow-up (17). The trial was conducted during the folic acid fortification era; baseline intakes of each micronutrient were high, with median intake of folate equivalent to the US recommended dietary allowance, and the daily dose of each micronutrient in the supplement was also high, with folic acid as high as 2.5 mg/day (46). The null results of the trial do not address the protective potential of folate in individuals with suboptimal intakes.

Additionally, the high intake of folate may imply high intakes of other nutrients, such as fiber. High intake of fiber is inversely associated with CRC (47). The inverse association of folate intake and CRC risk might reflect residual confounding by fiber intake. However, recent cohort studies with proper adjustment for fiber intake yielded similar inverse associations between folate intake and CRC risk (41, 48).

The etiology of CRC is complex and unlikely to be explained by a single factor. In clinical trials, researchers found that folic acid supplementation of 1 mg/day was not beneficial in adenoma patients with nutritionally adequate baseline folate status (15, 49). A potential explanation for this observation might be that an adenoma that develops in patients with moderate baseline folate status might actually go through a “folate-independent pathway” (15). Such an observation implies that the role of folate could vary according to the presence of other risk factors, such as smoking, alcohol consumption, diet, physical activity, and hormone-related factors. The CRC risk associated with folate might be different between people who smoke or drink alcohol heavily, or even between men and women.

In summary, our analyses suggest a null association between circulating folate concentrations and CRC risk. Interestingly, we observed a statistically significant inverse association between circulating folate and CRC risk in the studies that measured folate by radioimmunoassay, though the stronger association for the radioimmunoassay-based studies could reflect differences in cohorts and study designs rather than assay performance. Further investigations need to integrate more accurate measurements and modeling to explore the effects of folate in the presence of genetic, lifestyle, dietary, and hormone-related factors.

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