

Biomarkers of folate and vitamin B12, alcohol intake and breast cancer risk: report from the EPIC cohort

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Abstract

Background. B vitamin status and their interaction with alcohol were suggested to play a role in breast carcinogenesis; however, results from epidemiological studies have been inconsistent. We investigated the association between biomarkers of folate and vitamin B12 and the risk of breast cancer (BC) in a nested case-control study within the European Prospective Investigation into Cancer and Nutrition (EPIC) cohort.

Methods. Microbiological assays were used to determine plasma concentrations of folate and vitamin B12 in 2,491 BC cases individually matched to 2,521 controls among women participants to the EPIC study who provided baseline blood samples. Multivariable conditional logistic regression models were used to estimate odds ratios by quartiles of plasma B vitamins. Subgroup analyses by menopausal status, hormone receptor status of breast tumors (ER, PR, and HER2), levels of alcohol intake, and MTHFR polymorphisms (677C>T and 1298A>C) were also performed.

Results. Plasma concentrations of folate and vitamin B12 were not significantly associated with the overall risk of BC. No significant association emerged by hormone receptor status. A borderline positive association was observed between plasma concentrations of vitamin B12 and BC risk in women consuming above the median level of alcohol ($OR_{Q4-Q1} = 1.30$; 95% CI 1.03-1.64; $P_{trend} = 0.051$). Plasma concentrations of vitamin B12 were also marginally associated with BC risk in women with plasma folate levels below the median value ($OR_{Q4-Q1} = 1.26$; 95% CI 1.00–1.60; $P_{trend} = 0.014$). However, no significant heterogeneity between subgroups of alcohol intake ($P_{heterogeneity} = 0.14$) and plasma folate ($P_{heterogeneity} = 0.059$) was found. The association between MTHFR polymorphisms and BC risk in a subsample of this study population was not statistically significant.

Conclusions. The present study raises the possibility for a role of vitamin B12 in the etiology of BC, and provides support for potential interactions between nutrients involved in one-carbon metabolism.

Introduction

The etiology of BC is complex and results from the combination of lifetime reproductive events, genetics, dietary, and lifestyle factors (1). According to the latest Breast Cancer Report from the World Cancer Research Fund (WCRF), there is novel evidence that alcohol intake and factors that lead to a greater adult attained height are positively associated with postmenopausal and probably also premenopausal breast cancer (2). The European Prospective Investigation into Cancer and Nutrition (EPIC) study reported a decreased risk of BC associated with high intake of vegetable fiber (3) and a nutrient pattern high in micronutrients from vegetables, fruit and cereals (4), but an increased risk related to alcohol (5), high processed meat consumption (6), and biomarkers of industrial trans-monounsaturated fatty acids (7). Among micronutrients, dietary folate intake has also been suggested to play a role in breast carcinogenesis (8).

Vitamin B9 (folate) and vitamin B12 (cobalamin) are two water soluble B vitamins essentially involved in one-carbon metabolism (9), which generates substrates for DNA methylation and DNA synthesis (10). Thus, deficiencies of these nutrients may trigger both genetic and epigenetic pro-carcinogenic processes (11).

The association between dietary intake of folate and vitamin B12 with BC risk has been widely investigated. However, findings from prospective studies have been inconsistent, reporting either an inverse association (12;13), a positive association (14-16), or no significant association (17-19) between these vitamins and the overall risk of BC. The prospective investigation of the relationship between biomarkers of vitamin status and BC risk has also produced mixed results (20-24).

A number of factors have been suggested to influence the association between these nutrients estimated through dietary questionnaires or measurement of biomarkers and the risk of BC, including menopausal status (12;14;15;20;23;25), alcohol consumption (8;26;27), nutrient interactions (13), and methylenetetrahydrofolate reductase (MTHFR) gene polymorphisms (24;28). MTHFR is a key enzyme in one-carbon metabolism, where it balances the folate pool between synthesis and methylation of DNA (29). Thus,

functional polymorphisms in the gene coding for this enzyme may influence the risk of BC associated with folate status. Breast tumors are also subdivided into subgroups according to the expression of sex hormone receptors (ER, PR, and HER2), which have been differentially associated with both folate intake (8) and folate status (23).

We conducted a large nested case-control study within EPIC to evaluate the association between plasma concentrations of folate and vitamin B12, as biomarkers of vitamin status, and BC risk overall and stratified by potential risk factors. In addition, we examined the association between the MTHFR 677C>T (rs1801133) and 1298A>C (rs1801131) polymorphisms and BC risk using data from a subsample of the French part of the EPIC study.

Methods

Study design

The EPIC study is an on-going multi-centre European cohort study designed to investigate the role of dietary habits and lifestyle factors on the incidence of cancer of various sites, including BC (30). The cohort includes over 521,000 participants recruited between 1992 and 2000 from 23 centres in 10 European countries (Denmark, France, Germany, Greece, Italy, the Netherlands, Norway, Spain, Sweden, and the UK). Details of the recruitment procedures and data collection in the EPIC study have been previously described in details (31). Briefly, socio-demographic, lifestyle and dietary data were collected at baseline from all the cohort members by administration of country-specific questionnaires. Anthropometric measurements and peripheral blood samples of the participants were also collected. Methods of blood collection, processing, and storage are described in details elsewhere (32). All participants signed an informed consent for the use of their blood samples and data. The study was approved by the Ethical Review Board of the IARC and those of all national recruiting centres.

Selection of study subjects

Of 367,903 women recruited into the EPIC study, the present analysis excluded women with prevalent cancers at recruitment ($n = 19,853$) and missing diagnosis or censoring date ($n = 2892$). A total of 10,713 women with malignant primary BC were identified after a median follow-up of 11.5 years. A nested case-control study was designed among women who provided a blood sample and completed the lifestyle and dietary questionnaires at recruitment. Because of a flooding that occurred in the Danish Biobank, samples from Denmark were not included, leading to 2,491 BC cases with a confirmed first diagnosis of invasive BC during an average follow-up of 18 years between 1992 and 2010. Each case was individually matched to at least one control subject chosen randomly among cohort women with available

blood samples and free of cancer (except non-melanoma skin cancer) at the time of diagnosis of the corresponding case. Control subjects were matched to cases for study center, age at blood donation (± 3 months), time of the day at blood collection (± 1 hour), exogenous hormone use at blood collection (yes; no; unknown), menopausal status (pre; surgical post; natural post), fasting status (< 3 hrs; 3-6 hrs; >6 hrs) and phase of the menstrual cycle (early follicular; late follicular; periovulatory; mid luteal; other luteal) at recruitment.

Dietary and lifestyle data collection

Dietary data were obtained at enrolment using validated country-specific dietary history and food-frequency questionnaires (FFQs), designed to collect local dietary habits of the participants over the preceding year (31). Dietary intakes of folate and vitamin B12 were estimated using the updated EPIC Nutrient Database (ENDB) (33), following standardization from country-specific food composition tables (FCT) according to Bouckaert's recommendations (34). Details on dietary assessment have been discussed previously (8).

Participants also completed a baseline lifestyle questionnaire providing information on anthropometric and socio-demographic characteristics, reproductive history, family history of cancer, physical activity, alcohol use, smoking habits, use of oral contraceptives, hormone replacement therapy, and vitamin supplements in the year prior to enrolment date.

Outcome assessment

Participants were followed from the date of enrolment until first cancer diagnosis, death, emigration or end of the follow-up period, whichever occurred first. Incident cancer cases were identified through population cancer registries (Denmark, Italy except Naples, the Netherlands, Norway, Spain,

Sweden and the UK) or by a combination of methods including health insurance, cancer and pathology registries, and active follow-up through study subjects and their next-of-kin in three countries (France, Germany, Greece and Naples). Data on clinical and tumor characteristics were coded according to the 10th Revision of the International Statistical Classification of Diseases, Injuries and Causes of Death (ICD).

In the present study, 91% of BC cases were confirmed by histological or cytological examination, whereas the remaining 9% was diagnosed through clinical observation, ultrasound, autopsy, or death certificate. The most frequent subtype of BC was ductal carcinoma (71.5%), followed by lobular carcinoma (14.1%) and tubular carcinoma (2.7%). The remaining BC cases were classified as mixed (5.0%) or other (6.7%) subtypes.

Hormone receptor status determination

Determination of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor (HER2) status of BC cases was performed within each EPIC center. Information on hormone receptor status as well as on the methods for its determination was retrieved from each EPIC center using the same approaches used for collection of incident cases. To standardize the quantification of the receptor status collected across centers, the following criteria were applied for a positive receptor status: $\geq 10\%$ cells stained, any 'plus-system' description, ≥ 20 fmol/mg, an Allred score of ≥ 3 , an IRS ≥ 2 , or an H-score ≥ 10 (35). ER, PR, and HER2 status was available for 98%, 84%, and 44% of cases, respectively. For the remaining cases, hormone receptor status was not determined because of insufficient amount of tumor tissue available for histopathological evaluation. Furthermore, HER2 status could not be ascertained in the majority of cases because of the lack of a specific test in the nineties.

Laboratory measurements

All biochemical analyses were performed at the Bevital AS laboratory in Bergen, Norway (www.bevital.no). Microbiological assays were used to determine plasma concentrations of folate (36) and vitamin B12 (37). The assays were adapted to a microtiter plate format and carried out by a robotic workstation. Throughout all steps of the biochemical analysis, samples from each case-control set were analyzed within the same batch. The laboratory personnel were blinded to case-control status. To assess the measurement precision, each batch contained six quality control (QC) samples with known biomarker concentrations and four samples without biomarker (blanks). The six QC samples were three samples in parallels. The coefficient of variation calculated from the three duplicate sets of identical QC samples was 8.6% for folate and 5.0% for vitamin B12. Plasma concentrations of folate and vitamin B12 were determined for all study participants.

Genotyping analysis

Determination of the genotype status was carried out only for a subsample of 401 cases and 401 matched control individuals from the French part of the EPIC study. DNA extraction from white blood cells was carried out using Autopure LS kit (Gentra Systems, Minneapolis, MN). DNA concentration was quantified with Quant-iT PicoGreen dsDNA reagent (Molecular Probes).

The MTHFR 677C>T (rs1801133) and 1298A>C (rs1801131) single nucleotide polymorphisms (SNPs) were genotyped by Kaspar allelic discrimination assay using allele specific probes and fluorescent reporters (LGC Group, UK). Each reaction was carried out according to the manufacturer's instructions using supplied kits. Amplifications and end-point allele determination were performed in 96-well plates using a StepOne Plus system (Applied Biosystems). Each plate contained randomly placed case and control samples, while matched sets were analysed within the same plate. Genotyping success rates were 98.0% and 96.5% for rs1801131 and rs1801133, respectively. Samples not yielding genotypes were removed from further analyses.

Statistical methods

Lifestyle and dietary baseline characteristics of study participants were described using mean \pm standard deviation (SD) for continuous variables and percentages for categorical variables. Plasma concentrations of folate and vitamin B12 were log natural transformed to normalize their distribution. The paired t-test and Chi-square test from unadjusted conditional logistic regression were used to assess differences between cases and control individuals with regard to continuous and categorical variables, respectively.

Pearson's partial correlation coefficients adjusted for age, body mass index (BMI) and energy intake at baseline were computed to assess linear correlations between log natural transformed plasma concentrations of folate and vitamin B12 and estimated dietary intakes of these nutrients and alcohol.

Multivariable conditional logistic regression models were used to estimate odds ratios (ORs) and 95% confidence intervals (95% CIs) for overall BC and specific subgroups stratified by menopausal status at recruitment (dichotomized as natural/surgical postmenopausal and premenopausal) and by hormone receptor status (ER+/ER-, PR+/PR-, HER2+/HER2-). In addition, the association between each plasma biomarker and the risk of BC was examined using four-knot restricted cubic splines with the midpoint of the 5th decile of plasma vitamin B12 as the reference category (38;39).

Quartiles and tertiles of plasma levels of biomarkers for the overall and hormone receptor specific analyses, respectively, were determined on the basis of the distribution among control individuals. Tests for linear trends were performed by entering the categorical variable as continuous term in the multivariable models.

All multivariate models were adjusted by BMI, height, alcohol intake, total energy intake, educational attainment (primary school; technical/professional school; secondary school; university degree; 4.2% unknown), physical activity (inactive; moderately inactive; moderately active; active; 6.9% unknown), ever use of hormone replacement therapy (never; ever; 4.1% unknown), parity and age at first full-term birth combined (nulliparous; <30 y and 1-2 children; <30 y and \geq 3 children; >21–30 y; \geq 30 y;

3.6% unknown), and family history of BC (yes; no; 53.1% unknown). These variables were previously related to BC risk or blood measurements. Unknown categories of the above mentioned variables were included in the model using indicator variables.

Multivariate unconditional logistic regression models were used to investigate the association between plasma vitamin B12 and BC risk by levels of either alcohol intake or plasma folate (low and high levels based on median values). The joint effect of plasma folate (in tertiles) and categories of alcohol intake (0-3 g/d; 3-12 g/d; >12 g/d) on BC risk was evaluated by using the lowest tertile of plasma folate and highest category of alcohol intake as reference category, as previously assessed (8).

Tests for interaction between each plasma biomarker as continuous variable and potential risk factors were computed by likelihood ratio test. Formal tests of heterogeneity between ORs in menopausal and hormone receptor subgroups were based on Chi-square statistics, calculated as the deviations of logistic beta-coefficients observed in each of the subgroups relative to the overall beta-coefficient.

Genotypic (codominant) and dominant models were assumed for SNP effects. A trend test was conducted by treating the genotypes as equally spaced integer weights and entering the variable as a continuous term in the model. Effect modification of the SNP-BC association was investigated with conditional logistic regression stratifying on menopausal status instead of the matched sets and with age as covariate.

Specific sensitivity analyses were carried out by excluding women consuming multivitamin supplements and cases diagnosed within the first two years of follow-up (to reduce the chance of reverse causality).

Statistical tests were two-sided, and P values below .05 were considered statistically significant. All analyses were performed using STATA 12.1 (StataCorp. 2011, Stata Statistical Software: Release 11. College Station, TX: StataCorp LP).

Results

Table 1 summarizes the socio-demographic, reproductive and lifestyle characteristics of study participants by case-control status. Cases had slightly older age at menopause ($P = 0.026$) and at first live birth ($P=0.023$) than control individuals. A slightly higher BMI in cases compared with the control group was found among postmenopausal women ($P < 0.001$), but not among premenopausal women. Cases were also less physically active ($P = 0.0323$), more likely to have had a first-degree relative with breast cancer ($P = 0.009$), and had higher daily alcohol intake ($P = 0.002$). Both MTHFR SNPs were in Hardy-Weinberg equilibrium ($P = 0.298$ for C677T; $P = 0.823$ for A1298C), and the frequency of the minor allele among control individuals was 30.8% at locus C677T and 37.0% at locus A1298C (data not shown).

There was no significant association between plasma levels of folate and vitamin B12 and the overall risk of BC in either the continuous or categorical model (Table 2). Stratified analyses revealed a borderline increase in BC risk associated with increasing levels of plasma vitamin B12 among premenopausal women ($P_{\text{trend}} = 0.047$), while no suggestive association was found among postmenopausal women ($P_{\text{trend}} = 0.625$). However, the test of heterogeneity between the two subgroups was not statistically significant ($P_{\text{heterogeneity}} = 0.679$). No further association emerged after adjustment by MTHFR polymorphisms for the available subsample (data not shown).

Figure 1 shows a nonlinear model of the association between plasma concentrations of vitamin B12 and BC risk. There was a borderline significant trend ($P_{\text{trend}} = 0.071$) in increased risk associated with plasma concentrations of vitamin B12 higher than 360 pmol/l, while the odds ratio plateaued at levels ≥ 500 pmol/l. No dose dependent effect of plasma folate on the risk of BC was observed (data not shown).

No significant association emerged between plasma biomarkers of B vitamins and BC risk emerged according to ER, PR, or HER2 status (Table 3). However, a suggestive positive association between levels of plasma vitamin B12 and ER- BC risk was observed ($OR_{T3-T1} = 1.26$; 95% CI 0.85-1.86; $P_{\text{trend}} = 0.26$).

Figure 2 reports risk estimates by tertiles of plasma folate and categories of alcohol consumption. The association between plasma folate concentration and BC risk was not significantly modified by levels of alcohol intake ($P_{\text{interaction}} = 0.6943$).

The association between plasma levels of vitamin B12 and BC risk stratified by the median intake of alcohol is summarized in Table 4. There was a borderline significant increase in risk associated with the highest quartile of plasma vitamin B12 in women consuming at least 3.36 g/day of alcohol ($OR_{Q4-Q1} = 1.30$; 95% CI 1.03-1.64; $P_{\text{trend}} = 0.051$), while no significant association emerged in women drinking lower amounts of alcohol ($OR_{Q4-Q1} = 1.00$; 95% CI 0.80-1.26; $P_{\text{trend}} = 0.928$). However, no significant heterogeneity by alcohol intake was found ($P_{\text{heterogeneity}} = 0.14$). The multivariable risk estimates did not change appreciably after further adjustment by plasma folate concentration (data not shown).

A statistically significant interaction between plasma concentrations of folate and vitamin B12 on the risk of BC was observed ($P_{\text{interaction}} = 0.0418$; data not shown). To further explore this interaction, a stratification analysis by the median level of plasma folate was carried out (Table 4). A marginally increased risk of BC associated with increasing concentrations of plasma vitamin B12 was found in women with plasma levels of folate below 13.56 nmol/l ($OR_{Q4-Q1} = 1.26$; 95% CI 1.00–1.60; $P_{\text{trend}} = 0.014$), while no significant association occurred in women with higher levels of plasma folate ($P_{\text{trend}} = 0.806$). A borderline significant heterogeneity by plasma folate levels was also found ($P_{\text{heterogeneity}} = 0.059$).

Exclusion from analyses of women who consumed multivitamin supplements or cases diagnosed within the first two years of follow-up did not change the risk estimates in our study population (data not shown).

The association between MTHFR polymorphisms and BC risk was evaluated in a subsample of 401 cases and 401 matched control individuals from this nested case-control population. The 677C>T and 1298A>C SNPs were in strong linkage disequilibrium among both the cases ($D' = 1.00$) and control individuals ($D' = 0.98$). There was no significant association of either C677T ($OR_{TT \text{ vs. } CC} = 0.71$; 95% CI 0.42-1.19; $P_{\text{trend}} = 0.380$) or A1298C ($OR_{CC \text{ vs. } AA} = 0.97$; 95% CI 0.62-1.53; $P_{\text{trend}} = 0.915$) with the overall

risk of BC (data not shown). No further associations emerged in the dominant models, and further adjustment for the alternative SNP did not change the risk estimates (data not shown).

Discussion

We conducted a large case-control study nested within the EPIC study to assess the association of biomarkers of B vitamin status and MTHFR polymorphisms with the risk of BC. Circulating levels of folate and vitamin B12 were not significantly associated with the overall BC risk. However, we found borderline positive associations between plasma concentrations of vitamin B12 and BC risk restricted to women with either high alcohol intake or low folate status. The MTHFR C677T and A1298C SNPs were not significantly associated with the overall risk of BC in a subsample of this nested case-control study.

Consistent with our findings, a prospective study within EPIC reported a lack of significant association between dietary folate intake and the overall risk of BC risk (8). Some preliminary analyses from the French E3N cohort of the EPIC study have shown that correlations between dietary intakes and plasma levels of B vitamins are very low (0.19 for folate and 0.08 for vitamin B12), suggesting that the two approaches (intake versus status) might produce discordant results when looking at associations with BC (data not published). These results confirm the importance of considering both dietary intakes and blood biomarkers as complementary measures to investigate the association between B vitamins and BC risk.

Prospective investigations based on biomarkers of nutrient status reported inconsistent findings on the association between folate and BC risk (20-24). The correlation coefficient between plasma levels and dietary intake of folate in our study population (0.27) was low, but comparable with those reported in the Nurses' Health Study (0.33) and the Women's Health Study (0.26) which used different assays for blood folate measurement (22;23). Thus, the lack of consistency between studies is unlikely to be explained by differences in the methodology used for folate status measurement. The mean plasma folate concentration in our study population (14.2 nmol/l) was comparable to that reported in the Malmö Diet and Cancer cohort (12.8 nmol/l), which also reported a null association between plasma levels of folate and overall BC risk (24). However, our highest category of plasma folate (>19.8 nmol/l = 8.7 ng/ml) was substantially lower than that reported in the US population-based cohort from the Nurses' Health Study

(>14.0 ng/ml) in which a higher consumption of folic-acid containing foods and an inverse association between plasma folate levels and BC risk were observed (22). Thus, a minimal level of blood folate might be required for observing a beneficial effect of this nutrient on the risk of BC.

A recent dose-response meta-analysis of 16 prospective studies including a total of 26 205 BC patients identified a U-shaped relationship between energy-adjusted dietary folate intake and BC risk (40), supporting prior evidence of an increased risk of BC associated with folic acid fortification (41). The lack of data on consumption of folic acid-containing supplements within the EPIC population prevented us from testing whether folic acid intake might have been associated with high levels of plasma folate and an increased BC risk. However, the proportion of vitamin supplement users in our study population was only 23% among cases, suggesting that plasma levels of folate and other B vitamins were primarily attributable to natural food sources.

The lack of significant interaction between alcohol intake and plasma folate on BC risk in our analysis is consistent with results from previous prospective studies that used biomarkers of folate status (20-24). However, a recent prospective analysis within the EPIC study reported an inverse association between dietary folate intake and the risk of BC among heavy alcohol drinkers (8). Because alcohol may impair folate absorption (42), alcohol consumption behaviors are more likely to modify the risk of BC associated with dietary folate intake rather than plasma folate levels, which can be affected by a variety of other factors including genetic polymorphisms (43). Thus, women with high intake of folate and alcohol may not necessarily have a high folate status and consequently a reduced risk of BC.

The main sources of vitamin B12 are animal products, including meat, fish, dairy products, eggs and liver. Our finding of a positive association between plasma levels of vitamin B12 and BC risk in subgroup analyses is in the same direction of those from two previous prospective studies (16;23). However, an inverse association between biomarkers of vitamin B12 and the risk of BC has also been reported (20;22). The median value of plasma vitamin B12 in our study population (377 pmol/l = 511 pg/ml in cases) was not substantially different from those reported in other population-based prospective studies, ranging between 421 and 467 pg/ml (20;22;23). Thus, several other factors might have

contributed to the inconsistent findings between studies, including differences in alcohol consumption, genetic polymorphisms, and nutrient interactions in one-carbon metabolism (42;44).

As a cofactor required for the generation of methyl groups, a high vitamin B12 status could result in hypermethylation of CpG island promoters for tumor suppressor genes (45;46), which may lead to reduced expression of these cancer-related genes and ultimately promote breast carcinogenesis (47). These DNA methylation changes may also impair the proper expression and/or function of cell-cycle regulatory genes and thus confer a selective growth advantage to neoplastic cells (48).

In contrast to our finding, previous prospective studies that used either dietary intakes (15;16;18) or plasma biomarkers (20;22;23) failed to find a significant interaction between vitamin B12 and alcohol intake on the risk of BC. However, none of these studies reported relative risk estimates by strata of alcohol consumption. A randomized crossover trial suggested that moderate alcohol intake may diminish plasma vitamin B12 concentrations (49). In contrast, a case-control study found that plasma levels of vitamin B12 in heavy alcohol drinkers were significantly higher than those in light alcohol drinkers (50). Further studies are needed to clarify the modifying effect of alcohol on the vitamin B12-BC risk association.

The positive association between plasma levels of vitamin B12 and BC risk among women with low folate status is unexpected. Previous prospective studies found no evidence of an interaction between these two nutrients on the risk of BC (15-18;23;25;51). On the other hand, a prospective analysis within the French E3N cohort reported a strong joint protective effect of high intake of folate and vitamin B12 on BC risk (13). The almost exclusive form of folate in plasma is 5-methyl THF, which reflects the amount of folate available for DNA methylation (52;53). 5-methyl THF is converted to tetrahydrofolate (THF) via the vitamin B12-dependent enzyme methionine synthase. A high vitamin B12 status indicates that methionine synthase activity is increased, leading to depletion of 5-methyl THF and thus plasma folate concentration if not replaced by new 5-methyl THF from diet. In this situation, cells lack the substrate needed for methionine synthesis and DNA methylation is impaired. There is evidence that a low folate status may induce carcinogenesis through alteration of DNA methylation pathways (53). Thus, the

possibility that low plasma folate concentrations (mainly 5-methyl THF) as a consequence of high vitamin B12 status would impair DNA methylation might be suggested.

The MTHFR C677T and A1298C SNPs have been associated with decreased enzyme activity and reduced plasma folate concentration compared with the wild-type genotypes (54;55). Accordingly, we observed that individuals carrying the MTHFR 677T variant allele had a reduced plasma folate concentration and non-significantly increased risk of BC (data not shown). Our observation is in line with results from previous epidemiologic studies that used plasma biomarkers to measure folate status (24;56). However, a positive association between the MTHFR C677T polymorphism and BC risk at low dietary intake of folate was also reported (28;57-60). The effect of MTHFR polymorphisms on the association between folate status and BC risk is highly complex and may depend on the interaction with other lifestyle or genetic factors (61).

The present study is the largest prospective investigation to date to have examined the association between biomarkers of B vitamins and breast cancer risk. The high follow-up rates and large number of cases provided sufficient statistical power for most subgroup analyses. The major strength of our study is, however, the collection of blood samples before diagnosis and their use as biomarkers of exposure.

Major limitations of the present study include: 1) the single collection of blood samples at baseline, 2) the use of a non-reliable biomarker of long-term folate status, 3) the lack of complete hormone receptor status data, and 4) the insufficient statistical power for gene-nutrient interaction analyses.

In conclusion, the present study raises the possibility for a role of vitamin B12 in the etiology of BC, and provides support for potential interactions between nutrients involved in one-carbon metabolism. Prospective studies with extensive data on dietary vitamin B12 intake, MTHFR polymorphisms, and additional methyl-group donors such as methionine, betaine and choline are needed in order to confirm our findings and further explore gene-nutrient and nutrient-nutrient interactions in breast carcinogenesis.

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Table 1. Characteristics of study population*

	Cases, n (%)	Controls, n (%)	P difference†
No. of individuals, n (%)	2491 (49.7%)	2521 (50.3%)	
Mean age (y) at			
Blood collection§	54.1 ± 8.4	54.1 ± 8.4	
Diagnosis	60.2 ± 8.8		
Menopause	49.1 ± 4.7	48.7 ± 5.0	0.026
Menarche	13.0 ± 1.5	13.1 ± 1.6	0.010
Age at first birth and parity, n (%)			0.023
Nulliparous	349 (14.0)	318 (12.6)	
First birth before age 30 years, 1-2 children	1086 (43.6)	1129 (44.8)	
First birth before age 30 years, ≥3 children	590 (23.7)	652 (25.9)	
First birth after age 30 years	376 (15.1)	329 (13.0)	
Unknown≠	90 (3.6)	93 (3.7)	
Menopausal status, n (%)§			
Premenopause	761 (30.6)	770 (30.5)	
Postmenopause	1642 (65.9)	1665 (66.0)	
Perimenopause	88 (3.5)	86 (3.4)	
Ever use of menopausal hormones, n (%)			0.820
No	1687 (67.7)	1700 (67.4)	
Yes	703 (28.3)	714 (28.3)	
Unknown≠	101 (4.0)	107 (4.2)	
Ever use of contraceptive pill, n (%)			0.567
No	1136 (45.6)	1166 (46.2)	
Yes	1325 (53.2)	1325 (52.6)	
Unknown≠	30 (1.2)	30 (1.2)	
Anthropometric measures			
Adult weight (kg)	66.5 ± 11.7	65.2 ± 11.1	<0.001
Adult height (cm)	161.7 ± 6.5	161.3 ± 6.5	0.009
BMI in premenopause	24.6 ± 4.0	24.6 ± 4.1	0.699
BMI in postmenopause	26.0 ± 4.5	25.4 ± 4.1	<0.001
Waist/Hip Ratio (WHR)	0.792 ± 0.068	0.791 ± 0.066	0.552
Physical activity, n (%)			0.260
Inactive	333 (13.4)	293 (11.6)	
Moderately inactive	736 (29.5)	742 (29.4)	
Moderately active	1073 (43.1)	1109 (44.0)	
Active	180 (7.2)	201 (8.0)	
Unknown≠	169 (6.8)	176 (7.0)	
Alcohol intake, n (%)			0.002
Non-drinkers	440 (17.7)	458 (18.2)	
>0-3 g/d	716 (28.7)	777 (30.8)	
>3-12 g/d	658 (26.4)	713 (28.3)	

>12 g/d	677 (27.2)	573 (22.7)	
Family history of breast cancer, n (%)			0.009
No	998 (40.1)	1071 (42.5)	
Yes	159 (6.4)	122 (4.8)	
Unknown [≠]	1334 (53.5)	1328 (52.7)	
Smoking status, n (%)			0.752
Never	1432 (57.5)	1473 (58.4)	
Former	580 (23.3)	571 (22.6)	
Current	423 (17.0)	433 (17.2)	
Unknown [≠]	56 (2.2)	44 (1.8)	
Level of education, n (%)			0.090
Low	852 (34.2)	883 (35.0)	
Medium	998 (40.1)	1049 (41.6)	
High	541 (21.7)	479 (19.0)	
Unknown [≠]	100 (4.0)	110 (4.4)	
Dietary intake			
Energy intake (kcal)	1972.4 ± 549.8	1953.4 ± 555.0	0.211
Dietary folate (ug)	295.6 ± 112.1	296.5 ± 118.2	0.681
Dietary vitamin B12 (ug)	6.1 ± 3.5	6.2 ± 3.7	0.306
Vitamin supplement use, n (%)			0.870
No	878 (35.3)	879 (34.9)	
Yes	262 (10.5)	258 (10.2)	
Unknown [≠]	1351 (54.2)	1384 (54.9)	
Plasma concentrations			
Plasma folate (nmol/L) [‡]	14.1 ± 1.7	14.3 ± 1.8	0.512
Plasma vitamin B12 (pmol/L) [‡]	374.2 ± 1.5	370.0 ± 1.5	0.242
MTHFR C677T			0.377
C/C	197 (49.1)	194 (48.4)	
C/T	163 (40.7)	160 (39.9)	
T/T	29 (7.2)	42 (10.5)	
Unknown [≠]	12 (3.0)	5 (1.2)	
MTHFR A1298C			0.835
A/A	147 (36.7)	154 (38.4)	
A/C	188 (46.8)	178 (44.4)	
C/C	52 (13.0)	54 (13.5)	
Unknown [≠]	14 (3.5)	15 (3.7)	

*Data are presented as means (±SD) or percentages. Geometric means (±SD) of plasma folate and vitamin B12 are presented.

[†]Statistical significance for differences between cases and control individuals was tested using paired t-test for continuous variables and paired Chi-square test for categorical variables.

[‡]Differences in plasma concentration of folate and vitamin B12 were assessed on log natural transformed data. For all other variables, differences were assessed on crude data.

§P-difference was not computed for matching factors.

≠Unknown subjects are excluded from calculations.

Table 2. Multivariable odds ratios* and 95% confidence intervals for association of plasma folate and vitamin B12 with breast cancer risk overall and stratified by menopausal status at recruitment[‡]

Plasma concentration	Matched cases/controls (n) [†]	OR (95% CI)	P trend [‡]	P heterogeneity [§]
Folate (nmol/l)				
All women				
Continuous	2,491/2,521	0.93 (0.83; 1.05)		
<9.82	624/631	1 (ref)	0.797	
9.82-13.56	595/630	0.97 (0.82; 1.15)		
13.56-19.80	663/631	1.07 (0.90; 1.28)		
>19.80	609/629	0.94 (0.79; 1.13)		
Premenopausal women				
Continuous	736/747	0.99 (0.79; 1.23)		0.675
<9.34	198/187	1 (ref)	0.746	
9.34-12.81	160/187	0.80 (0.59; 1.09)		
12.81-18.68	189/187	1.01 (0.73; 1.39)		
>18.68	189/186	0.99 (0.71; 1.37)		
Postmenopausal women				
Continuous	1,615/1,634	0.93 (0.81; 1.07)		
<10.02	399/409	1 (ref)	0.535	
10.02-13.94	398/408	1.04 (0.84; 1.29)		
13.94-20.40	427/409	1.03 (0.83; 1.29)		
>20.40	391/408	0.93 (0.75; 1.17)		
Vitamin B12 (pmol/l)				
All women				
Continuous	2,489/2,519	1.10 (0.94; 1.29)		
<293.6	613/630	1 (ref)	0.242	
293.6-373.1	628/630	1.00 (0.85; 1.19)		
373.1-460.0	578/630	0.95 (0.80; 1.13)		
>460.0	670/629	1.14 (0.95; 1.36)		
Premenopausal women				
Continuous	735/746	1.06 (0.78; 1.45)	0.047	0.679
<291.3	168/187	1 (ref)		
291.3-371.0	174/186	1.04 (0.75; 1.45)		
371.0-465.3	203/187	1.29 (0.93; 1.78)		
>465.3	190/186	1.35 (0.96; 1.91)		
Postmenopausal women				
Continuous	1,614/1,633	1.15 (0.95; 1.39)	0.625	
<293.6	405/409	1 (ref)		
293.6-372.6	416/408	0.99 (0.80; 1.22)		
372.6-457.8	358/408	0.89 (0.72; 1.11)		
>457.8	435/408	1.09 (0.88; 1.37)		

*Subjects were matched by study center, age, menopausal status, exogenous hormone use, fasting status, phase of the menstrual cycle, and time of the day at blood collection. Models were adjusted by date at blood collection, education, BMI, height, physical activity, ever use of hormone replacement therapy, alcohol intake, parity and age at first full-term birth combined, total energy intake, and family history of breast cancer.

≠Menopausal status at recruitment dichotomized as natural/surgical postmenopausal and premenopausal.

†Cut points of quartiles determined on all, premenopausal, or postmenopausal control individuals.

‡Obtained by modeling the cut points of quartiles as continuous variable.

§Tests of heterogeneity between ORs in menopausal subgroups based on Chi-square statistics calculated as the deviations of logistic beta-coefficients observed in each of the subgroups (premenopausal and postmenopausal women) relative to the overall beta-coefficient.

Table 3. Multivariable odds ratios* and 95% confidence intervals for association between plasma B vitamins and breast cancer risk according to hormone receptor status†

Plasma concentration	Matched cases/controls (n)‡	OR (95% CI)	P trend≠	P heterogeneity§
Folate (nmol/l)				
ER+				
Continuous	1,987/2,009	0.96 (0.85; 1.09)		0.626
<10.96	630/677	1 (ref)	0.506	
10.96-17.85	674/662	1.11 (0.93; 1.31)		
>17.85	683/670	1.07 (0.90; 1.27)		
ER-				
Continuous	455/463	0.89 (0.67; 1.18)		
<10.96	162/153	1 (ref)	0.549	
10.96-17.85	160/159	0.97 (0.68; 1.39)		
>17.85	133/151	0.89 (0.62; 1.29)		
PR+				
Continuous	1,407/1,452	0.98 (0.84; 1.15)		0.932
<10.96	482/509	1 (ref)	0.667	
10.96-17.85	482/486	1.05 (0.85; 1.28)		
>17.85	443/430	1.05 (0.85; 1.30)		
PR-				
Continuous	690/696	0.97 (0.77; 1.22)		
<10.96	219/236	1 (ref)	0.911	
10.96-17.85	245/220	1.22 (0.91; 1.64)		
>17.85	226/240	1.03 (0.76; 1.38)		
HER2+				
Continuous	250/252	1.07 (0.72; 1.60)		0.714
<10.96	66/80	1 (ref)	0.633	
10.96-17.85	98/78	1.38 (0.81; 2.35)		
>17.85	86/94	1.16 (0.68; 1.99)		
HER2-				
Continuous	854/862	0.98 (0.80; 1.20)		
<10.96	294/314	1 (ref)	0.432	
10.96-17.85	287/298	1.10 (0.85; 1.43)		
>17.85	273/250	1.12 (0.85; 1.47)		
Vitamin B12 (pmol/l)				
ER+				
Continuous	1,986/2,008	1.06 (0.89; 1.26)		0.403
<323.1	693/684	1 (ref)	0.535	
323.1-426.0	607/668	0.90 (0.76; 1.06)		
>426.0	686/657	1.06 (0.89; 1.26)		
ER-				
Continuous	454/462	1.26 (0.86; 1.86)		
<323.1	140/147	1 (ref)	0.260	
323.1-426.0	150/154	1.16 (0.79; 1.68)		
>426.0	164/162	1.26 (0.85; 1.86)		
PR+				
Continuous	1,406/1,424	1.02 (0.82; 1.27)		0.431

<323.1	493/475	1 (ref)	0.888	
323.1-426.0	434/485	0.88 (0.72; 1.07)		
>426.0	479/465	1.02 (0.83; 1.25)		
PR-				
Continuous	689/695	1.18 (0.88; 1.60)		
<323.1	212/223	1 (ref)	0.279	
323.1-426.0	225/231	0.97 (0.72; 1.32)		
>426.0	252/242	1.18 (0.86; 1.62)		
HER2+				
Continuous	249/251	1.13 (0.70; 1.83)		0.979
<323.1	85/82	1 (ref)	0.906	
323.1-426.0	84/83	1.02 (0.60; 1.74)		
>426.0	80/87	1.03 (0.59; 1.81)		
HER2-				
Continuous	853/861	1.12 (0.84; 1.49)		
<323.1	328/311	1 (ref)	0.660	
323.1-426.0	244/281	0.85 (0.66; 1.10)		
>426.0	281/270	1.08 (0.82; 1.42)		

*Subjects were matched by study center, age, menopausal status, exogenous hormone use, fasting status, phase of the menstrual cycle, and time of the day at blood collection. Models were adjusted by date at blood collection, education, BMI, height, physical activity, ever use of hormone replacement therapy, alcohol intake, parity and age at first full-term birth combined, total energy intake, and family history of breast cancer.

†Classes of hormone receptors investigated: estrogen receptor positive/negative (ER+/-), progesterone receptor positive/negative (PR+/-), and human epidermal growth factor receptor 2 positive/negative (HER2+/-).

‡Cut points of tertiles determined on all control individuals.

≠Obtained by modeling the cut points of tertiles as continuous variable.

§Tests of heterogeneity between ORs in hormone receptor subgroups based on Chi-square statistics calculated as the deviations of logistic beta-coefficients observed in each of the subgroups (i.e. ER+ and ER- status) relative to the overall beta-coefficient.

Table 4. Multivariable odds ratios* with 95% confidence intervals for association of plasma vitamin B12 with breast cancer risk stratified by median level of alcohol intake and plasma folate

	Cases/controls (n)†	OR (95% CI)	P trend‡	P interaction§
Alcohol intake at recruitment≠				0.140
Below median value (<3.36 g/d)				
Continuous	1,205/1,266	1.07 (0.88; 1.30)		
<301.0	333/317	1 (ref)	0.928	
301.0-381.3	293/316	0.89 (0.71; 1.12)		
381.3-471.6	271/317	0.86 (0.68; 1.08)		
>471.6	308/316	1.00 (0.80; 1.26)		
Above median value (≥3.36 g/d)				
Continuous	1,284/1,255	1.23 (0.99; 1.54)		
<286.9	283/314	1 (ref)	0.051	
286.9-365.4	334/314	1.20 (0.96; 1.50)		
365.4-450.3	315/314	1.14 (0.90; 1.43)		
>450.3	352/313	1.30 (1.03; 1.64)		
Plasma folate at blood collection≠				0.059
Below median value (<13.56 nmol/l)				
Continuous	1,218/1,261	1.25 (1.03; 1.53)		
<279.5	276/316	1 (ref)	0.014	
279.5-351.5	277/315	1.01 (0.80; 1.28)		
351.5-442.6	336/315	1.26 (1.00; 1.59)		
>442.6	329/315	1.26 (1.00; 1.60)		
Above median level (≥13.56 nmol/l)				
Continuous	1,271/1,260	1.01 (0.81; 1.25)		
<315.0	355/315	1 (ref)	0.806	
315.0-390.4	296/315	0.84 (0.67; 1.05)		
390.4-478.6	287/315	0.85 (0.68; 1.07)		
>478.6	333/315	1.03 (0.82; 1.29)		

*Models were adjusted by matching factors (study center, age, menopausal status, exogenous hormone use, fasting status, phase of the menstrual cycle, and time of the day at blood collection), date at blood collection, education, BMI, height, physical activity, ever use of hormone replacement therapy, alcohol intake, parity and age at first full-term birth combined, total energy intake, and family history of breast cancer.

†Cut points of quartiles determined on control individuals below or above the median value of alcohol intake (3.36 g/d) or plasma folate (13.56 nmol/l).

‡Obtained by modeling the cut points of quartiles as continuous variable.

§Obtained by modeling the interaction term between plasma vitamin B12 in continuous and alcohol intake or plasma folate as dichotomous variable.

≠Alcohol intake and plasma folate dichotomized according to median value.

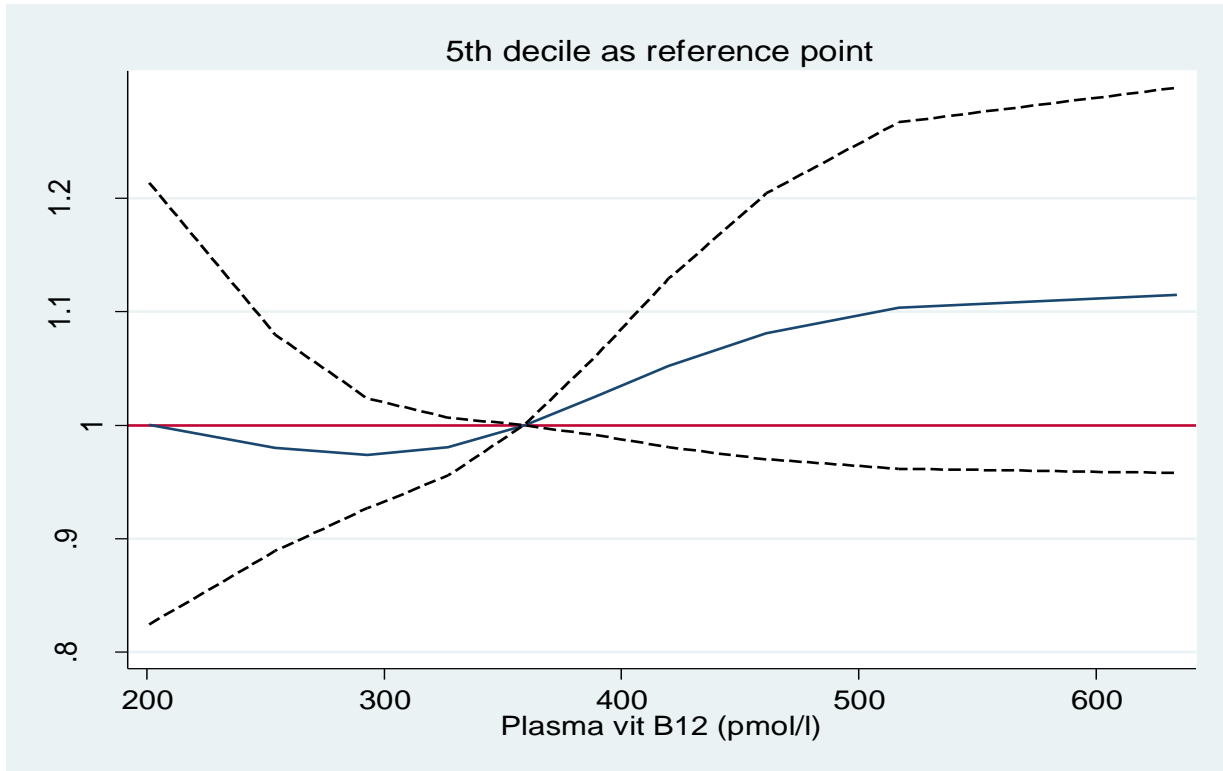
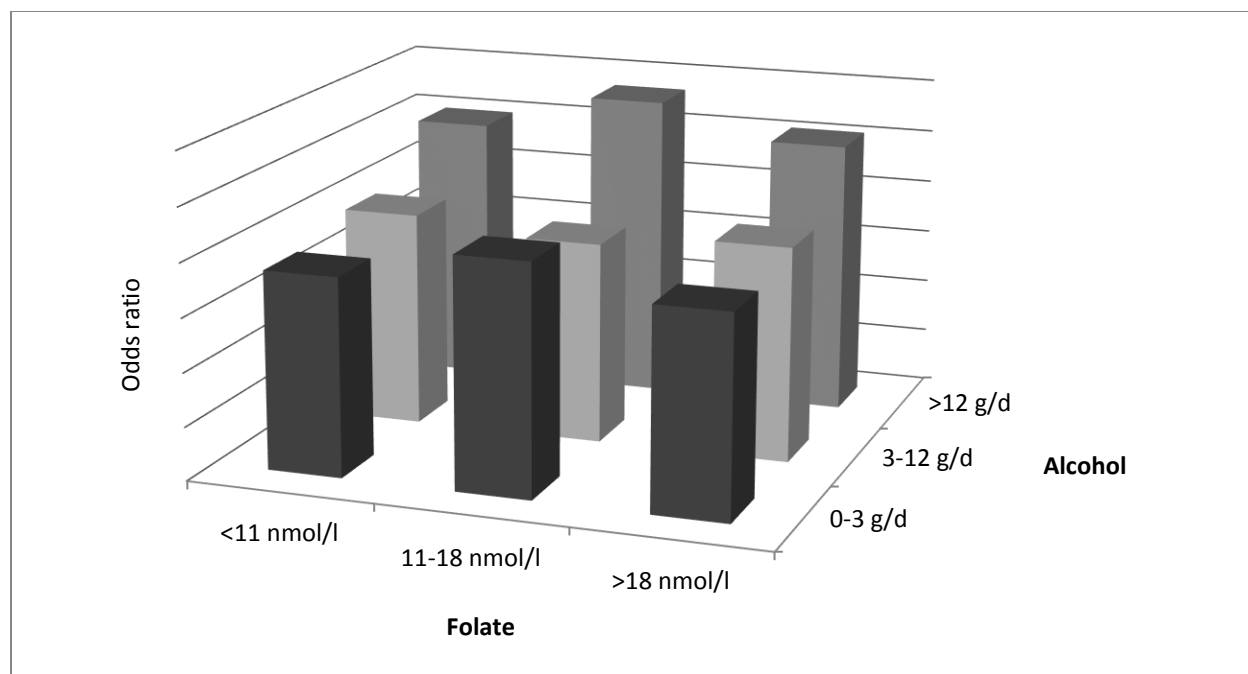


Figure 1. Nonlinear multivariable modeling of the association between plasma levels of vitamin B12 and breast cancer risk. Odds ratio and 95% confidence intervals obtained using four-knot restricted cubic splines with the median of the fifth decile of plasma vitamin B12 used as reference. Models were adjusted by matching factors (study center, age, menopausal status, exogenous hormone use, fasting status, phase of the menstrual cycle, and time of the day at blood collection), date at blood collection, education, BMI, height, physical activity, ever use of hormone replacement therapy, alcohol intake, parity and age at first full-term birth combined, total energy intake, and family history of breast cancer. **Continuous line** represent odds ratio; **dashed lines** represent 95% confidence intervals.



Alcohol intake‡	Plasma folate†		
	Low (<10.96 nmol/l)	Medium (10.96-17.85 nmol/l)	High (>17.85 nmol/l)
High (>12 g/day)	1 (ref)	1.14 (0.86; 1.53)	1.02 (0.76; 1.36)
BC cases	223	229	225
Medium (3-12 g/day)	0.80 (0.57; 1.11)	0.75 (0.54; 1.03)	0.80 (0.58; 1.11)
BC cases	185	242	231
Low (<3 g/day)	0.74 (0.54; 1.02)	0.86 (0.62; 1.19)	0.75 (0.54; 1.04)
BC cases	395	383	378
P interaction§	0.694		

Figure 2. Multivariable odds ratios (ORs)* and 95% confidence intervals (CIs) for association with breast cancer risk by levels of plasma folate (nmol/l)† and alcohol intake (grams/day)‡, including interaction test§. *Subjects were matched by study center, age, menopausal status, exogenous hormone use, fasting status, phase of the menstrual cycle, and time of the day at blood collection. Models were adjusted by date at blood collection, education, BMI, height, physical activity, ever use of hormone replacement therapy, alcohol intake, parity and age at first full-term birth combined, total energy intake, and family history of breast cancer. †Tertiles of plasma folate. ‡Categories of alcohol intake (0-3 g/d; 3-12 g/d; >12 g/d). §P interaction between plasma folate and alcohol intake as categorical variables. All statistical tests were two-sided.