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

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Abstract

Epidemiological studies have reported inconsistent findings for the association between B vitamins and breast cancer (BC) risk. We investigated the relationship between biomarkers of folate and vitamin B12 and the risk of BC in the European Prospective Investigation into Cancer and Nutrition (EPIC) cohort.

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

Plasma levels of folate and vitamin B12 were not significantly associated with the overall risk of BC or by hormone receptor status. A marginally positive association was found between vitamin B12 status and BC risk in women consuming above the median level of alcohol ($OR_{Q4\text{-}Q1}$ = 1.26; 95% CI 1.00-1.58; P_{trend} = 0.05). Vitamin B12 status was also positively associated with BC risk in women with plasma folate levels below the median value ($OR_{Q4\text{-}Q1}$ = 1.29; 95% CI 1.02–1.62; P_{trend} = 0.03).

Overall, folate and vitamin B12 status was not clearly associated with BC risk in this prospective cohort study. However, potential interactions between vitamin B12 and alcohol or folate on the risk of BC deserve further investigation.

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 BC (2). Among dietary factors, deficiencies of B vitamins related to Western dietary patterns have been suggested to play a role in breast carcinogenesis (3;4).

Vitamin B9 (folate) and vitamin B12 (cobalamin) are two water soluble B vitamins involved in one-carbon metabolism (5), which generates substrates for DNA methylation and DNA synthesis (6). Thus, deficiencies of these micronutrients may trigger both genetic and epigenetic pro-carcinogenic processes (7). Prospective studies that investigated the association between biomarkers of folate and BC risk have reported either an inverse association (8;9) or no association (10;11) overall. The prospective investigation of the relationship between biomarkers of vitamin B12 and BC risk has also produced mixed results (8-12). While studies found no evidence for an association between blood levels of vitamin B12 and BC risk in the overall population (9-11), an inverse association was independently reported among either postmenopausal women (10) or premenopausal women (9). However, a recent meta-analysis of prospective studies revealed no significant association between biomarkers of vitamin B12 and BC risk in the subgroup analysis by menopausal status (13).

A number of factors have been suggested to influence the association between B vitamins and the risk of BC, including menopausal status (11;14-16), alcohol consumption (15;17), nutrient interactions (18), and methylenetetrahydrofolate reductase (*MTHFR*) gene polymorphisms (12). *MTHFR* is a key enzyme in one-carbon metabolism where it balances the folate pool between synthesis and methylation of DNA (19). Although a number of *MTHFR* single nucleotide polymorphisms (SNPs) have been reported in the literature (20-22), only the C677T and A1298C SNPs have been consistently associated with decreased enzyme activity and reduced plasma folate levels compared with the wild-type genotypes (23;24). A prospective

study found a positive association between plasma folate concentration and postmenopausal BC among carriers of the *MTHFR* 677T allele (12). 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 (17) and folate status (11) among premenopausal women only.

We conducted a large nested case-control study within EPIC to evaluate the association between plasma concentrations of folate and vitamin B12 and BC risk overall and stratified by hormone receptor status and potential risk factors. In addition, we examined the interaction between the *MTHFR* 677C>T (rs1801133) and 1298A>C (rs1801131) polymorphisms and the two plasma B vitamins on the risk of BC using data from a subsample of this nested case-control population.

Materials and 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 (25). 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). Of 367 903 women (age 35-70 years) 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. The follow-up rate was very high (98.5%: 91.4% alive and 7.1% dead) and only 1.5% of women were lost to follow-up.

Details of the recruitment procedures and data collection in the EPIC study have been previously described in details (26). 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 (27). 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

A nested case-control study was designed among women who provided a blood sample and completed the lifestyle and dietary questionnaires at recruitment. A total of 2,491 BC cases with a confirmed

first diagnosis of invasive BC were identified 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), 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 enrollment using validated country-specific dietary history and food-frequency questionnaires (FFQs), designed to collect local dietary habits of the participants over the preceding year (26). Dietary intakes of folate and vitamin B12 were estimated using the updated EPIC Nutrient Database (ENDB) (28), following standardization from country-specific food composition tables (FCT) according to Bouckaert's recommendations (29). Details on dietary assessment have been discussed previously (17).

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 enrollment date.

Outcome assessment

Participants were followed from the date of enrollment 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: ≥10% cells stained, any 'plus-system' description, ≥20 fmol/mg, an Allred score of ≥3, an IRS ≥2, or an H-score≥10 (30). 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 (31) and vitamin B12 (32). 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 in a subsample of 401 cases and 401 matched control individuals from this nested case-control population. 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 ± 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 were used to assess differences between cases and control individuals with regard to continuous and categorical variables, respectively.

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-). Crude ORs were also presented to observe the effect of confounding on the risk estimates. In addition, the association between each plasma biomarker and the risk of BC was examined using four-knot restricted cubic splines with the mid-point of the 5th decile of plasma vitamin B12 as the reference category (33).

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 median value of each category 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 ≥3 children; >21–30 y; ≥30 y; 3.6% unknown), and family history of BC (yes; no; 53.1% unknown). These confounders were previously related to BC risk or blood measurements, and were chosen based on previous studies in the literature. 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 concentrations of folate and vitamin B12 and BC risk by levels of alcohol intake or plasma folate (low and high levels based on median values) and by *MTHFR* genotypes. 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 (17).

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.

The association between the SNPs and overall BC risk was evaluated by conditional logistic regression. 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.

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 more likely to have had a first-degree relative with BC (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 (Table 2) or by ER, PR, and HER2 status (Table 3). No further association emerged after adjustment by *MTHFR* polymorphisms for the available subsample (data not shown).

A nonlinear modelling of the association between plasma concentrations of vitamin B12 and BC risk showed a borderline significant trend (P_{trend} = 0.07) 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).

Because of the impaired folate absorption and altered one-carbon metabolism due to chronic alcohol consumption (34), we reported risk estimates by tertiles of plasma folate and categories of alcohol consumption (Figure 1). The association between plasma folate concentration and BC risk was not significantly modified by levels of alcohol intake ($P_{interaction}$ = 0.69). Similarly, no significant association between plasma folate and BC risk was observed by median level of alcohol consumption (data not shown).

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 ($OR_{Q4\text{-}Q1}$ = 1.26; 95% CI 1.00-1.58; P_{trend} = 0.05), while no significant association emerged in women drinking lower

amounts of alcohol ($OR_{Q4\text{-}Q1}$ = 1.08; 95% CI 0.86-1.35; P_{trend} = 0.56). However, no significant heterogeneity by alcohol intake was found ($P_{heterogeneity}$ = 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 ($P_{interaaction}$ = 0.04; 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 ($OR_{Q4\text{-}Q1}$ = 1.29; 95% CI 1.02–1.62; P_{trend} = 0.03), while no significant association occurred in women with higher levels of plasma folate (P_{trend} = 0.68). A borderline significant heterogeneity by plasma folate levels was also found ($P_{heterogeneity}$ = 0.05).

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 *MTHFR* 677C>T and 1298A>C SNPs were in low linkage disequilibrium among both the cases (r^2 = 0.24) and control individuals (r^2 = 0.25). There was no significant association between either C677T ($OR_{TT\ vs.\ CC}$ = 0.71; 95% CI 0.42-1.19; P_{trend} = 0.38) or A1298C ($OR_{CC\ vs.\ AA}$ = 0.97; 95% CI 0.62-1.53; P_{trend} = 0.91) and the overall risk of BC. The interaction between plasma folate or vitamin B12 and *MTHFR* SNPs was not statistically significant ($P_{interaction}$ > 0.05). Plasma concentrations of the two B vitamins were not significantly associated with BC risk in any of the genotypic classes (homozygous wild-type, heterozygous, homozygous variant) of each SNP (data not shown). No further association emerged in the dominant models, and adjustment for the alternative SNP did not change the risk estimates.

Discussion

In this large prospective European study, circulating levels of folate and vitamin B12 were not significantly associated with the overall risk of BC. 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 polymorphisms had no effect modification on the association between either plasma B vitamin and BC risk in a subsample of this nested case-control study.

A study was recently conducted to assess the reliability of plasma biomarkers involved in one-carbon metabolism in a subsample from the EPIC study (38 men and 35 women), which was estimated over a period of 2 to 5 years using an intraclass correlation coefficient (ICC) (35). The study showed that plasma vitamin B12 was a highly reliable biomarker (ICC = 0.75), while a modest reliability was observed for plasma folate (ICC = 0.45). Because our study was performed on a larger number of subjects and extended over 18 years of follow-up, it is difficult to predict whether and to what extent the single biomarker measurements may have led to attenuation of the risk estimates in our study. However, when the models were adjusted for the regression dilution using the ICCs as adjustment coefficients, no significant change in risk estimates was observed.

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 (17). Prospective investigations based on biomarkers of nutrient status reported inconsistent findings between folate and BC risk (8-12). 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 (12). However, our highest category of plasma folate (>19.8 nmol/l; 609 cases) was substantially lower than that reported in the US population-based cohort from the Nurses' Health Study (>14.0 ng/ml = 31.7 nmol/l; 120 cases) in which a higher consumption of folic-acid containing foods and

an inverse association between plasma folate levels and BC risk were observed (9). Thus, a minimal level of blood folate might be required for observing a beneficial effect of this nutrient on the risk of BC.

The high plasma folate concentrations reported in US based population studies is likely due to folic acid fortification of flour and cereal-grain products, which became mandatory in the United States since 1997 to prevent neural tube defects (36;37). On the other hand, no policy of folic acid fortification of foods has been implemented in European countries. Folic acid from fortified foods or supplementation is estimated to be approximately 1.7 times more bioavailable than natural folates (38). Because most of the enzymes that use folate as cofactor cannot use the synthetic form, there might be important perturbations in one-carbon metabolism and cellular processes that rely on this pathway. 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 (39), supporting prior evidence of an increased risk of BC associated with folic acid fortification (40). 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 a significant interaction between plasma folate levels and alcohol intake on BC risk in our analysis is consistent with results from previous prospective studies that used biomarkers of folate status (8-12). However, a recent prospective investigation within the EPIC study reported an inverse association between dietary folate intake and the risk of BC among heavy alcohol drinkers (17). Since alcohol may impair folate absorption (34)(39), 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 (41). Thus, women with high intake of both 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 accordance with two previous prospective studies that measured either dietary intake (42) or plasma levels (11) of this nutrient. However, an inverse association between biomarkers of vitamin B12 and the risk of BC has also been reported (9;10). 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 (9-11). Thus, several other factors might have contributed to the inconsistent findings, including differences in alcohol consumption, genetic polymorphisms, and nutrient interactions in one-carbon metabolism (34;43).

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 (44), which may lead to reduced expression of these cancer-related genes and ultimately promote breast carcinogenesis (45). 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 (46). A randomized crossover trial suggested that moderate alcohol intake may diminish plasma vitamin B12 concentrations (47). 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 (48). Further studies are needed to clarify the modifying effect of alcohol on the association between vitamin B12 and BC risk.

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 (16;42;49;50). 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 (18). The almost exclusive form of folate in plasma is 5-methyl THF, which reflects the amount of folate available for DNA methylation (51). 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 (52). 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.

Epidemiological studies provide support that the association between the *MTHFR* C677T polymorphism and BC risk is modified by intakes of some B vitamins, including folate and vitamin B12 (53-55). We observed no significant effect modification of *MTHFR* SNPs on the association between plasma folate or vitamin B12 and BC risk. The low power of these subgroup analyses prevented us from finding a potential interaction between *MTHFR* genotypes and B vitamin status on the risk of BC. Furthermore, the effect of *MTHFR* polymorphisms on plasma levels of B vitamins is highly complex and may depend on the interaction with other dietary and genetic factors (56).

The present study is the largest prospective investigation to date to have examined the association between biomarkers of folate and vitamin B12 and the risk of BC. 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 prior to diagnosis and the use as biomarkers of exposure as reflection of true vitamin status.

Major limitations include the single collection of blood samples at baseline and the measurement of a single biomarker of folate or vitamin B12 status. Folate concentration measured in plasma is considered to be a sensitive biomarker of recent dietary intake, and thus is not very informative for the assessment of long-term folate status (57). Plasma vitamin B12 is the most widely used biomarker of total cobalamin status, but not the most specific biomarker to characterize adequate vitamin concentrations (58). In order to obtain more reliable information on vitamin status, multiple measurements of plasma biomarkers should be taken over a period of time or a combination of different biomarkers should be used. Additional limitations include 1) the large percentage of missing data for family history of BC (53.1%) and supplement use (54.5%), 2) the determination of menopausal status at recruitment and not at diagnosis, 3) the lack of complete hormone receptor status data, and 4) the insufficient statistical power for gene-nutrient interaction

analyses. Because controlling for family history of BC and supplement use had minimal effect on the risk estimates, our results are unlikely to be explained by residual confounding by those factors.

In conclusion, no clear support for an association between plasma levels of folate and BC risk was found in this large prospective study. However, potential interactions between vitamin B12 and alcohol or folate on the risk of BC were observed. Our findings suggest a potential role of vitamin B12 in breast carcinogenesis and raise the possibility of important nutrient-nutrient and gene-nutrient interactions in the etiology of BC. The potential deleterious effect of high vitamin B12 status in combination with other risk factors for BC deserves further investigation. Given the inconsistent findings to date and the possibility that associations between folate and BC could be influenced by some factors yet to be identified, further studies based on novel biomarkers that take into account the effect of potential risk factors and genetic polymorphisms are warranted.

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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 (14.5)	318 (13.1)		
First birth before age 30 years, 1-2 children	1086 (45.2)	1129 (46.5)		
First birth before age 30 years, ≥3 children	590 (24.6)	652 (26.8)		
First birth after age 30 years	376(15.7)	329 (13.5)		
Unknown≠	90 (3.6)	93 (3.7)		
Menopausal status, n (%)				
Premenopause	761 (30.6)	770 (30.5)		
Postmenopause	1642 (65.9)	1665 (66.1)		
Perimenopause	88 (3.5)	86 (3.4)		
Ever use of menopausal hormones, n (%)				0.820
No	1687 (70.6)	1700 (70.4)		
Yes	703 (29.4)	714 (29.6)		
Unknown≠	101 (4.0)	107 (4.2)		
Ever use of contraceptive pill, n (%)				0.567
No	1136 (46.2)	1166 (46.8)		
Yes	1325 (53.8)	1325 (53.2)		
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 (14.3)	293 (12.5)		
Moderately inactive	736 (31.7)	742 (31.6)		
Moderately active	1073 (46.2)	1109 (47.3)		
Active	180 (7.7)	201 (8.6)		
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	573 (22.7)	677 (27.2)	
Family history of breast cancer, n (%)			0.009
No	998 (86.3)	1071 (89.8)	
Yes	159 (13.7)	122 (10.2)	
Unknown≠	1334 (53.5)	1328 (52.7)	
Smoking status, n (%)			0.752
Never	1432 (58.8)	1473 (59.5)	
Former	580 (23.8)	571 (23.0)	
Current	423 (17.4)	433 (17.5)	
Unknown≠	56 (2.2)	44 (1.8)	
Level of education, n (%)			0.090
Low	852 (35.6)	883 (36.6)	
Medium	998 (41.7)	1049 (43.5)	
High	541 (22.6)	479 (19.9)	
Unknown≠	100 (4.0)	110 (4.4)	
Dietary intake			
Energy intake (kcal)	1972.4 ± 549.8	1953.4 ± 555.0	0.210
Dietary folate (ug)	295.6 ± 112.1	296.5118.2	0.681
Dietary vitamin B12 (ug)	6.1 ± 3.5	6.2 ± 3.7	0.306
Vitamin supplement use, n (%)			0.870
No	878 (77.0)	879 (77.3)	
Yes	262 (23.0)	258 (22.7)	
Unknown≠	1351 (54.2)	1384 (54.9)	
Plasma concentrations			
Folate (nmol/L)‡	14.1 ± 1.7	14.3 ± 1.8	0.512
Vitamin B12 (pmol/L)‡	374.2 ± 1.5	370.0 ± 1.5	0.242
MTHFR C677T			0.337
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. Missing values are excluded from calculations.
†Statistical significance for differences between cases and controls 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.

Table 2. Crude and multivariable odds ratios* 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)†	Crude OR	Multivariable OR* (95% CI)	P trend‡	P heterogeneity§
Folate (nmol/l)					
All women					
Continuous≠	2,491/2,521	0.96	0.93 (0.83; 1.05)		
<9.82	624/631	1 (ref)	1 (ref)	0.80	
9.82-13.56	595/630	0.95	0.97 (0.82; 1.15)		
13.56-19.80	663/631	1.06	1.07 (0.90; 1.28)		
>19.80	609/629	0.98	0.94 (0.79; 1.13)		
Menopausal status at recruitment≠					
Premenopausal women					0.67
Continuous≠	736/747	0.98	0.99 (0.79; 1.23)		
<9.82	218/220	1 (ref)	1 (ref)	0.61	
9.82-13.56	168/192	0.88	0.88 (0.65; 1.20)		
13.56-19.80	201/179	1.16	1.27 (0.93; 1.75)		
>19.80	149/156	0.97	1.00 (0.72; 1.41)		
Postmenopausal women					
Continuous≠	1,615/1,634	0.97	0.93 (0.81; 1.07)		
<9.82	385/391	1 (ref)	1 (ref)	0.46	
9.82-13.56	393/406	0.98	1.01 (0.81; 1.26)		
13.56-19.80	418/408	1.04	1.01 (0.81; 1.25)		
>19.80	419/429	0.99	0.94 (0.75; 1.17)		
Vitamin B12 (pmol/l)					
All women					
Continuous≠	2,489/2,519	1.09	1.10 (0.94; 1.29)		
<293.6	613/630	1 (ref)	1 (ref)	0.24	
293.6-373.1	628/630	1.03	1.00 (0.85; 1.19)		
373.1-460.0	578/630	0.95	0.95 (0.80; 1.13)		
>460.0	670/629	1.13	1.14 (0.95; 1.36)		
Menopausal status at recruitment≠					0.68
Premenopausal women					
Continuous≠	735/746	1.01	1.06 (0.78; 1.45)	0.10	
<293.6	181/195	1 (ref)	1 (ref)		
293.6-373.1	176/191	1	0.98 (0.71; 1.35)		
373.1-460.0	187/171	1.22	1.23 (0.90; 1.71)		
>460.0	191/189	1.15	1.26 (0.90; 1.77)		
Postmenopausal women					
Continuous≠	1,614/1,633	1.13	1.15 (0.95; 1.39)	0.46	
<293.6	407/413	1 (ref)	1 (ref)		
293.6-373.1	421/408	1.05	1.00 (0.81; 1.23)		
373.1-460.0	356/412	0.88	0.88 (0.71; 1.09)		

>460.0	430/400	1.12	1.11 (0.89; 1.39)

*Subjects were matched by by study center, age, menopausal status, exogenous hormone use, fasting status, and phase of the menstrual cycle. 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 control individuals.

‡Obtained by modeling the median value of tertiles as continuous term in the multivariable model.

§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.

≠The OR (95% CI) in the continuous model corresponds to an increment of 2.7 units of folate (nmol/l) or vitamin B12 (pmol/l).

Table 3. Crude and multivariable odds ratios* for association of plasma folate and vitamin B12 with breast cancer risk according to hormone receptor status†

Plasma concentration	Matched cases/controls (n)‡	Crude OR	Multivariable OR* (95% CI)	P trend≠	P heterogeneity§
Folate (nmol/l)					
ER+					0.63
Continuous≠	1,987/2,009	0.99	0.96 (0.85; 1.09)		
<10.96	630/677	1 (ref)	1 (ref)	0.51	
10.96-17.85	674/662	1.11	1.11 (0.93; 1.31)		
>17.85	683/670	1.12	1.07 (0.90; 1.27)		
ER-					
Continuous≠	455/463	0.86	0.89 (0.67; 1.18)		
<10.96	162/153	1 (ref)	1 (ref)	0.55	
10.96-17.85	160/159	0.94	0.97 (0.68; 1.39)		
>17.85	133/151	0.82	0.89 (0.62; 1.29)		
PR+					0.93
Continuous≠	1,407/1,452	1.01	0.98 (0.84; 1.15)		
<10.96	482/509	1 (ref)	1 (ref)	0.67	
10.96-17.85	482/486	1.06	1.05 (0.85; 1.28)		
>17.85	443/430	1.11	1.05 (0.85; 1.30)		
PR-					
Continuous≠	690/696	0.96	0.97 (0.77; 1.22)		
<10.96	219/236	1 (ref)	1 (ref)	0.91	
10.96-17.85	245/220	1.21	1.22 (0.91; 1.64)		
>17.85	226/240	1.02	1.03 (0.76; 1.38)		
HER2+					0.71
Continuous≠	250/252	1.01	1.07 (0.72; 1.60)		
<10.96	66/80	1 (ref)	1 (ref)	0.63	
10.96-17.85	98/78	1.54	1.38 (0.81; 2.35)		
>17.85	86/94	1.13	1.16 (0.68; 1.99)		
HER2-					
Continuous≠	854/862	1.04	0.98 (0.80; 1.20)		
<10.96	294/314	1 (ref)	1 (ref)	0.43	
10.96-17.85	287/298	1.05	1.10 (0.85; 1.43)		
>17.85	273/250	1.2	1.12 (0.85; 1.47)		
Vitamin B12 (pmol/l)					
ER+					0.40
Continuous≠	1,986/2,008	1.05	1.06 (0.89; 1.26)		
<323.1	693/684	1 (ref)	1 (ref)	0.54	
323.1-426.0	607/668	0.9	0.90 (0.76; 1.06)		
>426.0	686/657	1.05	1.06 (0.89; 1.26)		
ER-					
Continuous≠	454/462	1.22	1.26 (0.86; 1.86)		
<323.1	140/147	1 (ref)	1 (ref)	0.26	
323.1-426.0	150/154	1.05	1.16 (0.79; 1.68)		
>426.0	164/162	1.09	1.26 (0.85; 1.86)		
PR+					0.43

Continuous≠	1,406/1,424	1.02	1.02 (0.82; 1.27)		
<323.1	493/475	1 (ref)	1 (ref)	0.89	
323.1-426.0	434/485	0.86	0.88 (0.72; 1.07)		
>426.0	479/465	1.01	1.02 (0.83; 1.25)		
PR-					
Continuous≠	689/695	1.13	1.18 (0.88; 1.60)		
<323.1	212/223	1 (ref)	1 (ref)	0.30	
323.1-426.0	225/231	1.05	0.97 (0.72; 1.32)		
>426.0	252/242	1.12	1.18 (0.86; 1.62)		
HER2+					0.98
Continuous≠	249/251	1.15	1.13 (0.70; 1.83)		
<323.1	85/82	1 (ref)	1 (ref)	0.90	
323.1-426.0	84/83	0.97	1.02 (0.60; 1.74)		
>426.0	80/87	0.87	1.03 (0.59; 1.81)		
HER2-					
Continuous≠	853/861	1.04	1.12 (0.84; 1.49)		
<323.1	328/311	1 (ref)	1 (ref)	0.66	
323.1-426.0	244/281	0.82	0.85 (0.66; 1.10)		
>426.0	281/270	1	1.08 (0.82; 1.42)		

*Subjects were matched by study center, age, menopausal status, exogenous hormone use, fasting status, and phase of the menstrual cycle. 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 median value of tertiles as continuous term in the multivariable model.

§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.

≠The OR (95% CI) in the continuous model corresponds to an increment of 2.7 units of folate (nmol/l) or vitamin B12 (pmol/l).

Table 4. Crude and multivariable odds ratios* for association of plasma folate and vitamin B12 with breast cancer risk stratified by levels of alcohol intake and plasma folate

Plasma vitamin B12 (pmol/l)	Cases/controls (n)†	Crude OR	Multivariable OR* (95% CI)	P trend‡	P interaction§
Alcohol intake at recruitment≠					0.14
Below median value (<3.36 g/d)					
Continuous≠	1,205/1,266	0.99	1.06 (0.87; 1.29)		
<293.6	301/297	1 (ref)	1 (ref)	0.56	
293.6-373.1	296/307	0.95	0.96 (0.76; 1.21)		
373.1-460.0	264/319	0.82	0.85 (0.67; 1.07)		
>460.0	344/343	0.99	1.08 (0.86; 1.35)		
Above median value (≥3.36 g/d)					
Continuous≠	1,284/1,255	1.2	1.21 (0.97; 1.51)		
<293.6	312/334	1 (ref)	1 (ref)	0.05	
293.6-373.1	332/323	1.1	1.09 (0.87; 1.37)		
373.1-460.0	312/311	1.07	1.07 (0.86; 1.35)		
>460.0	328/287	1.22	1.26 (1.00; 1.58)		
Plasma folate at blood collection≠					0.05
Below median value (<13.56 nmol/l)					
Continuous≠	1,218/1,261	1.2	1.25 (1.02; 1.52)		
<293.6	322/377	1 (ref)	1 (ref)	0.03	
293.6-373.1	329/334	1.15	1.15 (0.92; 1.42)		
373.1-460.0	282/278	1.19	1.22 (0.97; 1.53)		
>460.0	285/272	1.23	1.29 (1.02; 1.62)		
Above median level (≥13.56 nmol/l)					
Continuous≠	1,271/1,260	0.93	0.99 (0.79; 1.22)		
<293.6	291/254	1 (ref)	1 (ref)	0.68	
293.6-373.1	299/296	0.88	0.90 (0.71; 1.13)		
373.1-460.0	294/352	0.73	0.75 (0.59; 0.95)		
>460.0	387/358	0.94	1.02 (0.81; 1.28)		

*Models were adjusted by matching factors (study center, age, menopausal status, exogenous hormone use, fasting status, and phase of the menstrual cycle,), 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.
†Cutpoints of quartiles determined on control individuals.
‡Obtained by modeling the median value of tertiles as continuous term in the multivariable model.
§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.
≠The OR (95% CI) in the continuous model corresponds to an increment of 2.7 units of folate (nmol/l) or vitamin B12 (pmol/l).

Figure Legends

Figure 1. 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, and phase of the menstrual cycle. 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.