Research Article

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# Vitamins B2 and B6 and Genetic Polymorphisms Related to One-Carbon Metabolism as Risk Factors for Gastric Adenocarcinoma in the European Prospective Investigation into Cancer and Nutrition

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## Abstract

B vitamins and polymorphisms in genes coding for enzymes involved in one-carbon metabolism may affect DNA synthesis and methylation and thereby be implicated in carcinogenesis. Previous data on vitamins B2 and B6 and genetic polymorphisms other than those involving *MTHFR* as risk factors for gastric cancer (GC) are sparse and inconsistent. In this case-control study nested within the European Prospective Investigation into Cancer and Nutrition cohort, cases ($n = 235$) and controls ($n = 601$) were matched for study center, age, sex, and time of blood sampling. B2 and B6 species were measured in plasma, and the sum of riboflavin and flavin mononucleotide was used as the main exposure variable for vitamin B2 status, whereas the sum of pyridoxal 5′-phosphate, pyridoxal, and 4-pyridoxic acid was used to define vitamin B6 status. In addition, we determined eight polymorphisms related to one-carbon metabolism. Relative risks for GC risk were calculated with conditional logistic regression, adjusted for *Helicobacter pylori* infection status and smoking status. Adjusted relative risks per quartile (95% confidence interval, $P_{trend}$) were 0.85 (0.72-1.01, 0.06) for vitamin B2 and 0.78 (0.65-0.93, <0.01) for vitamin B6. Both relations were stronger in individuals with severe chronic atrophic gastritis. The polymorphisms were not associated with GC risk and did not modify the observed vitamin-cancer associations. In summary, results from this large European cohort study showed an inverse association between vitamin B2 and GC risk, which is borderline significant, and a significant inverse association between vitamin B6 and GC risk. *Cancer Epidemiol Biomarkers Prev; 19(1); 28–38. ©2010 AACR.*

## Introduction

Although the incidence of gastric cancer (GC) has declined in industrialized countries over the last century, it is still the second most important cause of cancer mortality in many developing countries (1). *Helicobacter pylori* (Hp) infection of the gastric mucosa has been associated with noncardia GC, whereas severe chronic atrophic gastritis (SCAG) has been associated with cardia GC (2). The relatively high prevalence of *Hp* infection in populations with

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low GC risk (3) suggests that also other factors, such as diet and genetic predisposition, may play an etiologic role.

One-carbon metabolism (Fig. 1) is of special interest with respect to carcinogenesis. The activity of enzymes involved can be affected by low concentrations of B vitamin cofactors and by genetic polymorphisms. Derangement of one-carbon metabolism (4) might be implicated in carcinogenesis via impaired synthesis and methylation of DNA (5). Folate (6-9) and cobalamin (9-13) are among the B vitamins that have been studied most frequently in relation to GC risk and show inconsistent associations. Riboflavin (vitamin B2) and pyridoxine (vitamin B6) have been studied less frequently. Only a few case-control studies have investigated associations of vitamin B2 (10, 13-21) and B6 (10, 13, 15-18) intake with GC risk. The results suggest either no association (10, 13-17), an increased (18, 19) or decreased (20, 21) risk with higher vitamin B2 intake, or a decreased risk with higher vitamin B6 intake (10, 16-18). The vitamins B2 and B6 are interrelated because the interconversion of some vitamin B6 species requires the vitamin B2 species flavin mononucleotide (FMN) and flavin dinucleotide as cofactors (22, 23). In one-carbon metabolism, vitamin B2 serves as a cofactor for the enzymes methylenetetrahydrofolate reductase (MTHFR) and methionine synthase reductase (MTRR), whereas vitamin B6 serves as a cofactor for the enzyme cystathionine β-synthase (CBS) and cystathionine γ-lyase. The majority of previous research on effects of genetic variation and GC risk has focused on *MTHFR 677C→T* and *1298C→A* polymorphisms (9, 24), whereas few results have been published on the *CBS 844ins68* (25), *MTRR 66A→G* (26, 27), methionine synthase (*MTR) 2756A→G* (25, 27), methylenetetrahydrofolate dehydrogenase (*MTHFD1*) *1958G→A* (28), and reduced folate carrier [solute carrier family 19 (*SLC19A1*)] *80G→A* (29) polymorphisms. An association with GC risk has previously been observed for variant genotypes of the *MTHFR* 677C→*T* (24), *MTHFR 1298C→A* (9, 24), and the *MTHFD1 1958G→A* (28) polymorphisms.

Most previous studies on vitamins B2 (10, 13-21) and B6 (10, 13, 15-18) in GC have been relatively small, and none measured plasma concentrations of vitamin B2 and B6 species. Moreover, in all previous studies, intake of vitamin B2 and B6 was reported after diagnosis of the disease, which may have affected the results. To obtain a clearer understanding of the role of prediagnostic plasma concentrations of vitamin B2 and B6, and their possible interactions with eight one-carbon polymorphisms in GC etiology, this case-control study nested within the European Prospective Investigation into Cancer and Nutrition (EPIC; refs. 30, 31) was conducted.

## Materials and Methods

### Study Population and Collection of Blood Samples

The design and methods of the EPIC study have previously been described in detail (30, 31). Briefly, the EPIC cohort consists of 23 centers in 10 European countries (Denmark, France, Greece, Germany, Italy, Netherlands, Norway, Spain, Sweden, and United Kingdom). Between 1992 and 1998, country-specific dietary questionnaires, standardized lifestyle and personal history questionnaires, anthropometric data, and blood samples were collected from the majority of the participants.

The present study includes GC cases, which were diagnosed after blood collection, and matched control cohort members free of cancer from all EPIC countries. In each of the recruitment centers, fasting and nonfasting blood samples of at least 30 mL were drawn from all participants and stored at 5°C to 10°C while protected from light and transported to local laboratories for processing and aliquoting, as previously described (30, 31), except for the EPIC-Oxford center. The EPIC-Oxford center collected blood samples from a network of general practitioners and health conscious individuals, and blood samples were transported to a central laboratory in Oxford via mail. While protected from light, the whole blood samples from Oxford were exposed to ambient temperatures for up to 48 h. As B vitamins are partly degraded by such handling, all EPIC-Oxford (three cases, nine controls) samples were excluded from the present analyses. GC cases were not diagnosed among the Norwegian cohort members.

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

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1.0 mL were stored locally in Nunc tubes at −150°C under nitrogen vapor. In Sweden, samples were stored in −70°C freezers.

### Follow-up for Cancer Incidence

In EPIC, follow-up is based on population cancer registries (Denmark, Italy, Netherlands, Norway, Spain, Sweden, and the United Kingdom), health insurance records, pathology registries, and active contact of study subjects or next of kin (France, Germany, and Greece). The follow-up period for the present study was for cases included in reports received at IARC until the end of October 2002, representing complete follow-ups until either December 2000 or December 2001 for all centers using cancer registry data and until 2002 for France, Germany, and Greece. Cancers of the stomach included cancers coded as C16 (10th Revision of the International Statistical Classification of Diseases, Injury and Causes of Death). The diagnosis, tumor site classification, and morphology (according to ICD02 and Lauren classifications) of each identified cancer were confirmed and validated by an independent panel of pathologists with a representative from each EPIC country and a coordinator (32). The pathologist panel reviewed original histologic slides and/or recuts from the paraffin blocks and original histopathology reports that were provided by each EPIC center.

### Nested Case-Control Study Design and Selection of Study Subjects

Incident GC cases were cohort members who developed cancer after recruitment into EPIC. The present study includes a total of 221 gastric adenocarcinomas and 14 adenocarcinomas of the gastroesophageal junction (GEJ). In this study, these 235 cases are grouped together. For each identified cancer case, control subjects with available blood samples were randomly selected from all cohort members who were alive and free of cancer (except nonmelanoma skin cancer) at the time of diagnosis of the case patient. Controls ($n$ = 601) were matched by gender, age group (±2.5 y), study center, and date of blood sample collection (±45 d). GC cases were divided into three groups according to anatomic subsite: (*a*) tumors originating from the gastric cardia ($n$ = 62), combining tumors that reached the GEJ, either crossing the GEJ or located below the GEJ (all 14 GEJ cancers) or not; (*b*) noncardial tumors ($n$ = 102) grouping cases from other sites in the stomach; and (*c*) tumors from unknown or mixed sites ($n$ = 71). When divided by histologic subtype, of the 235 cancer cases, 82 were classified as diffuse and 81 as intestinal according to the Lauren classification. The remaining cases ($n$ = 72) were of unknown or mixed histologic types. All gastric lymphomas, gastric stump cancers, other gastric nonadenocarcinoma, esophageal nonadenocarcinomas, and otherwise unspecified malignant neoplasms of the stomach were excluded from this analysis. This study was approved by the Ethical Review Board of the IARC and those of all individual EPIC centers.

### Laboratory Measurements

Vitamin B2 measures included citrate plasma concentrations of riboflavin and FMN, and vitamin B6 measures included pyridoxal 5′-phosphate (PLP), pyridoxal (PL), and 4-pyridoxic acid (PA). All vitamin species were determined by liquid chromatography–tandem mass spectrometry assay in the same laboratory in Bergen, Norway (33). Within- and between-day coefficients of variation (CV) of vitamin B2 and B6 species were 3% to 18% and 6% to 22%, respectively. The largest CVs are for FMN (within CV = 18%; between CV = 22%), which is present in low concentrations and has a relatively low signal (33).

Eight polymorphisms (reference, amino acid change) of genes coding for enzymes involved in one-carbon metabolism were determined by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (34) in Bergen, Norway. These included *CBS (699C→T;* rs 234706, $Tyr^{233}Tyr$*)* and, in addition, the *CBS 844ins68 insertion, MTHFD1 (1958G→A;* rs 2236225, $Arg^{653}Gln$*), MTR (2756A→G;* rs 1805087, $Asp^{919}Gly$*)*, MTRR *(66A→G;* rs 1801394, $Ile^{22}Met$*)*, *SLC19A1 (80G→A;* rs 1051266, $Arg^{27}His$*)*, and transcobalamin II (*TCN2 67A→G;* rs RsaI, $Ile^{23}Val$ and *TCN2 776C→G;* rs 1801198, $Pro^{259}Arg$*)*. These single-nucleotide polymorphisms (SNP) are "nonsynonymous" and are often called "potentially functional" (35). Quantification of anti-*Hp* (Hp) antibodies in stored plasma samples was done by an ELISA technique using incubation with lysates of the CCUG Hp strain (36). Pepsinogen A (PGA) was assayed in plasma by a commercial microplate-based quantitative ELISA kit (Biohit). SCAG was serologically defined as a PGA circulating concentration lower than 22 μg/L (36).

### Statistical Methods

Because riboflavin and FMN are interconvertible (37-40), as are PLP and PL (41, 42), and PA is formed from PL, we calculated the sum of riboflavin and FMN as an index for vitamin B2 status and the sum of PLP, PL, and PA as an index of vitamin B6 status. In the present study, these sums of B2 and B6 vitamers are therefore considered as the main exposure variables for vitamin B2 and B6 status, respectively. In addition, results are also presented for the individual B2 and B6 species.

The Mann-Whitney $U$ test was used to assess potential differences in plasma concentrations of vitamin B2 and B6 species according to sex, age (<60 y versus >60 y), European region (north versus central), Hp infection status, and SCAG status, whereas the Kruskall-Wallis test was used to assess differences in concentration among categories of smoking categories (nonsmokers, ex-smokers, current smokers, and missing) in the 601 controls. Differences in plasma concentrations between cases and controls were investigated by the Mann-Whitney $U$ test.

Relative risks (RR) and 95% confidence intervals (95% CI) for GC in relation to indices of vitamin B2 and B6 status were calculated by conditional logistic regression using the SAS LOGISTIC procedure (SAS statistical

software, version 9.1; SAS Institute) stratified by the case-control set. In our study, the RR indicated the incidence rate ratio, which is reflected by the odds ratio calculated from conditional logistic regression (43). Risk estimates were adjusted for Hp infection status and smoking categories. The RRs on GC were examined by quartiles with cutoff points based on the distribution of B2 and B6 indices in all 601 controls combined. Likelihood ratio tests were used to assess linear trends in RRs across the categories using values for quartile categories as the quantitative score of exposure. Models were also carried out separately for each anatomic subsite (cardia versus noncardia), histologic subtype (diffuse versus intestinal), European region [north-central Europe (Sweden, Denmark, Germany, the Netherlands, United Kingdom, and France) versus southern Europe (Italy, Greece and Spain)], time from blood donation to cancer diagnosis (<1 y versus ≥1 y), age at recruitment (<60 y versus >60 y), sex, and Hp status (infection or no infection). Heterogeneity was tested by adding the product term of the vitamin indices and potential effect modifiers in the model. In addition, subgroup analyses by SCAG (yes or no) were done for cases with and without SCAG and their matched controls.

The associations between the polymorphisms and GC risk were studied with conditional logistic regression but by stratifying on country instead of the matched sets and with age and sex as covariates. The risk estimates were calculated with the wild-type as the reference category. An ordinal level variable with equally spaced integer weights (0, 1, 2) for the genotypes was used to test for trend to summarize the effect of each polymorphism. Effect modification of the SNP-GC associations by vitamin concentrations was studied with conditional logistic regression.

## Results

### Characteristics of the Study Population

Selected characteristics of the 235 GC cases and 601 matched controls are summarized in Table 1. Forty-one percent of the cases were female, mean age at diagnosis was 62 years, and the mean time from blood donation to cancer diagnosis was 3.2 years. GC cases had a higher prevalence of Hp infection, SCAG, and current smoking than their matched controls.

### Concentrations of Vitamins B2 and B6

The distributions of vitamin B2 and B6, and the individual vitamers, in the control group were skewed, with a longer tail at higher concentrations (Table 2). Plasma concentrations of all vitamin B6 species correlated strongly with each other after adjustment for age, sex, and study center (correlation coefficients ranged from 0.51 to 0.74; $P$ for all correlations < 0.01), and the correlation between plasma concentrations of riboflavin and FMN was 0.32 ($P$ < 0.01; data not shown).

Table 2 shows that median vitamin B2 concentrations were higher in females, whereas vitamin B6 concentrations were higher in males. Furthermore, concentrations of vitamin B2 and B6 were lower in southern European countries compared with northern European countries ($P_{difference}$ < 0.01) and in smokers compared with former and never smokers ($P_{trend}$ < 0.01). Concentrations did not differ with respect to age (<60 y versus ≥60 y), Hp infection (yes versus no), and SCAG (yes versus no) in controls (Table 2). A trend toward higher concentrations of all B6 species was observed for the homozygote *GG* variants of the *MTRR* ($P_{trend}$ < 0.05) and *TCN2* ($P_{trend}$ < 0.05) polymorphisms, whereas concentrations of the B2 and B6 species did not differ across other polymorphic genetic variants (data not shown).

Median concentrations (nmol/L) of the vitamin B2 sum, riboflavin, FMN, the vitamin B6 sum, PLP, PL, and PA in the GC cases were 15.6, 12.1, 2.9, 51.9, 22.1, 13.4, and 15.6 nmol/L, respectively. Furthermore, concentrations did not differ significantly among cases and controls with Hp infection ($P_{difference}$ > 0.05) and SCAG ($P_{difference}$ > 0.05; data not shown).

### Associations between Plasma Indices of Vitamin B2 and B6 Status with GC

GC risk decreased borderline significantly with increasing vitamin B2 sum (RR per increase in quartile of 0.85; $P_{trend}$ = 0.06) and with strongest associations for FMN ($P_{trend}$ = 0.01). The vitamin B6 sum was significantly inversely associated with GC risk (RR per increase in quartile of 0.78; $P_{trend}$ < 0.01), and among the separate B6 species, only high PA concentrations were significantly associated with lower GC risk ($P_{trend}$ = 0.01; Table 3). Exclusion of cohort members with extremely high concentrations of the B6 species, attributable to vitamin B6 supplements as indicated by the presence of pyridoxine in plasma ($n$ = 81; ref. 44), did not alter any of the observed associations (data not shown).

We also analyzed the associations between vitamin B2 and B6 indices with GC risk separately in cases with ($n$ = 42) and without ($n$ = 191) SCAG (Table 4). Generally, associations of the vitamins with GC risk were more pronounced in those with SCAG compared with those without SCAG. Associations were stronger, with RR/quartile ($P_{trend}$) of 0.38 (<0.01) for FMN and 0.51 (0.01) for PA. Moreover, the difference in associations in those with and without SCAG was significant for FMN ($P_{interaction}$ = 0.03; Table 4). Because gastritis was not a matching criterion, 2% of controls matched to cases without SCAG had SCAG, and 32% of controls matched to cases with SCAG had SCAG, which may have biased these associations. Therefore, we additionally analyzed these associations stratified for gastritis comparing cases and controls with or without SCAG. These analyses revealed even stronger inverse associations between vitamin B2 and B6 and GC in individuals with SCAG (data not shown).

We further studied whether the associations between GC and vitamin B2 and B6 were modified by *Hp* infection status (yes or no), time between blood sampling and cancer diagnosis (<1st year or ≥1st year), sex, age

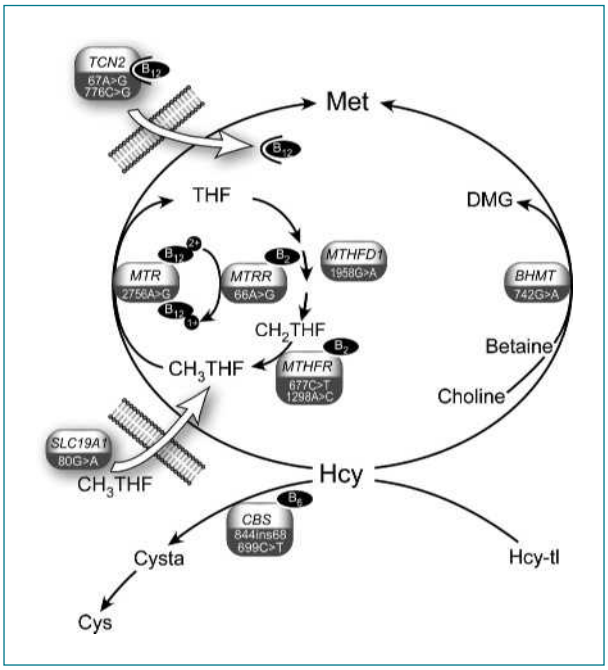


**Figure 1.** One-carbon metabolism and related enzymes and genetic polymorphisms. *BHMT*, betaine homocysteine methyltransferase (remethylation of homocysteine to methionine); *Cysta*, cystathionine; *Cys*, cysteine; *$CH_2THF$*, methylenetetrahydrofolate; *$CH_3THF$*, methyltetrahydrofolate; *DMG*, dimethylglycine; *Hcy*, homocysteine; *Hcy-tl*, homocysteine thiolactone; *Met*, methionine; *THF*, tetrahydrofolate.

(<60 y or ≥60 y), anatomic subsite (cardia or noncardia), histologic subtype (diffuse or intestinal), or European region (north or south), and associations between vitamin B2 and B6 and GC risk were not modified by these factors (data not shown).

### Polymorphisms in Enzymes with Vitamin B2 and Vitamin B6 as Cofactors and Their Association with GC

All the SNPs in *MTHFR, MTRR, MTR*, and *CBS* genes were in Hardy-Weinberg equilibrium ($P > 0.05$, $\chi^2$ test, for all SNPs). In this EPIC cohort, the *MTHFR 677C→T* polymorphism was not associated with GC risk, whereas the variant CC genotype of the *MTHFR 1298A→C* polymorphism showed a 47% increased GC risk compared with the AA genotype ($P = 0.04$), as presented elsewhere (9). The genotype distributions did not differ among cases and controls (Table 5). Overall, homozygote variant genotypes of all polymorphisms were more prevalent in northern European countries compared with southern European countries ($P < 0.03$ for all SNPs), with most pronounced differences for *MTRR 66A→G* (31% in north versus 20% in south). None of the *MTRR, MTR*, and *CBS* polymorphisms was associated with GC risk ($P_{trend}$ for all > 0.05; Table 5). However, European region significantly modified overall associations for the *CBS 844ins68* ($P_{interaction} = 0.03$), showing opposite associations in northern and southern European countries (Table 5).

We further assessed potential effect modification by vitamin B2 and vitamin B6 status of the associations between cancer and the *MTHFR, MTRR, MTR*, and *CBS* polymorphisms. None of the associations between the SNPs and GC risk was statistically significantly modified by variable vitamin B2 and vitamin B6 status ($P_{interaction} > 0.06$ for all relevant interactions; data not shown).

### Polymorphisms in Enzymes with Folate and Vitamin B12 as Cofactors and Their Association with GC

Overall, homozygote variant genotypes of the *TCN2 776C→G*, *TCN2 67A→G*, *MTHFD1 1958G→A*, and *SLC19A1 180G→A* polymorphisms were more prevalent in northern European countries than in southern European countries ($P < 0.03$ for all SNPs), with most pronounced differences for *MTHFD1 1958G→A* (13% in north versus 6% in south). None of these polymorphisms was associated with GC risk ($P_{trend}$ for all > 0.05). However, *GG* variant of the *TCN2 776* gene showed an increased risk in northern European countries and a decreased risk in southern countries ($P_{interaction} = 0.04$), whereas the *AA* variant of the *SLC19A1* gene showed an increased risk in southern and decreased risk in northern countries

**Table 1.** Characteristics of cohort members

| | Cases (*n* = 235) | Controls (*n* = 601) |
|---|---|---|
| Sex (female), *n* (%) | 96 (41) | 245 (41) |
| Age at recruitment, mean (range) | 58.9 (31.8-76.3) | 58.7 (28.5-76.6) |
| Age at diagnosis, mean (range) | 62.1 (34.3-77.7) | n.a. |
| Years between blood donation and diagnosis, mean (range) | 3.2 (0.01-9.69) | n.a. |
| Infection with *Helicobacter pylori*, *n* (%) | 200 (84) | 404 (67) |
| Severe atrophic chronic gastritis, *n* (%)* | 42 (18) | 45 (8) |
| Current/former smokers, *n* (%) | 67 (28)/88 (37) | 136 (23)/198 (33) |

Abbreviation: n.a., not applicable.
*Defined as plasma PGA concentrations <22 μg/L.

($P_{interaction}$ = 0.05). None of the associations between the SNPs and GC risk was statistically significantly modified by folate and vitamin B12 status ($P_{interaction}$ > 0.19 for all relevant interactions; data not shown).

## Discussion

The present nested case-control study investigated the association of GC risk with indices of vitamin B2 and B6 status, and nine polymorphisms in genes encoding for enzymes involved in one-carbon metabolism. Overall analyses indicated a tendency for an inverse association between vitamin B2 and GC risk and a significant inverse association between vitamin B6 and GC risk. Furthermore, the associations were more pronounced in individuals with SCAG. None of the studied polymorphisms was related to GC risk, nor did they modify the observed associations for vitamin B2 and B6.

**Table 2.** Vitamins B2 and B6 [mean/median (5-95 percentile)] in relation to demographic characteristics and risk factors of GC in control cohort members (*n* = 601)

| | | *n* | Vitamin B2 sum | Riboflavin | FMN | Vitamin B6 sum | PLP | PL | PA |
|---|---|---|---|---|---|---|---|---|---|
| | Overall | 601 | 23.9/16.6 (7.6-59.1) | 18.8/12.8 (5.0-46.0) | 5.1/3.2 (1.2-12.6) | 89/56 (28-174) | 32.4/23.5 (8.5-78.0) | 23.6/14.5 (7.2-36.9) | 33.0/16.4 (8.7-66.5) |
| Sex | Male | 356 | 24.1/15.3 (7.7-55.6) | 18.7/12.2 (5.0-43.3) | 5.4/2.9 (1.2-13.2) | 98/59 (30-178) | 34.2/26.2 (9.0-79.8) | 26.2/14.7 (7.4-36.9) | 37.4/17.1 (8.4-71.4) |
| | Female | 245 | 23.6/18.5 (7.6-61.6) | 18.9/14.6 (5.3-50.8) | 4.7/3.5 (1.2-12.2) | 76/55 (26-164) | 29.7/20.6 (7.3-71.3) | 20.0/13.8 (7.2-37.0) | 26.7/16.0 (8.9-60.9) |
| | $P_{difference}$* | | <0.01 | <0.01 | 0.08 | <0.01 | <0.01 | 0.16 | 0.13 |
| Age | <60 y | 289 | 23.9/15.6 (7.5-62.9) | 18.8/12.0 (5.1-50.8) | 5.1/3.2 (1.2-13.2) | 97/54 (27-148) | 33.8/23.9 (8.9-73.1) | 27.6/14.2 (7.1-33.2) | 35.9/15.1 (7.8-51.9) |
| | ≥60 y | 312 | 23.8/17.6 (7.7-51.5) | 18.8/13.5 (5.0-44.7) | 5.1/3.0 (1.1-11.4) | 81/58 (28-185) | 31.1/23.4 (7.9-78.0) | 20.0/14.6 (7.4-38.2) | 30.3/18.7 (9.3-76.8) |
| | $P_{difference}$* | | 0.12 | 0.06 | 0.41 | 0.05 | 0.92 | 0.23 | <0.01 |
| European region† | North | 338 | 27.5/19.0 (9.3-60.6) | 22.1/14.9 (6.8-49.0) | 5.4/3.2 (1.3-14.4) | 113/64 (32-236) | 37.8/27.2 (10.0-90.5) | 30.0/15.7 (8.0-51.1) | 44.9/20.8 (9.6-97.8) |
| | South | 263 | 19.3/14.4 (6.7-47.7) | 14.6/10.7 (4.2-38.9) | 4.6/3.2 (1.2-12.1) | 59/48 (25-101) | 25.5/20.0 (7.0-55.6) | 15.5/12.9 (6.7-24.5) | 17.8/14.1 (7.8-28.3) |
| | $P_{difference}$* | | <0.01 | <0.01 | 0.87 | <0.01 | <0.01 | <0.01 | <0.01 |
| Smoking status | Never | 256 | 25.5/18.2 (7.6-58.0) | 20.7/13.8 (5.4-48.2) | 4.7/3.4 (1.4-13.2) | 103/59 (28-189) | 33.4/24.7 (8.5-79.8) | 29.0/15.1 (7.2-35.4) | 40.5/17.2 (9.7-59.4) |
| | Former | 198 | 27.8/18.0 (7.8-69.5) | 21.1/13.8 (5.8-53.2) | 6.7/3.3 (1.2-17.0) | 89/59 (33 -174) | 35.4/26.7 (9.0-82.4) | 21.5/14.8 (7.8-37.3) | 31.8/17.6 (9.9-76.8) |
| | Current | 136 | 15.7/13.1 (6.3-39.0) | 12.2/9.9 (4.3-30.2) | 3.5/2.7 (0.9-8.0) | 65/47 (24-148) | 26.3/19.1 (7.3-62.5) | 17.5/12.8 (6.6-38.2) | 21.4/13.7 (6.5-52.4) |
| | Unknown | 11 | 15.7/14.2 (5.4-26.9) | 12.1/11.7 (4.4-20.5) | 3.6/2.4 (0.7-8.1) | 69/51 (21-232) | 29.9/20.1 (5.5-89.7) | 15.3/11.6 (6.5-54.7) | 23.5/16.1 (8.9-87.7) |
| | $P_{trend}$‡ | | <0.01 | <0.01 | <0.01 | <0.01 | <0.01 | <0.01 | <0.01 |
| *Hp* infection | No | 196 | 25.0/16.5 (7.4-69.5) | 19.1/12.8 (5.1-53.5) | 5.8/2.8 (1.3-16.3) | 107/57 (31-168) | 32.8/25.1 (9.4-78.0) | 30.7/14.3 (7.4-35.3) | 43.7/18.1 (9.6-71.4) |
| | Yes | 404 | 23.3/16.6 (7.7-51.5) | 18.6/12.8 (5.0-43.8) | 4.7/3.3 (1.1-12.1) | 80/56 (27-185) | 32.1/22.3 (7.6-78.0) | 20.2/14.6 (7.2-38.2) | 27.8/16.1 (8.2-66.5) |
| | $P_{difference}$* | | 0.71 | 0.57 | 0.18 | 0.36 | 0.30 | 0.70 | 0.13 |
| SCAG | No | 548 | 24.5/16.9 (7.6-60.5) | 19.3/12.9 (5.1-48.2) | 5.2/3.2 (1.2-13.0) | 91/56 (28-172) | 32.7/23.9 (8.9-72.1) | 24.3/14.5 (7.4-36.5) | 34.2/16.5 (8.4-64.8) |
| | Yes | 45 | 17.2/15.0 (7.9-31.8) | 13.3/11.0 (4.4-28.4) | 3.7/3.0 (0.9-8.2) | 68/48 (26-232) | 29.2/18.8 (7.0-110.0) | 16.6/13.2 (7.0-50.7) | 22.3/15.0 (9.1-76.3) |
| | $P_{difference}$* | | 0.12 | 0.11 | 0.20 | 0.08 | 0.08 | 0.24 | 0.35 |

*$P$ for difference based on median concentrations.
†North: Scandinavia, United Kingdom, and Central Europe; south: Italy, Spain, and Greece.
‡Category unknown not included in the test for trend.

### Study Design

An important strength of our study is the availability of blood samples before cancer diagnosis. One could argue that a mean lag time of 3 years between blood donation and cancer diagnosis may be too short to obtain unbiased associations between the vitamins and GC. Vitamin concentrations measured shortly before diagnosis should be considered with caution because low vitamin status may be a consequence of malignancy rather than causing it. In this regard, we previously observed that cobalamin was inversely associated with GC risk, particularly among individuals with severe atrophic chronic gastritis (9). This agrees with a reduced bioavailability of cobalamin due to infection with *H. pylori* and atrophic gastritis (45), which may precede GC (46). Notably, in the present study, the inverse association between cancer risk and concentrations of vitamin B2 and B6 was strongest in cases with SCAG. This may suggest that impaired vitamin B2 and B6 status contributes to the development of GC or that atrophic gastritis may adversely affect the status of these vitamins. In line with the latter possibility, reduced PLP concentrations are consistently associated with inflammation (47-49). In the present study, vitamin B2 and B6 concentrations did not differ significantly from people with and without SCAG, and correction for low pepsinogen concentrations did not confound the association between the vitamins and GC. It should be noted that low PGA concentrations are a marker for severe gastritis (50). Results of the present study do therefore only apply to severe gastritis and not for milder forms of gastritis. Previous studies on the association of the vitamins B2 (10, 13-21) and B6 (10, 13, 15-18) with GC investigated the effects of dietary intake rather than plasma concentrations. Blood sampling is less likely to result in bias due to measurement errors that are inherent to food frequency questionnaires. Within this multicenter study, all study centers collected and stored blood samples according to a standardized protocol (31), and all biochemical analyses were carried out in one laboratory, thereby optimizing sample treatment during analysis. Moreover, the present study included plasma concentrations of several B2 and B6 species. Although individual vitamin B2 and B6 species reflect vitamin B2 (51) and B6 (47) status, there is no clear consensus as to which vitamin specie is the best marker. We also used the sum of the individual species as indices of vitamin B2 and B6 status because the B2 species (37-40) and the B6 species (41, 42) are interconvertible, and the plasma concentrations are positively correlated, as shown here and by others (44).

### Vitamin B2 and B6 Intake and GC

The most important dietary sources of vitamin B2 are milk and dairy products (52). Contrary to our results showing an inverse association of plasma vitamin B2 with GC risk, other studies observed that vitamin B2 (18, 19) and milk intake (15, 53) are associated with increased risk. Although information on dairy intake was not taken into account in the present study, it is possible that milk intake is increased to reduce gastric pain during disease progression. Vitamin B6 may be obtained from various food groups, including fruit, vegetables, and meat (41), and once ingested, all vitamin B6 species are converted into PLP and PL (41, 42). No strong overall evidence was observed for a protective role of fruit and vegetable intake in GC in our study population (54) or in other cohort studies (55, 56). In contrast, meat intake has been found to be associated with increased risk of the noncardia subsite of GC in the EPIC cohort (57).

### Mechanism

Studies in riboflavin-deficient rats show an increased GC risk, increased induction of DNA repair enzymes (58), and increased carcinogen binding to DNA (59) compared with rats on a riboflavin-replete diet. Moreover,

**Table 3.** RRs (95% CI) for GC risk by quartiles of plasma vitamins B2 and B6 indices

| | Case/control | Q1* | Q2 | Q3 | Q4 | RR/quartile | $P_{trend}$ |
|---|---|---|---|---|---|---|---|
| Vitamin B2 sum | 231/590 | 1 | 0.78 (0.48-1.27) | 0.71 (0.43-1.17) | 0.61 (0.36-1.03) | 0.85 (0.72-1.01) | 0.06 |
| Riboflavin | 231/591 | 1 | 1.02 (0.64-1.64) | 0.73 (0.44-1.23) | 0.71 (0.42-1.19) | 0.87 (0.74-1.03) | 0.09 |
| FMN | 231/590 | 1 | 1.18 (0.76-1.85) | 0.64 (0.38-1.08) | 0.55 (0.32-0.96) | 0.79 (0.67-0.95) | 0.01 |
| Vitamin B6 sum | 233/596 | 1 | 0.70 (0.44-1.11) | 0.59 (0.35-0.98) | 0.46 (0.26-0.80) | 0.78 (0.65-0.93) | 0.006 |
| PLP | 235/600 | 1 | 0.82 (0.51-1.30) | 0.61 (0.36-1.05) | 0.64 (0.36-1.13) | 0.85 (0.71-1.02) | 0.08 |
| PL | 234/599 | 1 | 1.02 (0.65-1.60) | 0.56 (0.33-0.95) | 0.74 (0.44-1.26) | 0.86 (0.72-1.02) | 0.07 |
| PA | 234/599 | 1 | 0.87 (0.56-1.35) | 0.81 (0.50-1.30) | 0.48 (0.28-0.83) | 0.81 (0.68-0.96) | 0.01 |

NOTE: Calculated by conditional logistic regression, stratified by the case-control set and adjusted for Hp infection status and smoking. Controls were matched to cases by age, sex, study center, and date of blood sample collection. The cutoff values for the quartiles were as follows: vitamin B2 sum, 11.88, 16.63, and 25.15 μmol/L; riboflavin, 8.52, 12.80, and 20.00 μmol/L; FMN, 2.13, 3.15, and 5.21 μmol/L; vitamin B6 sum, 40.8, 55.7, and 80.6 μmol/L; PLP, 14.90, 23.50, and 36.60 μmol/L; PL, 10.60, 14.50, and 18.97 μmol/L; and PA, 12.60, 16.40, and 24.90 μmol/L.
*Reference category.

**Table 4.** RRs (95% CI) for GC risk by quartiles of plasma concentrations of vitamin B2 and B6 indices in cases with and without SCAG

| | SCAG* | Case/control | Q1† | Q2 | Q3 | Q4 | RR/quartile | $P_{trend}$ | $P_{interaction}$ |
|---|---|---|---|---|---|---|---|---|---|
| Vitamin | Yes | 42/106 | 1 | 1.10 (0.32-3.85) | 0.46 (0.12-1.78) | 0.31 (0.07-1.39) | 0.64 (0.40-1.04) | 0.07 | |
| B2 sum | No | 187/474 | 1 | 0.76 (0.44-1.31) | 0.76 (0.44-1.33) | 0.77 (0.43-1.37) | 0.92 (0.77-1.11) | 0.40 | 0.23 |
| Riboflavin | Yes | 42/106 | 1 | 1.49 (0.47-4.79) | 0.61 (0.15-2.50) | 0.33 (0.07-1.45) | 0.65 (0.41-1.02) | 0.06 | |
| | No | 187/475 | 1 | 0.91 (0.54-1.55) | 0.74 (0.42-1.30) | 0.89 (0.50-1.58) | 0.94 (0.78-1.13) | 0.53 | 0.21 |
| FMN | Yes | 42/106 | 1 | 0.66 (0.19-2.33) | 0.11 (0.02-0.61) | 0.06 (0.01-0.44) | 0.38 (0.20-0.73) | <0.01 | |
| | No | 187/474 | 1 | 1.25 (0.77-2.02) | 0.79 (0.45-1.39) | 0.73 (0.41-1.32) | 0.87 (0.73-1.05) | 0.16 | 0.03 |
| Vitamin | Yes | 41/105 | 1 | 0.44 (0.14-1.42) | 0.57 (0.15-2.09) | 0.20 (0.04-0.94) | 0.64 (0.40-1.03) | 0.07 | |
| B6 sum | No | 190/481 | 1 | 0.74 (0.44-1.25) | 0.58 (0.32-1.05) | 0.52 (0.28-0.96) | 0.80 (0.66-0.98) | 0.03 | 0.78 |
| PLP | Yes | 42/106 | 1 | 0.84 (0.28-2.56) | 0.55 (0.15-2.03) | 0.50 (0.13-1.96) | 0.78 (0.50-1.22) | 0.27 | |
| | No | 191/484 | 1 | 0.82 (0.49-1.38) | 0.63 (0.34-1.15) | 0.65 (0.34-1.24) | 0.86 (0.69-1.05) | 0.14 | 0.95 |
| PL | Yes | 42/105 | 1 | 0.80 (0.25-2.55) | 0.97 (0.29-3.22) | 0.50 (0.11-2.19) | 0.85 (0.54-1.33) | 0.47 | |
| | No | 190/484 | 1 | 1.09 (0.65-1.80) | 0.47 (0.26-0.87) | 0.81 (0.44-1.46) | 0.86 (0.71-1.04) | 0.12 | 0.59 |
| PA | Yes | 42/105 | 1 | 0.46 (0.16-1.27) | 0.35 (0.10-1.20) | 0.10 (0.02-0.62) | 0.51 (0.30-0.86) | 0.01 | |
| | No | 190/484 | 1 | 1.04 (0.63-1.71) | 1.02 (0.60-1.74) | 0.63 (0.35-1.15) | 0.88 (0.73-1.06) | 0.19 | 0.15 |

NOTE: RR (95% CI) calculated by conditional logistic regression, stratified by the case-control set and adjusted for Hp infection status and smoking. Of the 45 matched controls with SCAG, 32% (34 of 106) were matched to cases with SCAG and 2% (11 of 484) to cases without SCAG. The cutoff values for the quartiles were as follows: vitamin B2 sum, 11.88, 16.63, and 25.15 μmol/L; riboflavin, 8.52, 12.80, and 20.00 μmol/L; FMN, 2.13, 3.15, and 5.21 μmol/L; vitamin B6 sum, 40.8, 55.7, and 80.6 μmol/L; PLP, 14.90, 23.50, and 36.60 μmol/L; PL, 10.60, 14.50, and 18.97 μmol/L; and PA, 12.60, 16.40, and 24.90 μmol/L.

*SCAG defined as PGA concentrations <22 μg/L.

†Reference category.

some carcinogens are metabolized by flavin-dependent enzymes, and vitamin B2 status may modify effects of the carcinogen (58). PLP catalyzes ~100 essential enzymatic reactions in human metabolism (60). Although it is unclear whether low vitamin concentrations are a cause or a consequence of the disease, low PLP concentrations have been associated with high concentrations of inflammatory markers (47-49), which may explain our finding of stronger associations among those cases with SCAG. Furthermore, low PLP concentrations cause a decreased enzyme activity of serine hydroxymethyltransferase, which results in a lack of methylene groups for 5,10-methylenetetrahydrofolate production (61). This may lead to increased chromosome strand breaks (62), impaired DNA repair (63, 64), and DNA hypomethylation (65, 66), which has been observed in different types of tumors.

The majority of previous studies on genetic variations related to one-carbon metabolism in GC focused on the *MTHFR* gene and were mainly conducted in Asian populations (24). The overall result of a meta-analysis shows that in East Asian populations, but not in Caucasians, the risk of GC increases with the number of T alleles of the *MTHFR 677C→T* polymorphism (24). Published results for this EPIC population showed no association between the *MTHFR 677C→T* polymorphism and GC (9), and the null findings in the present report are in line with previous studies investigating polymorphisms of the *MTRR 66A→G* (26, 27), *MTR 2756A→G* (27), and *SLC19A1 180G→A* (29) genes, but not with one study showing increased GC risk with variant genotypes of the *MTHFD1 1958G→A* (28) polymorphism. Notably, we observed opposite trends for the *CBS 844ins68*, the *TCN2 776*, and the *SLC19A1 180* polymorphisms in northern and southern European countries. Furthermore, none of the polymorphisms modified the associations of the vitamins B2 and B6 with GC risk. However, sample sizes might have been too small to detect genetic associations with GC risk, interactions between genes and European region, and interactions between genes and vitamins and might have resulted in chance findings.

In summary, this large prospective European multicenter study revealed that higher concentrations of vitamin B2 suggest to decrease GC risk, whereas vitamin B6 were associated with a decreased GC risk, with more pronounced associations in a subgroup with SCAG. Furthermore, none of the polymorphisms related to one-carbon metabolism was associated with GC risk, nor did they modify the associations of vitamin B2 and B6 with this type of cancer.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

**Table 5.** Distribution of genotypes by European region and their associations with GC risk

| Gene and SNP | Genotype | GC (all) | | GC (northern Europe) | | GC (southern Europe) | |
|---|---|---|---|---|---|---|---|
| | | Case/control | OR (95% CI) | Case/control | OR (95% CI) | Case/control | OR (95% CI) |
| *CBS 699* | *CC* | 103/282 | 1 | 62/147 | 1 | 41/135 | 1 |
| | *CT* | 118/251 | 1.26 (0.91-1.74) | 72/141 | 1.18 (0.77-1.81) | 46/110 | 1.37 (0.84-2.24) |
| | *TT* | 16/69 | 0.58 (0.31-1.08) | 11/37 | 0.62 (0.28-1.37) | 5/32 | 0.52 (0.19-1.43) |
| | | MAF = 0.32 | $P_{trend}$ = 0.61 | MAF = 0.33 | $P_{trend}$ = 0.65 | MAF = 0.31 | $P_{trend}$ = 0.81 |
| *CBS** | *0 insertions* | 210/536 | 1 | 128/312 | 1 | 82/224 | 1 |
| | *1 insertion* | 34/91 | 0.96 (0.62-1.49) | 24/40 | 1.44 (0.82-2.53) | 10/51 | 0.54 (0.26-1.12) |
| | *2 insertions* | 3/4 | 2.00 (0.43-9.35) | 2/1 | 4.40 (0.36-53.5) | 1/3 | 1.10 (0.11-10.8) |
| | | MAF = 0.08 | $P_{trend}$ = 0.81 | MAF = 0.07 | $P_{trend}$ = 0.10 | MAF = 0.09 | $P_{trend}$ = 0.15 |
| *MTR 2756* | *AA* | 171/415 | 1 | 104/219 | 1 | 68/196 | 1 |
| | *AG* | 64/188 | 0.80 (0.57-1.12) | 42/108 | 0.77 (0.49-1.21) | 22/80 | 0.82 (0.48-1.40) |
| | *GG* | 8/13 | 1.58 (0.62-3.99) | 4/11 | 0.76 (0.23-2.47) | 4/2 | 13.1 (1.43-120) |
| | | MAF = 0.17 | $P_{trend}$ = 0.56 | MAF = 0.18 | $P_{trend}$ = 0.26 | MAF = 0.15 | $P_{trend}$ = 0.62 |
| *MTRR 66* | *AA* | 58/156 | 1 | 33/72 | 1 | 24/84 | 1 |
| | *AG* | 100/286 | 0.96 (0.65-1.41) | 55/149 | 0.82 (0.48-1.40) | 45/137 | 1.13 (0.65-1.98) |
| | *GG* | 81/165 | 1.25 (0.82-1.91) | 59/109 | 1.20 (0.70-2.07) | 22/56 | 1.26 (0.64-2.48) |
| | | MAF = 0.52 | $P_{trend}$ = 0.28 | MAF = 0.57 | $P_{trend}$ = 0.39 | MAF = 0.46 | $P_{trend}$ = 0.51 |

NOTE: RR (95% CI) calculated by conditional logistic regression, stratified by country and adjusted for age and sex.
Abbreviation: MAF, minor allele frequency (cases and controls combined).
*Significant interaction term ($P$ = 0.03) for *CBS 844ins68* with European region.

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