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EPIDEMIOLOGY

Plasma B-vitamins and one-carbon metabolites and the risk of breast cancer in younger women

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Abstract

Purpose We examined the association of plasma B-vitamins and metabolites, and related genetic variants, with risk of breast cancer among predominantly premenopausal women.

Methods We conducted a nested case–control study within the Nurses' Health Study II. From blood samples collected in 1996–1999 and follow-up through 2007, plasma measures were available for 610 cases and 1207 controls. Unconditional multivariable logistic regression was used to estimate relative risks (RR) of breast cancer and 95% confidence intervals (CIs). We examined whether associations varied by methylenetetrahydrofolate reductase (MTHFR) and dihydrofolate reductase polymorphisms, breast cancer risk factors, or tumor characteristics.

Results Plasma vitamin B_{12} was associated with a 64% higher risk of breast cancer comparing the highest versus lowest quintile (95% CI 1.17–2.29, p-trend = 0.02). Plasma folate (comparable RR = 1.18, 95% CI 0.84–1.66), pyridoxal 5′-phosphate (RR = 1.18, 95% CI 0.85–1.64), homocysteine (RR = 0.93, 95% CI 0.67–1.28), cysteine (RR = 1.14, 95% CI 0.81–1.62), and cysteinylglycine (RR = 0.93, 95% CI 0.66–1.31) were not associated with overall breast cancer risk. Folate was significantly positively associated with invasive and estrogen receptor-positive/progesterone receptor-positive breast cancer, and this association was suggestively stronger for bloods collected post-fortification. Several nutrient/breast cancer associations varied across subgroups defined by age, smoking, alcohol, multivitamin use, and MTHFR status (p-interaction < 0.05).

Conclusions Overall, plasma B-vitamins and metabolites were not associated with lower breast cancer risk. Plasma vitamin B-12 was positively associated with higher risk of overall breast cancer, and plasma folate was positively associated with risk of invasive breast cancer. Additionally, there may be associations in subgroups defined by related genetic variants, breast cancer risk factors, and tumor factors. Further studies in younger women and in the post-fortification era are needed to confirm these findings.

Keywords B-vitamins · Folate · Breast cancer subtype · *MTHFR* · *DHFR*

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Introduction

Folate (B_9), vitamin B_6, and vitamin B_{12} are involved in one-carbon metabolism where they play important roles in DNA synthesis and methylation, which are linked to carcinogenesis [1–3]. Naturally occurring food folate is predominately in the fully reduced form, 5-methyl-tetrahydrofolate (5-methyl-THF). In contrast, folic acid, a synthetic version found in supplements and fortified foods needs to be reduced by dihydrofolate reductase (DHFR) before being used in one-carbon metabolism [1, 3, 4]. A 19-bp pair deletion on intron 1 of the *DHFR* gene has been suggested to increase gene expression [5–7]. A second enzyme, methylenetetrahydrofolate reductase (MTHFR), regulates the irreversible conversion of 5,10-methylene-THF to 5-methyl-THF, balancing DNA synthesis and DNA methylation [2]. Two well-known polymorphisms in the *MTHFR* gene, *MTHFR* C677T (rs1801133) and *MTHFR* A1298C (rs1801131), reduce MTHFR activity [3, 8, 9], which would lead to lower levels of 5-methyl-THF thus favoring DNA synthesis [10].

Folate and vitamins B_6 and B_{12} also are involved in several reactions in the transulfuration pathway [11, 12]. Cysteine is the rate-limiting substrate in the biosynthesis of glutathione, the principal intracellular antioxidant that protects against oxidative stress by scavenging free radicals and detoxifying reactive oxygen species [13, 14], which have been linked to breast cancer [15]. Cysteinylglycine, a product of extracellular glutathione catabolism, is involved in the generation of reactive oxygen species and induces oxidative stress when it interacts with iron ions [16].

Prior epidemiologic cohort studies have generally found no associations of folate and other B-vitamins with breast cancer overall, but differences by menopause status have been suggested [17–19]. However, prior studies have been limited by small numbers to examine the relationship between different plasma measures and related polymorphisms and breast cancer risk among younger women. Therefore, we conducted a prospective study to evaluate plasma levels of folate, B_{12}, pyridoxal 5′-phosphate (PLP; the principal active form of B_6), homocysteine, cysteine, and cysteinylglycine and breast cancer risk in the Nurses' Health Study II, a large cohort among younger women. We secondarily examined the effect of three polymorphisms of folate metabolism genes (*MTHFR* C677T, *MTHFR* A1298C, and *DHFR* 19-bp deletion) on these relations.

Methods

Study population

The Nurses' Health Study II (NHS2) was established in 1989 when 116,429 female nurses aged 25 to 42 years returned a mailed baseline questionnaire that obtained information on reproductive history, medical history, and health behaviors. Subsequently, the women have completed follow-up questionnaires every 2 years, updating information on risk factors, medical history, and self-reported disease status. Additionally, diet has been assessed every 4 years since 1991 using semi-quantitative food frequency questionnaires. The study protocol was approved by the institutional review boards of the Brigham and Women's Hospital and Harvard T.H. Chan School of Public Health, and those of participating registries as required.

Blood samples were collected between 1996 and 1999 from 29,611 women aged 32 to 53 years and details regarding collection methods have been published previously [20]. Briefly, women had blood drawn in the morning and were fasting when possible (61% fasting). Premenopausal women who were not pregnant, breastfeeding, or on oral contraceptives provided two timed samples ($n = 18{,}521$), a "follicular" sample during the 3rd–5th day of their menstrual cycle and a second "luteal" sample 7–9 days before the anticipated start of their next cycle. An additional 11,090 women, mostly postmenopausal, provided a single untimed sample. All samples were returned to the lab on ice; 94% of luteal and untimed samples were received within 26 h of collection. Upon arrival at the laboratory, the blood was separated into plasma, and red blood cell and white blood cell components and stored in liquid nitrogen freezers. The current study uses the luteal and untimed blood samples. The women completed a questionnaire recording the 1st day of their menstrual cycle during which blood samples were drawn and other information including date and timing of the blood draw, fasting status, and current weight. The follow-up rate among women who donated a blood sample was > 90% through 2007.

Identification of cases and controls and molecular subtyping of breast cancer tumors

Incident breast cancers (invasive and in situ), post blood collection through 2007, were self-reported on the biennial questionnaires and confirmed via medical records, where pathology reports were used to ascertain tumor invasiveness, grade, and hormone receptor status. Cases were then matched, using incidence density sampling, to two controls by year of birth, month and year of blood draw, time of blood draw, fasting status, luteal day of menstrual cycle at blood collection, menopausal status at blood collection and diagnosis, and race/ethnicity.

Collection of the breast cancer tumor tissue, construction of the tissue microarrays (TMAs), and immunohistochemistry staining for molecular subtyping breast cancers have been reported in detail previously [21, 22]. Briefly,

formalin-fixed paraffin-embedded breast tumor tissue was obtained for primary incident breast cancers. TMAs were constructed using three 0.6 mm cores from each tumor and 5 µm sections of the TMA, using a Dako Autostainer (Dako Corporation, Carpinteria, CA), were immunohistochemical stained for estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor 2 (HER2). The study pathologist manually evaluated each core for expression of the markers to classify molecular subtypes. Positivity was defined as any nuclear staining for ER and PR, and greater than 10% of cells with moderate or strong membrane staining for HER2 overexpression in any of the three cores. Luminal A breast cancers defined as ER positive and/or PR positive and HER2 negative and histologic grade 1 or 2; while Luminal B breast cancers were either (a) ER positive and/or PR positive and HER2 positive or (b) ER positive and/or PR positive and HER2 negative and grade 3.

Laboratory analyses

Plasma concentrations of cysteine, cysteinylglycine, and homocysteine were measured using high-performance liquid chromatography with fluorescence detection at the Jean Mayer USDA Human Nutrition Research Center on Ageing at Tufts University [23]. Plasma concentrations of folate and vitamin B_{12} were determined using radio assay kits (Bio-Rad, Richmond, CA). Plasma PLP concentrations were determined using the tyrosine decarboxylase apoenzyme method of Shin-Buehring [24]. All specimens were deidentified and matched case–control sets were assayed in the same analytical run (case–control status was unknown to the laboratory personnel). Samples were assayed in four batches; within each batch, pooled plasma quality control samples were interspersed to assess laboratory precision. The mean coefficients of variation for the plasma measures were all $\leq 15\%$ (range 2 to 15%).

DNA analyses

DNA was extracted from blood using QIAmp (Qiagen, Chatsworth, CA) and DNA was amplified using Genomiphi (GE Healthcare, Piscataway, NJ). Genotyping of *MTHFR* C677T (rs1801133) and A1298C (rs1801131) for 1332 (follow-up through 2005 only) and 1787 women, respectively, and the *DHFR* 19-bp deletion for 1787 women was performed at the Dana Farber/Harvard Cancer Center High-Throughput Genotyping Core by the 5′ nuclease assay (Taqman®) on the Applied Biosystems Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, California). The percentage of missing genotype data was < 3%. The genotype distribution among controls did not deviate from Hardy–Weinberg equilibrium ($P \geq 0.14$ for all). *MTHFR* C677T and A1298C were in high linkage disequilibrium ($D' = 0.98$, $r^2 = 0.50$).

Statistical analysis

A total of 610 cases and 1207 matched controls with assays of plasma measures were included in this analysis. Thirteen cases had only one matched control. All four batches were recalibrated to an average standard batch using the method by Rosner et al. [25] and plasma concentrations were categorized into quantiles based upon the distribution of control participants. Continuous plasma concentrations were natural log (ln) transformed to improve normality and standardized to a 1-unit increase in the standard deviation (SD). Spearman correlations were calculated between plasma and dietary measures.

Unconditional logistic regression was used to estimate relative risks (RR) and 95% confidence intervals (CI) for the analysis of plasma concentrations in relation to risk of breast cancer adjusting for matching factors. Results from unconditional models were very similar overall to conditional models. In multivariable analysis, we further controlled for age at menarche (continuous), parity/age at first birth (nulliparous, 1–2 children/first birth < 25 years, 1–2 children/first birth 25 + years, ≥ 3 children/first birth < 25 years, ≥ 3 children/first birth 25 + years), history of breast cancer in mother or a sister (yes vs. no), history of benign breast disease (yes vs. no), height (continuous), body mass index at age 18 (continuous), weight change since age 18 (< 5 kg, 5 to < 20 kg, 20 + kg), and alcohol intake (continuous). We conducted stratified analyses according to age at blood collection (median split ~ 45 years), alcohol intake (< 10 g/day and ≥ 10 g/day), smoking (current vs. never/past), current multivitamin use, (current vs. non-current at blood collection), and body mass index (BMI, < 25 kg/m^2 vs. ≥ 25 kg/m^2). Missing covariate information was filled in using median values of all participants for continuous values (2% for alcohol intake and ≤ 1% for age at menarche, height, and body mass index at age 18) and the most common value for categorical values (≤ 1% for parity/age at first birth and weight change since 18). Additionally, since 45% of the women gave blood before fortification was mandatory in 1998, we conducted stratified analyses to examine whether there were differences for overall and invasive breast cancer before (blood draw before 1998) and after fortification (blood draw 1998 and later). Tests for trend were conducted by modeling the quantile medians as a continuous variable. Tests for tumor subtype heterogeneity were preformed using methods of Wang et al. [26] Tests for interaction were performed using likelihood ratio tests of models with and without a multiplicative interaction term for a 1 SD increase in the natural log-transformed plasma measure with the binary stratification variable.

Among those with available information on the three genetic polymorphisms, we examined whether the polymorphism itself was associated with breast cancer using multivariable unconditional logistic regression as noted above. We then examined whether associations of plasma folate and the other plasma measures with breast cancer varied by strata of the genetic polymorphisms. Additionally, to examine whether factors known to increase levels of plasma folate (i.e., natural folate from diet, synthetic folate from fortified foods and supplements, MTHFR and DHFR genotypes, other) explained any association between plasma folate and breast cancer risk, we regressed plasma folate on all these factors in the same model. The regression coefficients were then multiplied by the value for each component to obtain predicted plasma folate levels explained by each component. We then examined the association between the predicted plasma folate explained by each component and risk of breast cancer [27].

All *P* values were two-tailed and 0.05 was considered statistically significant. Analyses were conducted using SAS 9.3 (SAS Institute Inc., Cary, NC, USA) for UNIX.

Results

Descriptive characteristics

The time from blood collection to diagnosis of breast cancer for the 610 cases ranged from 1 month to 150 months (median 57). Compared with their matched controls, women with breast cancer tended to consume more alcohol and were more likely to have a family history of breast cancer and a previous history of benign breast disease (Table 1). Reproductive factors, including age at menarche, age at first birth, and use of postmenopausal hormones did not differ significantly between cases and controls. Most plasma levels of nutrients were not significantly different in between breast cancer cases and their matched controls, except plasma levels of vitamins PLP and B_{12} which were higher in cases. However, dietary intake of vitamins B_{12}, B_6, and use of multivitamins did not differ between cases and controls.

Correlations between plasma B-vitamins and dietary intake

Plasma levels of folate, B_{12}, and PLP tended to be moderately correlated with each other ($r = 0.41$ to 0.43), whereas homocysteine was inversely correlated ($r = -0.16$ to -0.29; Supplementary Table 1). Cysteine was most strongly correlated to homocysteine ($r = 0.40$) and cysteinylglycine was slightly correlated with homocysteine and cysteine ($r = 0.12$ and 0.27, respectively). In general, correlations of plasma folate, B_{12}, and PLP were stronger for total dietary intake including supplements ($r = 0.21$ to 0.37) than with foods sources only ($r = 0.10$ to 0.23). Dietary intakes of folate, B_{12}, and B_6 were inversely correlated with homocysteine and not correlated with plasma cysteine or cysteinylglycine.

Relation of plasma B-vitamins and breast cancer risk

Plasma levels of folate, PLP, homocysteine, cysteine, and cysteinylglycine were not significantly associated with risk of breast cancer (Table 2). The multivariable adjusted relative risks (95% CI) of total breast cancer comparing the highest quintile to the lowest quintile were 1.18 (0.84–1.66) for plasma folate, 1.18 (0.85–1.64) for plasma PLP, 0.93 (0.67–1.28) for plasma homocysteine, 1.14 (0.81–1.62) for plasma cysteine, and 0.93 (0.66–1.31) for plasma cysteinylglycine. In contrast, a positive association between plasma vitamin B_{12} and risk of breast cancer was observed (comparable multivariable RR = 1.64 (1.17–2.29); p for trend = 0.02). In exploratory analyses, the estimates for plasma B_{12} and risk of breast cancer were unchanged when we additionally controlled for intake of animal fat or red meat (data not shown). The association between plasma B_{12} and breast cancer risk was attenuated, but still significantly positive, when we removed cases diagnosed within 1 year of blood collection (RR for highest quintile versus lowest = 1.47, 95% CI 1.04–2.10, p for trend = 0.04) or 2 years of blood collection (RR for highest quintiles versus lowest = 1.43, 95% CI 0.99–2.06, p for trend = 0.04).

Plasma levels of PLP, homocysteine, and cysteine were not significantly associated with any subgroups of breast cancer and tests of heterogeneity were non-significant for all nutrients (Table 3). Plasma folate and B_{12} were positively associated with several tumor subtypes. For the highest tertile compared to the lowest, plasma folate was associated with a 47% increase in invasive breast cancer (p for trend = 0.01), 60% increased risk in ER+/PR+ tumors (p for trend = 0.01), and a 58% increase in luminal A tumors (p for trend = 0.06). For plasma B_{12}, comparing the highest tertile of plasma B_{12} to the lowest, the relative risks (95% CI) were 1.34 (1.00–1.81) for invasive tumors, 1.32 (0.87–1.99) for in situ, 1.39 (0.98–1.97) for ER+/PR+ tumors, and 2.11 (1.24–3.59) for luminal B tumors. Results were significant in continuous models. Plasma cysteinylglycine was not significantly associated with invasive or in situ breast tumors separately, nor with luminal A or luminal B tumors. However, in continuous analyses, plasma cysteinylglycine was positively associated with ER+/PR− tumors (RR = 1.45, 95% CI 1.01–2.07) and borderline inversely associated with ER−/PR− tumors (RR = 0.76, 95% CI 0.59–1.00).

Results did not vary by fortification period for any of the nutrients with overall breast cancer (P-interaction for all ≥ 0.13). However, plasma folate had a significant

Table 1 Characteristics of breast cancer cases and matched controls at blood collection in the Nurses' Health Study II

Characteristics	Cases ($n=610$)	Controls ($n=1207$)
	Mean (SD)	Mean (SD)
Age (years)	45.0 (4.4)	44.9 (4.4)
Age at menarche (years)	12.4 (1.3)	12.4 (1.4)
Age at first birth[a] (years)	26.5 (4.6)	26.2 (4.6)
Parity[a]	2.2 (0.9)	2.3 (0.9)
Body mass index at age 18 (kg/m^2)	20.7 (2.7)	21.1 (3.0)
Body mass index at blood collection (kg/m^2)	25.2 (4.9)	25.9 (6.1)
Alcohol Intake (g/day)	4.1 (7.1)	3.5 (6.3)
Age at menopause (years)	43.2 (5.2)	42.4 (3.8)
Characteristics	Cases ($n=610$)	Controls ($n=1207$)
	%	%
Family history of breast cancer	28	17
Personal history of benign breast disease	56	49
Current use of multivitamins	44	44
Postmenopausal	13	15
Postmenopausal hormone use, current[b]	89	83
Characteristics	Cases ($n=610$)	Controls ($n=1207$)
Plasma measures	Median (5–95th percentile)	Median (5–95th percentile)
Folate (ng/mL)	16.4 (7.1–32.8)	16.0 (6.0–33.2)
Vitamin B_{12} (pg/mL)	443 (221–1012)	424 (207–896)
Pyridoxal 5′-phosphate (pmol/mL)	49.9 (19.8–321.5)	48.8 (19.9–272.9)
Homocysteine (nmol/mL)	9.3 (6.0–15.1)	9.2 (6.2–15.0)
Cysteine (nmol/mL)	244.1 (195.5–310.7)	242.7 (191.8–315.9)
Cysteinylglycine (nmol/mL)	243.8 (163.8–369.7)	245.5 (159.3–376.8)
Characteristics	Cases ($n=610$)	Controls ($n=1207$)
Dietary intake	Median (5–95th percentile)	Median (5–95th percentile)
Total folate (μg/day)	366 (185–941)	366 (176–929)
Folate from foods only (μg/day)	290 (178–506)	290 (170–488)
Total B_{12} (μg/day)	7.0 (3.0–23.0)	7.0 (3.0–21.0)
B_{12} from foods only (μg/day)	5.2 (2.7–12.0)	5.2 (2.7–10.8)
Total B_6 (mg/day)	2.5 (1.5–55.8)	2.5 (1.5–43.4)
B_6 from foods only (mg/day)	2.0 (1.5–3.0)	2.0 (1.5–2.8)

[a]Among parous women only

[b]Among postmenopausal women only

positive association with invasive breast cancer post-fortification (pre-fortification: RR [1 SD increase ln-folate] = 1.06, 95% CI 0.88–1.28; post-fortification: RR = 1.21, 95% CI 1.00–1.45; p-interaction = 0.18).

Relation of plasma B-vitamins and breast cancer risk stratified by other risk factors

Associations varied significantly by age at blood collection only for plasma B_{12} and plasma homocysteine (Table 4). Among those less than 45 years, higher plasma B_{12} had significantly higher risk (RR = 1.31, 95% CI 1.12–1.54) than those 45 and older (RR = 1.10, 95% CI 0.96–1.27) (p for interaction = 0.02). Conversely, for homocysteine, women younger than 45 appeared to have a non-significant lower risk of breast cancer while older women had no association with breast cancer with increasing homocysteine levels. Significant interactions with current smoking status at blood collection were seen for plasma B_{12} and cysteinylglycine. Among never or past smokers, plasma B_{12} had a significant

Table 2 Relative risks and 95% confidence intervals (CI) of total breast cancer by quintiles of plasma measures

	Quintile of plasma measure					*P* for
	1	2	3	4	5	Trend
Folate, ng/mL	< 10.2	10.2 to 13.8	13.9 to 17.9	18.0 to 23.6	≥ 23.7	
Cases/Controls	109/240	103/242	143/240	125/241	129/240	
Simple relative risk[a] (95% CI)	1.00 (ref.)	0.96 (0.68, 1.35)	1.24 (0.90, 1.72)	1.12 (0.81, 1.56)	1.25 (0.90, 1.74)	0.13
Multivariable relative risk[b] (95% CI)	1.00 (ref.)	0.99 (0.70, 1.40)	1.20 (0.86, 1.67)	1.11 (0.79, 1.55)	1.18 (0.84, 1.66)	0.28
Vitamin B_{12}, pg/mL	< 281	281 to 371	372 to 472	473 to 619	≥620	
Cases/Controls	93/240	124/241	123/241	118/241	150/241	
Simple relative risk[a] (95% CI)	1.00 (ref.)	1.42 (1.02, 1.98)	1.53 (1.09, 2.14)	1.40 (1.00, 1.96)	1.71 (1.23, 2.37)	0.008
Multivariable relative risk[b] (95% CI)	1.00 (ref.)	1.42 (1.01, 2.01)	1.57 (1.11, 2.21)	1.42 (1.01, 2.00)	1.64 (1.17, 2.29)	0.02
Pyridoxal 5′-phosphate, pmol/mL	< 30.0	30.0 to 41.8	41.9 to 58.6	58.7 to 96.1	≥96.2	
Cases/Controls	112/241	123/242	120/241	122/242	133/241	
Simple relative risk[a] (95% CI)	1.00 (ref.)	1.15 (0.83, 1.58)	1.13 (0.82, 1.57)	1.16 (0.84, 1.61)	1.30 (0.95, 1.79)	0.14
Multivariable relative risk[b] (95% CI)	1.00 (ref.)	1.13 (0.82, 1.58)	1.02 (0.73, 1.42)	1.06 (0.75, 1.48)	1.18 (0.85, 1.64)	0.4
Homocysteine, nmol/mL	< 7.5	7.5 to 8.6	8.7 to 9.7	9.8 to 11.3	≥ 11.4	
Cases/Controls	128/239	121/241	109/239	124/240	127/240	
Simple relative risk[a] (95% CI)	1.00 (ref.)	0.90 (0.65, 1.24)	0.82 (0.59, 1.13)	0.90 (0.65, 1.23)	0.93 (0.68, 1.28)	0.81
Multivariable relative risk[b] (95% CI)	1.00 (ref.)	0.88 (0.64, 1.22)	0.80 (0.57, 1.11)	0.90 (0.65, 1.24)	0.93 (0.67, 1.28)	0.85
Cysteine, nmol/mL	< 218.0	218.0 to 235.7	235.8 to 249.9	250.0 to 268.1	≥ 268.2	
Cases/Controls	99/241	125/241	125/242	138/241	121/241	
Simple relative risk[a] (95% CI)	1.00 (ref.)	1.15 (0.83, 1.60)	1.15 (0.83, 1.61)	1.22 (0.88, 1.69)	1.16 (0.83, 1.62)	0.42
Multivariable relative risk[b] (95% CI)	1.00 (ref.)	1.11 (0.79, 1.56)	1.13 (0.80, 1.58)	1.18 (0.84, 1.65)	1.14 (0.81, 1.62)	0.46
Cysteinylglycine, nmol/mL	< 200.0	200.0 to 229.7	229.8 to 260.6	260.7 to 304.6	≥ 304.7	
Cases/Controls	111/239	123/240	131/240	134/240	102/240	
Simple relative risk[a] (95% CI)	1.00 (ref.)	1.04 (0.75, 1.44)	1.13 (0.82, 1.56)	1.12 (0.81, 1.55)	0.87 (0.62, 1.22)	0.46
Multivariable relative risk[b] (95% CI)	1.00 (ref.)	1.04 (0.75, 1.45)	1.12 (0.81, 1.56)	1.09 (0.79, 1.52)	0.93 (0.66, 1.31)	0.69

[a]Unconditional logistic regression with adjustments for matching factors

[b]Unconditional logistic regression with the adjustments for matching factors, age at menarche, parity/age at first birth, history of breast cancer in mother or a sister, history of benign breast disease, height, body mass index at age 18, weight change since 18, and alcohol intake

positive association (RR = 1.24, 95% CI 1.11–1.38) and among current smokers there was a non-significant inverse association (RR = 0.77, 95% CI 0.52–1.15). Cysteinylglycine similarly was inversely associated with breast cancer among those that were current smokers at blood collection (RR = 0.53, 95% CI 0.33–0.83) but not associated with risk among past or never smokers. When stratified by alcohol intake, among those who consumed less than 10 g/day, there were significant positive associations with breast cancer with increasing plasma folate and plasma B_{12}; however, only plasma folate had a significant *P* for interaction ($p = 0.03$). There was a significant interaction for plasma cysteine and cysteinylglycine with current multivitamin use at blood collection; however, neither stratum for cysteinylglycine reached significance. With higher plasma cysteine levels, among current multivitamin users, there was a significantly higher breast cancer risk (RR = 1.22, 95% CI 1.04–1.44) and among non-users, there was a non-significant inverse association (RR = 0.90, 95% CI 0.78–1.05). Plasma measures did not vary by BMI status (results not shown), except homocysteine (*P*-interaction = 0.002). However, strata were only borderline significant with women < 25 kg/m^2 (RR = 0.89, 95% CI 0.78–1.02) and women ≥ 25 kg/m^2 (RR = 1.12, 95% CI 0.95–1.32).

Plasma B-vitamins, MTHFR & DHFR, and breast cancer risk

As expected, adjusted mean folate plasma levels declined with each additional minor allele of *MTHFR* 677C > T and the difference between TT genotype (mean: 14.4 ng/ml) and CC genotype (mean: 16.3 ng/ml) was statistically significant (Supplementary Table 2). The adjusted mean plasma folate plasma levels were similar for *MTHFR* 1298A > C across genotypes. Lastly, for each additional deletion on the *DHFR* gene, the adjusted mean plasma folate levels increased, and having two deletions (mean: 16.8 ng/ml) compared to not having a deletion (mean: 14.7 ng/ml) was statistically significant.

Table 3 Multivariable relative risks and 95% confidence intervals of breast cancer subtypes by tertiles of plasma measures

	Cases/controls	Tertile of plasma measure			*P* for Trend	Continuous[†,‡]
		1	2	3		
Folate, ng/mL		< 12.7	12.7 to 19.6	≥ 19.7		
Invasive breast cancer	423/1203	1.00 (ref.)	1.19 (0.87, 1.62)	1.47 (1.09, 2.00)	0.01	1.16 (1.02, 1.31)
In situ breast cancer	166/1203	1.00 (ref.)	1.14 (0.75, 1.72)	0.90 (0.58, 1.39)	0.56	1.00 (0.85, 1.19)
ER+/PR+ tumors	291/1203	1.00 (ref.)	1.32 (0.92, 1.89)	1.60 (1.12, 2.29)	0.01	1.15 (0.99, 1.32)
ER+/PR− tumors	38/1203	1.00 (ref.)	1.03 (0.42, 2.54)	1.54 (0.65, 3.66)	0.28	1.17 (0.82, 1.68)
ER−/PR− tumors	72/1203	1.00 (ref.)	1.08 (0.57, 2.04)	1.20 (0.63, 2.28)	0.56	1.18 (0.90, 1.55)
Luminal A	190/1203	1.00 (ref.)	1.49 (0.98, 2.29)	1.58 (1.03, 2.45)	0.06	1.08 (0.91, 1.28)
Luminal B	109/1203	1.00 (ref.)	0.95 (0.54, 1.64)	1.59 (0.95, 2.67)	0.04	1.23 (0.99, 1.53)
Vitamin B_{12}, pg/mL		<338	338 to 516	≥517		
Invasive breast cancer	422/1204	1.00 (ref.)	1.47 (1.09, 1.98)	1.34 (1.00, 1.81)	0.09	1.20 (1.07, 1.36)
In situ breast cancer	166/1204	1.00 (ref.)	1.02 (0.66, 1.56)	1.32 (0.87, 1.99)	0.16	1.20 (1.01, 1.42)
ER+/PR+ tumors	291/1204	1.00 (ref.)	1.53 (1.08, 2.17)	1.39 (0.98, 1.97)	0.10	1.24 (1.08, 1.42)
ER+/PR− tumors	37/1204	1.00 (ref.)	2.26 (0.92, 5.57)	1.49 (0.58, 3.85)	0.60	1.11 (0.78, 1.59)
ER−/PR− tumors	72/1204	1.00 (ref.)	0.98 (0.53, 1.83)	1.09 (0.60, 1.98)	0.77	1.05 (0.82, 1.35)
Luminal A	190/1204	1.00 (ref.)	1.41 (0.94, 2.10)	1.03 (0.68, 1.55)	0.96	1.10 (0.94, 1.29)
Luminal B	109/1204	1.00 (ref.)	1.68 (0.96, 2.93)	2.11 (1.24, 3.59)	0.008	1.43 (1.16, 1.77)
Pyridoxal 5′-phosphate, pmol/mL		<37.5	37.5 to 67.0	≥67.1		
Invasive breast cancer	423/1207	1.00 (ref.)	0.96 (0.72, 1.29)	1.01 (0.75, 1.35)	0.89	1.06 (0.94, 1.20)
In situ breast cancer	167/1207	1.00 (ref.)	1.13 (0.74, 1.72)	1.04 (0.68, 1.61)	0.99	1.09 (0.91, 1.29)
ER+/PR+ tumors	291/1207	1.00 (ref.)	0.98 (0.70, 1.38)	1.02 (0.72, 1.44)	0.88	1.05 (0.91, 1.21)
ER+/PR− tumors	38/1207	1.00 (ref.)	1.29 (0.55, 3.02)	1.20 (0.50, 2.85)	0.79	1.24 (0.90, 1.71)
ER−/PR− tumors	72/1207	1.00 (ref.)	0.65 (0.35, 1.21)	0.84 (0.46, 1.53)	0.79	1.01 (0.78, 1.31)
Luminal A	190/1207	1.00 (ref.)	0.97 (0.65, 1.45)	0.93 (0.62, 1.40)	0.75	1.11 (0.94, 1.30)
Luminal B	109/1207	1.00 (ref.)	1.04 (0.62, 1.75)	1.14 (0.68, 1.91)	0.61	1.01 (0.81, 1.25)
Homocysteine, nmol/mL		<8.3	8.3 to 10.0	≥10.1		
Invasive breast cancer	423/1199	1.00 (ref.)	0.78 (0.58, 1.04)	0.88 (0.66, 1.18)	0.48	0.95 (0.84, 1.07)
In situ breast cancer	166/1199	1.00 (ref.)	0.90 (0.59, 1.38)	1.13 (0.75, 1.69)	0.50	1.01 (0.85, 1.20)
ER+/PR+ tumors	291/1199	1.00 (ref.)	0.65 (0.46, 0.92)	0.83 (0.59, 1.15)	0.38	0.91 (0.79, 1.05)
ER+/PR− tumors	38/1199	1.00 (ref.)	0.72 (0.33, 1.58)	0.47 (0.19, 1.14)	0.10	0.78 (0.54, 1.12)
ER−/PR− tumors	72/1199	1.00 (ref.)	1.33 (0.71, 2.51)	1.36 (0.72, 2.58)	0.38	1.15 (0.89, 1.48)
Luminal A	190/1199	1.00 (ref.)	0.66 (0.44, 1.00)	0.83 (0.56, 1.24)	0.49	0.86 (0.72, 1.01)
Luminal B	109/1199	1.00 (ref.)	0.78 (0.47, 1.28)	0.72 (0.43, 1.20)	0.22	0.97 (0.79, 1.19)
Cysteine, nmol/mL		<231.4	231.4 to 253.8	≥253.9		
Invasive breast cancer	422/1206	1.00 (ref.)	0.95 (0.70, 1.28)	1.06 (0.78, 1.43)	0.68	1.01 (0.89, 1.15)
In situ breast cancer	166/1206	1.00 (ref.)	1.04 (0.68, 1.59)	1.37 (0.90, 2.09)	0.13	1.07 (0.90, 1.27)
ER+/PR+ tumors	291/1206	1.00 (ref.)	0.76 (0.53, 1.08)	0.96 (0.68, 1.36)	0.93	0.98 (0.84, 1.13)
ER+/PR− tumors	38/1206	1.00 (ref.)	1.41 (0.60, 3.35)	1.26 (0.51, 3.14)	0.66	1.19 (0.83, 1.71)
ER−/PR− tumors	71/1206	1.00 (ref.)	1.13 (0.61, 2.09)	1.05 (0.55, 2.01)	0.89	0.94 (0.70, 1.26)
Luminal A	190/1206	1.00 (ref.)	0.85 (0.56, 1.29)	0.92 (0.61, 1.39)	0.73	0.89 (0.74, 1.06)
Luminal B	109/1206	1.00 (ref.)	0.91 (0.55, 1.51)	0.93 (0.55, 1.57)	0.80	1.07 (0.85, 1.34)
Cysteinylglycine, nmol/mL		<219.8	219.8 to 272.6	≥272.7		
Invasive breast cancer	419/1199	1.00 (ref.)	1.17 (0.87, 1.58)	1.08 (0.80, 1.47)	0.68	1.03 (0.91, 1.17)
In situ breast cancer	162/1199	1.00 (ref.)	1.01 (0.68, 1.51)	0.77 (0.50, 1.19)	0.23	0.90 (0.76, 1.08)
ER+/PR+ tumors	288/1199	1.00 (ref.)	1.11 (0.79, 1.57)	1.10 (0.78, 1.55)	0.63	1.03 (0.89, 1.19)
ER+/PR− tumors	38/1199	1.00 (ref.)	1.38 (0.55, 3.50)	1.85 (0.76, 4.54)	0.17	1.45 (1.01, 2.07)
ER−/PR− tumors	72/1199	1.00 (ref.)	1.13 (0.64, 2.00)	0.56 (0.29, 1.10)	0.09	0.76 (0.59, 1.00)
Luminal A	190/1199	1.00 (ref.)	1.25 (0.83, 1.89)	1.14 (0.76, 1.73)	0.60	1.02 (0.86, 1.20)
Luminal B	106/1199	1.00 (ref.)	1.17 (0.70, 1.97)	1.07 (0.63, 1.82)	0.85	1.05 (0.84, 1.31)

Unconditional logistic regression with adjustments for matching factors, age at menarche, parity/age at first birth, history of breast cancer in mother or a sister, history of benign breast disease, height, body mass index at age 18, weight change since 18, and alcohol intake

[†] 1 SD increase in natural log-transformed plasma measure

[‡] All p for heterogeneity > 0.05; Comparisons: (1) invasive vs. in situ; (2) ER+/PR+ vs. ER+/PR− vs. ER−/PR− tumors; (3) luminal A vs. luminal B

We observed no significant associations of *MTHFR* C677T, *MTHFR* A1298C, or *DHFR* 19-bp deletion polymorphisms with breast cancer risk (results not shown). However, when the relationship between plasma measures and breast cancer risk was stratified by genotype of the *MTHFR* C677T polymorphism, there were significant

Table 4 Multivariable relative risks and 95% confidence intervals of breast cancer for a 1 SD increase in natural log-transformed plasma measure by age at blood, smoking status, alcohol intake, and current multivitamin use at blood collection

Plasma measure	Cases/Controls	Per 1-SD increase in natural log-transformed concentrations RR (95% CI)	Cases/Controls	Per 1-SD increase in natural log-transformed concentrations RR (95% CI)	*P* for interaction
	Age at blood < 45 years		Age at blood ≥ 45 years		
Folate, ng/mL	281/591	1.13 (0.96, 1.32)	328/614	1.04 (0.89, 1.21)	0.15
Vitamin B_{12}, pg/ml	280/591	1.31 (1.12, 1.54)	328/615	1.10 (0.96, 1.27)	0.02
Pyridoxal 5′-phosphate, pmol/ml	281/592	1.16 (0.98, 1.37)	329/617	1.04 (0.91, 1.19)	0.20
Homocysteine, nmol/ml	281/589	0.90 (0.77, 1.06)	328/612	1.04 (0.90, 1.21)	0.04
Cysteine, nmol/ml	281/592	1.00 (0.85, 1.19)	327/616	1.06 (0.92, 1.22)	0.32
Cysteinylglycine, nmol/ml	276/588	1.00 (0.85, 1.18)	325/613	0.99 (0.86, 1.15)	0.92
Plasma measure	Cases/Controls	Per 1-SD increase in natural log-transformed concentrations RR (95% CI)	Cases/Controls	Per 1-SD increase in natural log-transformed concentrations RR (95% CI)	*P* for interaction
	Never/Past Smoker at Blood		Current Smoker at blood		
Folate, ng/mL	553/1108	1.09 (0.97, 1.22)	56/96	1.01 (0.68, 1.50)	0.70
Vitamin B_{12}, pg/ml	552/1109	1.24 (1.11, 1.38)	56/96	0.77 (0.52, 1.15)	0.001
Pyridoxal 5′-phosphate, pmol/ml	553/1112	1.06 (0.95, 1.19)	57/96	1.47 (1.02, 2.12)	0.12
Homocysteine, nmol/ml	553/1106	0.99 (0.89, 1.10)	56/94	0.79 (0.52, 1.19)	0.17
Cysteine, nmol/ml	552/1111	1.05 (0.93, 1.17)	56/96	0.96 (0.66, 1.38)	0.49
Cysteinylglycine, nmol/ml	546/1105	1.04 (0.93, 1.17)	55/95	0.53 (0.33, 0.83)	0.0001
Plasma measure	Cases/Controls	Per 1-SD increase in natural log-transformed concentrations RR (95% CI)	Cases/Controls	Per 1-SD increase in natural log-transformed concentrations RR (95% CI)	*P* for interaction
	Alcohol < 10 g/day		Alcohol ≥ 10 g/day		
Folate, ng/mL	530/1080	1.14 (1.01, 1.28)	79/125	0.83 (0.58, 1.18)	0.03
Vitamin B_{12}, pg/ml	529/1081	1.22 (1.09, 1.36)	79/125	0.99 (0.70, 1.39)	0.13
Pyridoxal 5′-phosphate, pmol/ml	531/1084	1.10 (0.99, 1.23)	79/125	0.92 (0.66, 1.28)	0.12
Homocysteine, nmol/ml	530/1076	0.96 (0.85, 1.07)	79/125	1.23 (0.89, 1.70)	0.08
Cysteine, nmol/ml	530/1083	1.02 (0.91, 1.14)	78/125	1.22 (0.86, 1.72)	0.07
Cysteinylglycine, nmol/ml	523/1076	0.93 (0.81, 1.07)	78/125	0.94 (0.66, 1.34)	0.78
Plasma measure	Cases/Controls	Per 1-SD increase in natural log-transformed concentrations RR (95% CI)	Cases/Controls	Per 1-SD increase in natural log-transformed concentrations RR (95% CI)	*P* for interaction
	Non-current multivitamin user at blood		Current multivitamin user at blood		
Folate, ng/mL	343/672	1.08 (0.93, 1.26)	266/533	1.15 (0.97, 1.36)	0.35
Vitamin B_{12}, pg/ml	342/672	1.15 (1.00, 1.33)	266/534	1.29 (1.09, 1.53)	0.17
Pyridoxal 5′-phosphate, pmol/ml	343/673	1.14 (0.98, 1.32)	267/536	1.05 (0.90, 1.23)	0.33
Homocysteine, nmol/ml	343/669	0.95 (0.83, 1.10)	266/532	1.02 (0.86, 1.20)	0.24
Cysteine, nmol/ml	342/673	0.90 (0.78, 1.05)	266/535	1.22 (1.04, 1.44)	<0.0001
Cysteinylglycine, nmol/ml	340/670	0.93 (0.81, 1.07)	261/531	1.09 (0.92, 1.29)	0.009

Unconditional logistic regression with adjustments for matching factors, age at menarche, parity/age at first birth, history of breast cancer in mother or a sister, history of benign breast disease, height, body mass index at age 18, weight change since 18, and alcohol intake

positive associations among women with the minor allele (i.e., CT, TT) for folate, B_{12}, and PLP with breast cancer (Table 5). Among women with the TT genotype, the relative risks (95% CI) for a one SD increase in natural log-transformed plasma measures were 2.01 (1.21–3.34) for folate, 1.72 (1.08–2.72) for B_{12}, and 1.78 (1.14–2.77) for PLP. However, only B_{12} and PLP had significant *P* for interactions comparing those having a T allele versus not having a T allele. The associations between the plasma measures and breast cancer did not vary when comparing those with a *MTHFR* 1298A > C minor allele (i.e., AC, CC) to those without; though plasma folate and B_{12} had significant associations within genotype strata. Folate and the other plasma measures, with the exception of B_{12}, did not vary by *DHFR* 19-bp deletion genotypes. Among those with a 19-bp deletion, there was a positive association between B_{12} and breast cancer; however, the *P* for interaction was non-significant.

The RR (95% CI) of breast cancer was 0.86 (0.58–1.26) for a 1 ng/ml increase in plasma folate explained by intake of natural folate, 1.14 (0.85–1.51) for a 1 ng/ml increase in plasma folate explained by intake of synthetic folate, 1.93 (0.44–8.46) for a 1 ng/ml increase in plasma folate explained by MTHFR and DHFR genotypes, and 1.16 (0.97–1.39) for a 1 ng/ml increase in plasma folate explained by other factors (i.e., residual).

Discussion

In this large prospective study, higher plasma levels of vitamin PLP, homocysteine, cysteine, and cysteinylglycine were not associated with risk of breast cancer overall among

Table 5 Relative risks and 95% confidence intervals of breast cancer according to 1 SD increase in natural log-transformed plasma measures and polymorphisms

MTHFR 677C > T	CC Cases/Controls (166/360)	CT Cases/Controls (225/397)	TT Cases/Controls (*n* = 51/119)	*P* for interaction*	CT + TT Cases/Controls (*n* = 276/516)	*P* for interaction†
Plasma folate, ng/mL	1.03 (0.83–1.27)	1.06 (0.88–1.28)	2.01 (1.21–3.34)	0.28	1.21 (1.02–1.42)	0.13
Plasma vitamin B_{12}, pg/ml	1.12 (0.90–1.39)	1.22 (1.01–1.47)	1.72 (1.08–2.72)	0.45	1.30 (1.10–1.53)	0.04
Plasma pyridoxal 5′-phosphate, pmol/ml	0.92 (0.74–1.15)	1.11 (0.92–1.35)	1.78 (1.14–2.77)	0.01	1.18 (1.00–1.40)	0.05
Plasma homocysteine, nmol/ml	1.03 (0.83–1.27)	1.05 (0.88–1.26)	0.91 (0.59–1.39)	0.25	1.02 (0.87–1.20)	0.67
Plasma cysteine, nmol/ml	0.95 (0.77–1.18)	1.16 (0.97–1.39)	1.33 (0.89–1.98)	0.82	1.16 (0.99–1.36)	0.01
Plasma cysteinylglycine, nmol/ml	0.97 (0.80–1.18)	1.04 (0.87–1.25)	1.28 (0.82–1.98)	0.21	1.04 (0.89–1.23)	0.15
MTHFR1298A > C	AA Cases/Controls (279/530)	AC Cases/Controls (*n* = 266/511)	CC Cases/Controls (*n* = 50/100)	*P* for interaction*	AC + CC Cases/Controls (*n* = 316/611)	*P* for interaction†
Plasma folate, ng/mL	1.22 (1.04–1.44)	1.02 (0.86–1.20)	1.18 (0.70–1.99)	0.03	1.03 (0.88–1.20)	0.09
Plasma vitamin B_{12}, pg/ml	1.34 (1.15–1.56)	1.10 (0.93–1.29)	2.09 (1.16–3.76)	0.001	1.15 (0.99–1.34)	0.07
Plasma pyridoxal 5′-phosphate, pmol/ml	1.12 (0.96–1.30)	1.10 (0.93–1.29)	1.06 (0.66–1.71)	0.01	1.10 (0.94–1.28)	0.93
Plasma homocysteine, nmol/ml	0.98 (0.84–1.14)	0.97 (0.83–1.14)	1.04 (0.65–1.68)	0.31	0.97 (0.83–1.12)	0.83
Plasma cysteine, nmol/ml	1.15 (0.98–1.35)	0.96 (0.81–1.14)	1.35 (0.82–2.22)	0.23	0.96 (0.82–1.12)	0.02
Plasma cysteinylglycine, nmol/ml	1.01 (0.86–1.19)	0.96 (0.81–1.13)	1.13 (0.73–1.75)	0.77	0.99 (0.85–1.15)	0.83
DHFR WT > del	WT/WT Cases/Controls (*n* = 188/377)	WT/del Cases/Controls (*n* = 280/576)	del/del Cases/Controls (*n* = 125/205)	P for interaction*	WT/del + del/del Cases/Controls (*n* = 405/781)	P for interaction†
Plasma folate, ng/mL	1.10 (0.89–1.37)	1.05 (0.90–1.23)	1.09 (0.85–1.40)	0.75	1.07 (0.94–1.22)	0.38
Plasma vitamin B_{12}, pg/ml	1.18 (0.96–1.44)	1.09 (0.93–1.27)	1.50 (1.16–1.93)	0.44	1.22 (1.07–1.38)	0.88
Plasma pyridoxal 5′-phosphate, pmol/ml	1.08 (0.88–1.34)	1.09 (0.93–1.26)	0.99 (0.77–1.27)	0.50	1.07 (0.94–1.21)	0.73
Plasma homocysteine, nmol/ml	1.01 (0.83–1.24)	0.99 (0.84–1.15)	0.95 (0.75–1.20)	0.85	0.97 (0.85–1.10)	0.54
Plasma cysteine, nmol/ml	1.03 (0.83–1.27)	1.02 (0.87–1.19)	1.13 (0.87–1.47)	0.37	1.06 (0.93–1.20)	0.72
Plasma cysteinylglycine, nmol/ml	1.04 (0.84–1.29)	1.03 (0.88–1.20)	0.90 (0.70–1.17)	0.69	0.99 (0.87–1.13)	0.66

**P* for interaction compares all three genotypes using ordinal coding

†*P* for interaction compares any minor allele to no minor allele

predominantly premenopausal women. In contrast, we observed a significant positive association between plasma levels of vitamin B_{12} and risk of breast cancer overall. While we noted no association between plasma folate and breast cancer overall and no significant heterogeneity by tumor subtype, we did observe significant positive associations with invasive, ER+/PR+, and luminal A tumors. Further, we found that several of the nutrient/breast cancer associations appeared to vary by age, smoking status, and genotype.

Four prior studies have examined plasma folate among premenopausal women. The Women's Health Study reported a significant positive association with invasive breast cancer risk (RR quintile 5 vs 1 = 1.99, 95% CI 1.01–3.93, *P* for trend = 0.04) [19] and the three other studies found no significant association [18, 28, 29]. While the study by Kim et al. was non-significant among premenopausal women (OR high vs low = 1.88, 95% CI 0.35–10.07), the overall association (regardless of menopausal status) was significantly positive (OR high vs low = 3.20, 95% CI 1.03–9.92) [28]. Both positive studies [19, 28] were conducted in populations consuming fortified foods, whereas the other studies either were done prior to fortification [18] or in countries where food is not fortified [29]. Furthermore, in the present study among women who provided blood samples after 1998, there was a suggestive increased risk of invasive breast cancer with higher folate levels, similar to the WHS [19].

In the present study, we observed an unexpected positive association between plasma B_{12} and breast cancer risk. Among studies that have examined the relationship between plasma B12 and breast cancer risk among premenopausal women, one study found no association [18] and two found non-significant positive associations [19, 29]. Comparing the highest versus lowest category, the RR (95% CI) was 1.63 (95% CI 0.83–3.19, *p* for trend = 0.08) in the Women's Health Study [19] and 1.26 (95% CI 0.90–1.77, *p* for trend = 0.10) in the EPIC cohort [29]. Our findings are unlikely to be explained by consumption of red meat, which is a good source of B_{12} and has been previously been associated with higher risk of breast cancer in this cohort [31–33]. However, it is possible that our observed association may be due to other unknown confounders. A limited number of studies have suggested that elevated levels of plasma B_{12} or haptocorrin, a B_{12} binding protein, may indicate undiagnosed (subclinical) cancer, primarily upper gastrointestinal, liver, pancreas, lung, and myeloid cancers [34–36], though a weak association with breast cancer was recently noted [36]. However, the positive association between plasma B_{12} and breast cancer risk in the present study was only modestly attenuated after removing cases diagnosed within one or 2 years of blood collection and thus reverse causation likely does not explain the association.

We observed no association between plasma PLP and overall breast cancer risk similar to two of three other prospective studies that examined premenopausal breast cancer risk [18, 19]. In the third study, a significant inverse association was observed among premenopausal women (OR 1SD increase = 0.66, 95% CI 0.48–0.92) [17]. Similar to our results, prior studies found no significant associations with either plasma homocysteine [17, 18] or cysteinylglycine [37] among premenopausal women. Lastly, no prior studies have reported results for cysteine among premenopausal women separately.

Among prospective studies that have examined plasma folate and other B-vitamins by menopausal status [17–19, 28, 29, 37], only one study had more than 200 cases among premenopausal women [29] limiting further stratification by tumor subtypes or other risk factors. As no prior study has examined whether associations between plasma folate and other B-vitamins and breast cancer vary by subtypes among premenopausal women, only general comparisons can be made. Similar to other studies of plasma PLP, homocysteine, and cysteine, we did not see significant heterogeneity by ER/PR status [17, 19, 30, 38]. Our finding that plasma folate was positively associated with ER+/PR+ and luminal A breast tumors is similar to the Women's Health Study where borderline positive associations, but no significant heterogeneity, were reported with ER+ and PR+ tumors [19]. While we did not observe significant heterogeneity by ER/PR status similar to other prospective studies, [19, 29, 30] we did observe a significant positive association for plasma B_{12} among ER+/PR+ and luminal B breast tumors. Similar to NHS1 [30], we did not observe significant heterogeneity in associations by hormone receptor status for cysteinylglycine; however, suggestive findings among the small number of ER+/PR− (positive association) and ER−/PR− (inverse association) cases warrant follow-up with larger case numbers.

In the present study, the relationship between plasma B_{12} and breast cancer varied by current smoking status, with positive associations among never/past smokers and a suggestive inverse among current smokers. This finding is consistent with the findings in the NHS1 [30]. Additionally, there was a significant interaction with smoking and cysteinylglycine on breast cancer risk, with an inverse association among those that were current smokers [30], which was not observed in NHS1 or WHS [30, 37, 38]. Prior studies have found no significant heterogeneity in the association of plasma folate and breast cancer by alcohol intake [18, 19, 29], including the NHS1 [30], though prior studies have shown an inverse association between dietary folate and breast cancer among higher alcohol consumers [39], similar to our current finding. The significant interactions of cysteine and cysteinylglycine with current multivitamin use on breast cancer risk were unexpected; in the only prior study to assess this, Lin et al. found no significant heterogeneity by multivitamin intake [37, 38]. Both cysteine and

cysteinylglycine have been shown to act as pro-oxidants [40, 41]. It is conceivable that multivitamin use could have altered the oxidative stress balance, as seen previously in a low-dose multivitamin intervention [42], leading cysteine and cysteinylglycine to act as pro-oxidants, particularly with intake of multivitamins containing iron. Given the limited data to date, additional assessments are warranted.

Similar to our results, most previous studies, but not all, have observed no association of either *MTHFR* 677C > T or *MTHFR* 1298A > C with risk of breast cancer [43]. Additionally, similar to the Long Island Breast Cancer Study Project, we found no overall association with the *DHFR* 19-bp deletion polymorphism and breast cancer risk [5]. Only two studies have examined the plasma folate/breast cancer association stratified by *MTHFR* 677C > T and/or *MTHFR* 1298A > C [10, 29]. European Prospective Investigation into Cancer and Nutrition found no evidence of interaction with either SNP [29], whereas the Malmo Diet Cancer Study found a similar positive trend with tertiles of plasma folate (P for trend = 0.03) for women with minor alleles (CT + TT) for *MTHFR* 677C > T [10].

In our exploratory analysis of predicted plasma folate and breast cancer risk explained by folate intakes and MTHFR and DHFR genotypes, we saw that the increase in plasma folate from dietary folate intake, either natural or synthetic, was not associated with breast cancer. An increase in plasma folate from the MTHFR and DHFR genotypes had strong but not statistically significant positive relation with breast cancer risk. However, as the analysis was exploratory and estimates imprecise, this should be evaluated further.

Those with *MTHFR* polymorphisms have reduced conversion of 5,10-methylene-THF to 5-methyl-THF, which can lead to less DNA methylation and increased DNA synthesis, which in turn has been suggested to allow early neoplastic lesions to progress to breast cancer in animal studies [44, 45]. Additionally, high plasma levels of folate, particularly among those taking folic acid supplements, have been shown to saturate this conversion mechanism (i.e., 5,10-methylene-THF to 5-methyl-THF) resulting in detectable levels of circulating unmetabolized folic acid in plasma [46]. In the Framingham Offspring Cohort, 81% of supplement users were found to have detectable levels of unmetabolized folic acid in their plasma in the post-fortification era [47]. Therefore, with our findings suggesting that the association between plasma folate (which includes unmetabolized folic acid) and breast cancer is primarily found among those with the MTHFR minor alleles, future assessments of unmetabolized folic acid may be of particular interest.

The prospective design and high follow-up rates in our study minimize the possibility that our findings result from methodologic biases. Controlling for established risk factors for breast cancer had minimal effect on relative risks; therefore, results are unlikely to be explained by residual confounding due to these factors. Our focus on younger, predominantly premenopausal women is also a strength.

The analysis examining associations stratified by date of blood collection (before/after mandatory folic acid fortification implementation in 1998) may be non-differentially misclassified, as some food manufacturers began implementing fortification prior to the mandatory date. Additionally, we were unable to distinguish between the different forms of plasma folate, particularly unmetabolized folate levels. Although our study was large overall, a number of our subgroup analyses had limited statistical power.

In summary, PLP, homocysteine, cysteine, and cysteinylglycine were not associated with overall breast cancer, though there were suggestions of associations among subgroups defined by related genetic variants, breast cancer risk factors, and tumor characteristics. Plasma folate and vitamin B_{12} may be positively associated with risk of breast cancer, particularly invasive breast cancer, among younger women. In novel and preliminary analyses, the higher risk of invasive breast cancer with plasma folate was suggestively stronger in the post-fortification period, and the association between folate and invasive breast cancer appeared due mainly to genetic variation. Additional prospective studies among younger women are warranted to evaluate whether our findings for plasma folate and vitamin B_{12} and other subgroups can be replicated or are due to chance. Additionally, future epidemiologic studies should examine the role of unmetabolized folic acid in breast carcinogenesis.

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Data availability Data are from the Nurses' Health Study II. Requests for data access can be made to the Data Access Committee via http://www.nurseshealthstudy.org/researchers.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical standards The study protocol was approved by the institutional review boards of the Brigham and Women's Hospital and Harvard T.H. Chan School of Public Health, and those of participating registries as required.

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