A Prospective Study on Folate, B12, and Pyridoxal 5′-Phosphate (B6) and Breast Cancer[1]

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Abstract

To investigate the incidence of breast cancer and prediagnostic serum levels of folate, B12, and pyridoxal 5′-phosphate (B6), we conducted a nested case-control study using resources from the Washington County (Maryland) serum bank. In 1974, 12,450 serum specimens were donated, and in 1989, 14,625 plasma specimens were donated by female residents of Washington County. One hundred ninety-five incident breast cancer cases and 195 controls were matched by age, race, menopausal status at donation, and cohort participation as well as by date of blood donation. In both cohorts and all menopausal subgroups, median B12 concentrations were lower among cases than controls. Differences reached statistical significance only among women who were postmenopausal at donation (1974 cohort, 413 *versus* 482 pg/ml, $P = 0.03$; 1989 cohort, 406 *versus* 452 pg/ml, $P = 0.02$). Among women postmenopausal at blood donation, observed associations of B12 suggested a threshold effect with increased risk of breast cancer in the lowest one-fifth compared to the higher four-fifths of the control distribution [lowest *versus* highest fifth: 1974 cohort, matched odds ratio = 4.00 (95% confidence interval = 1.05–15.20); 1989 cohort, matched odds ratio = 2.25 (95% confidence interval = 0.86–5.91)]. We found no evidence for an association between folate, B6, and homocysteine and breast cancer. Findings suggested a threshold effect for serum B12 with an increased risk of breast cancer among postmenopausal women in the lowest one-fifth compared to the higher four-fifths of the control distribution. These results should stimulate further investigations of potentially modifiable risk factors, such as these B-vitamins, for prevention of breast cancer.

Introduction

Most accepted risk factors for breast cancer, such as early age at first menarche, late age at menopause, and late age at first full-term pregnancy, are either very difficult or even impossible to alter. For this reason, it is important to search for potentially modifiable risk factors, such as dietary risk factors (1). Micronutrients, especially the antioxidant vitamins C and E and β-carotene, have been investigated for a potential protective role against breast cancer, but results have been inconclusive (2–5). Other vitamins, such as the B-vitamins folate, B12, and B6, have been hypothesized to be involved in carcinogenesis, but associations between these B-vitamins and breast cancer risk have rarely been investigated. Plausible biological mechanisms for the involvement of folate, B12, and PLP,[3] the principal active form of B6 (6), in carcinogenesis include their proposed roles in DNA synthesis, DNA methylation (folate and B12), and steroid hormone action (B6; Refs. 7–12).

The first of these proposed mechanisms stems from the role of these vitamins in purine and thymidine nucleotide synthesis. Deficiency of those B-vitamins can result in reduced synthesis of those nucleotides leading to an impairment in DNA synthesis and DNA repair mechanisms, which, in turn, might increase mutation rate (7, 8).

Another hypothesized mechanism for an involvement of folate and B12 in carcinogenesis is their role in DNA methylation. Folate and B12 are involved in a reaction that provides *de novo* methyl groups for many methylation processes, including DNA methylation (7, 9, 10). DNA hypomethylation has been observed in several human tumors (13), although the specific role of DNA hypomethylation in breast cancer is uncertain (14–18).

The hypothesized involvement of PLP in the regulation of steroid hormone action (11) is the third potential mechanism for a possible role of these B-vitamins in carcinogenesis. One study (12) showed that, after receiving estrogen, ovariectomized B6-deficient female rats suppressed luteinizing hormone to a greater extent than ovariectomized controls whose diets had been supplemented with B6. The authors concluded from these findings and other experiments that there might be a possible association between borderline deficient B6 status and in-

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[3] The abbreviations used are: PLP, pyridoxal 5′-phosphate; OR, odds ratio; CI, confidence interval.

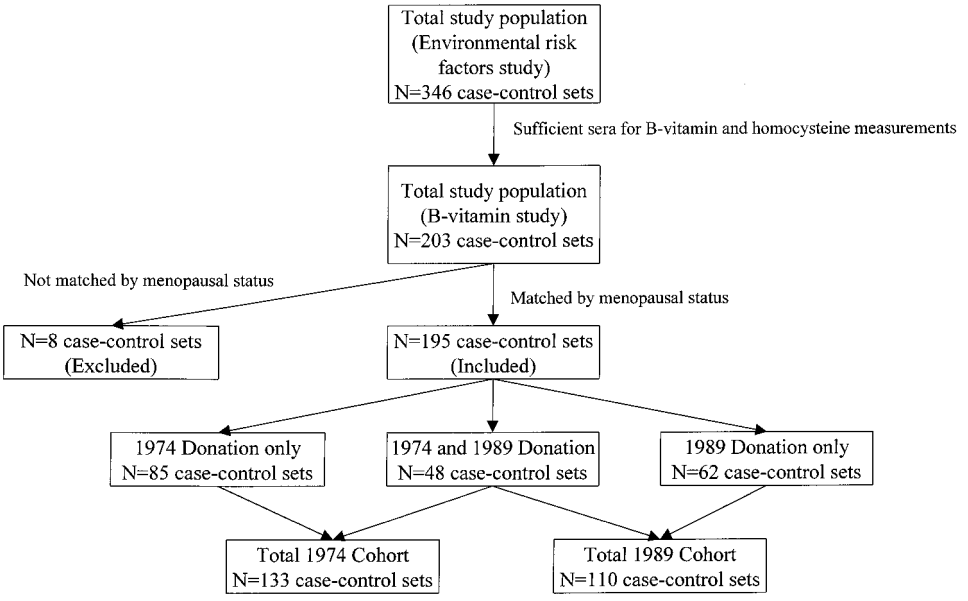

Fig. 1. Cohort participation in study population.

creased sensitivity to steroid hormones, which could have implications for breast, prostate, and uterine carcinogenesis.

We conducted a nested case-control study to investigate the association between prediagnostic serum concentrations of those vitamins and subsequent breast cancer risk. Measurement of serum homocysteine was also included because of findings suggesting that increased homocysteine concentrations might be a marker for deficient folate, B12, and B6 status (19). We used the resources of a serum bank in Washington County, Maryland.

Materials and Methods

In 1974, 12,450 serum samples were collected, and in 1989, 14,625 plasma samples were collected from female residents of Washington County for a serum bank. Samples were stored at −70°C. More detailed information on the Washington County serum bank has been published previously (20). In both programs, a short questionnaire was administered at the time of blood donation. The information collected included smoking history, education, marital status, hours since last meal, use of medications and vitamin supplements during the 48 h prior to blood donation, and days since last menstrual period. The questionnaire in 1989 also collected information on self-reported height and weight. Information on the date of the onset of menses subsequent to blood donation in 1989 was obtained from telephone interviews administered after blood donation to premenopausal participants.

Incident cases were identified by linkage to the Washington County Registry. Completeness of ascertainment of cases by the registry was estimated by comparing observed cases to that expected based on rates from the National Cancer Institute's Surveillance, Epidemiology, and End Results registry (21). Between 1975 and 1992, 1146 incident breast cancer cases were reported to the Washington County cancer registry; 1012 incident breast cancer cases would have been expected in this population, resulting in an observed:expected ratio of 1.13:1. Results were similar using cancer registry data from the recently established Maryland Cancer Registry (22). In 1993, 86 incident female breast cancer cases were reported to the Maryland Cancer Registry and the Washington County cancer registry recorded 89 breast cancer cases.

Cases were defined as women for whom breast cancer was the first cancer (International Classification of Diseases: ICD-8 174 and ICD-9 174), other than nonmelanoma skin cancer or *in situ* cancer of the cervix, diagnosed between January 1, 1975, and July 1, 1994. Cases who had donated in both programs had to be diagnosed with breast cancer after their donation in 1989. One female control, who at the time of diagnosis of the case was not known to have died or to have been diagnosed with cancer (except for possible nonmelanoma skin cancer or *in situ* cancer of the cervix), was selected for each case, matching for age, race, menopausal status at blood donation, cohort participation, and date of blood donation. To preserve scarce serum from cases for future studies, no case could be used as a control for a different case in this study. Premenopausal cases and controls were also matched by time of menstrual cycle using information on days since last menstrual period, for those who donated in 1974, and by dates of last and next menstrual period, for those who donated in 1989. Women whose last menstrual period was 365 days or more prior to blood donation were initially classified as postmenopausal at time of blood donation.

Cases and controls for this study were a subset of cases and controls from another study on environmental risk factors and breast cancer. From the 346 matched case-control sets in the parent study, there were 203 case-control sets with sufficient sera available for the B-vitamin and homocysteine assays (Fig. 1). All but one case-control set from the original 1989 cohort was included in this study. When we compared baseline characteristics of the 1974 cohort in the parent study to our study population, case-control sets who had donated in 1974 and were included in this study were slightly younger than the original 1974 case-control sets (49.9 years *versus* 51.7 years, $P = 0.02$) but were similar with regard to education, marital status, smoking status, time since last meal, and month of blood donation (data not shown).

In 1995, a questionnaire was mailed to both breast cancer cases and controls or their next of kin to obtain additional information on risk factors for breast cancer. Questions in-

Table 1 Baseline characteristics for cases and controls in total study population[a]

	Cases (n = 195)	Controls (n = 195)	P (χ^2 test)
Year(s) of blood donation			Matching variable
1974 only	85 (43.6%)	85 (43.6%)	
1989 only	62 (31.8%)	62 (31.8%)	
1974 and 1989	48 (24.6%)	48 (24.6%)	
Age (yr)			Matching variable
18–44	41 (21.0%)	42 (21.5%)	
45–54	67 (34.4%)	73 (37.4%)	
55–64	55 (28.2%)	49 (25.1%)	
67–74	27 (13.9%)	25 (12.8%)	
75–90	5 (2.6%)	6 (3.1%)	
Menopausal status at donation			Matching variable
Premenopausal	86 (44.1%)	86 (44.1%)	
Postmenopausal	109 (55.9%)	109 (55.9%)	
Education (yr)			
>12	60 (30.8%)	39 (20.0%)	
≤12	135 (69.2%)	156 (80.0%)	0.02
Smoking status			
Never	122 (62.6%)	119 (61.0%)	
Past	36 (18.5%)	31 (15.9%)	
Current	37 (19.0%)	45 (23.1%)	0.55
Time since last meal[b] (h)			
0–1	47 (24.2%)	53 (27.2%)	
2–3	85 (43.8%)	73 (37.4%)	
4–5	36 (18.6%)	38 (19.5%)	
6–7	5 (2.6%)	11 (5.6%)	
8+	21 (10.8%)	20 (10.3%)	0.46
Time from donation to diagnosis (yr)			
≤2	28 (14.4%)		
3–5	39 (20.0%)		
7–10	33 (16.9%)		
11–15	25 (12.8%)		
≥16	70 (35.9%)		

[a] Individuals who donated in both 1974 and 1989 were included once using 1974 baseline data.
[b] Information missing for one case.

cluded a detailed reproductive history, family history of breast cancer, hormone use, physical activity, and dietary vitamin supplementation, including supplementation with folate, B12, B6, or B-vitamin complexes. More cases than controls responded to the questionnaire (88.7 *versus* 75.9%, $P = 0.001$). Of the 334 returned questionnaires, 31 were answered by surrogates of cases, and 14 were answered by surrogates of controls. Respondents and nonrespondents were similar with respect to education and marital status. Respondents were somewhat more likely to be nonsmokers and to be older than nonrespondents (data not shown).

Information from the 1995 questionnaire was used to refine menopausal status at blood donation and diagnosis. Some women with missing information on reproductive history on the 1995 questionnaire (*i.e.*, missing or nonrespondents) who, according to their last date of menstrual period, were considered postmenopausal at time of donation might have actually been premenopausal because of a possible hysterectomy without a bilateral ovariectomy. However, we expect this number to be very small and hardly enough to change our conclusions. Eight sets were found not to be adequately matched with regard to menopausal status at donation and were excluded because of reported differences in homocysteine concentrations by menopausal status (23). Menopausal status at diagnosis was determined based on age at last menstrual period or, if that information was unavailable, age at diagnosis (age of <50 years was considered premenopausal). A total of 85 case-control sets who donated only in 1974, 62 sets who donated only in 1989, and 48 sets who donated in both programs were included in the final analysis. For the rest of this report, the "1974 cohort" will include all 133 case-control sets who donated in 1974 and were properly matched on menopausal status, including the 48 sets who donated in both programs. References to the "1989 cohort" will include all of the 110 case-control sets who donated in 1989, including all 48 sets who donated in both programs (Fig. 1).

There is evidence suggesting that risk factors for the development of pre- and postmenopausal breast cancer might differ (24). Susceptibility to carcinogens may also vary with hormonal status (25). When menopausal status at the time of donation and diagnosis was considered, three different menopausal subgroups could be defined: (*a*) "pre-pre" group, premenopausal at donation and at diagnosis of the case; (*b*) "pre-post" group, premenopausal at donation and postmenopausal at diagnosis of case; and (*c*) "post-post" group, postmenopausal at donation and postmenopausal at diagnosis. Of the 133 cases in the 1974 cohort, 13 cases were assigned to the pre-pre group, 57 cases were assigned to the pre-post group, and 63 cases were assigned to the post-post group. Among the 110 cases who participated in 1989, 22 cases were assigned to the pre-pre group, 3 cases were assigned to the pre-post group, and 85 cases were assigned to the post-post group. Controls were assigned the same menopausal subgroups as their matched cases. Analyses were conducted stratified by year of blood donation and menopausal status at donation and diagnosis. Because of small numbers in the 1974 pre-pre group and the 1989 pre-post group, analyses of these subgroups were not performed.

This study was approved by the Committee on Human Research of the Johns Hopkins University School of Hygiene and Public Health, and all participants signed an informed consent form at the time of blood donation.

Laboratory Assays. The word "serum" refers to serum from samples donated in 1974 and plasma from samples donated in 1989, unless otherwise specified. Serum samples were grouped in case-control sets. Thawing was performed in ice water and under dim yellow light. After aliquotting, specimens were immediately refrozen at −70°C. The radiometric tyrosine decarboxylase method was used to determine PLP (26). A method reported by Araki and Sako (27) was applied to measure total serum homocysteine. RIA (Bio-Rad Quantaphase II kit) was used to measure serum folate and B12. Laboratory personnel were unaware of case-control status, and each case-control set was analyzed in the same batch using the same reagents. A total of 26 pooled quality control sets, each containing two samples, were distributed randomly among the specimens. For the 1974 samples, calculated mean intrapair coefficients of variation were as follows: folate, 4.0%; B12, 10.1%; PLP, 4.3%; and homocysteine, 5.5%. For the 1989 samples, the coefficients of variation were as follows: folate, 4.9%; B12, 3.2%; PLP, 6.2%; and homocysteine, 11.1%.

Statistical Analysis. Concentrations of the measured analytes were skewed to the right. The data were log-transformed and log mean concentrations between cases and controls were compared using paired t tests. ORs and 95% CIs were calculated using conditional logistic regression analysis to assess the association between known risk factors for breast cancer and breast cancer risk and to determine dose-response relationships between serum concentrations of the measured analytes and breast cancer risk. When sample size permitted, the serum concentrations of analytes were divided into fifths, based on the distribution among controls. For smaller numbers, serum con-

Table 2 Matched ORs and 95% CIs for selected risk factors for breast cancer for the 1974 and 1989 cohorts[a]

	1974 cohort			1989 cohort		
	Cases (n = 133)	Controls (n = 133)	Matched OR (95% CI)	Cases (n = 110)	Controls (n = 110)	Matched OR (95% CI)
Family history of breast cancer						
None	86	78	1.00	71	82	1.00
Mother or sister	20	9	2.12 (0.87–5.19)	22	10	2.74 (1.14–6.56)
Not stated[b]	27	46	0.55 (0.32–0.98)	17	18	1.05 (0.53–2.12)
Age at menarche (yr)						
≤11	16	18	1.00	19	15	1.00
12–13	59	44	1.46 (0.64–3.34)	60	52	0.84 (0.37–1.87)
≥14	26	25	1.19 (0.48–2.93)	17	27	0.49 (0.19–1.27)
Not stated	32	46	0.70 (0.28–1.76)	14	16	0.68 (0.24–1.89)
Age at first full-term birth (yr)						
<20	21	23	1.00	26	24	1.00
20–29	65	45	1.60 (0.74–3.46)	53	52	0.89 (0.45–1.77)
≥30	10	7	1.41 (0.42–4.76)	1	5	0.18 (0.02–1.74)
No conception or full-term birth	14	14	0.96 (0.33–2.83)	15	13	0.96 (0.36–2.58)
Not stated	23	44	0.48 (0.19–1.20)	15	16	0.87 (0.36–2.13)
Oral contraceptive use						
Never	93	70	1.00	73	72	1.00
Ever	19	22	0.64 (0.28–1.50)	25	24	1.03 (0.50–2.13)
Not stated	21	41	0.36 (0.18–0.70)	12	14	0.85 (0.37–1.96)
Hormone replacement therapy[c]						
None	82	68	1.00	75	67	1.00
Estrogen and progesterone	6	3	1.80 (0.43–7.46)	8	7	0.92 (0.32–2.66)
Estrogen alone	17	17	0.90 (0.39–2.10)	11	19	0.48 (0.20–1.16)
Not stated	28	45	0.48 (0.26–0.89)	16	17	0.85 (0.39–1.85)
Education (yr)[d]						
>12	41	18	1.00	37	27	1.00
≤12	92	115	0.34 (0.18–0.66)	73	83	0.67 (0.38–1.17)
Number of drinks per week						
<1/week	85	66	1.00	68	70	1.00
1–3/week	11	13	0.77 (0.32–1.85)	16	15	1.21 (0.56–2.61)
≥4/week	18	10	1.50 (0.62–3.65)	15	9	1.83 (0.74–4.54)
Not stated	19	44	0.31 (0.16–0.63)	11	16	0.69 (0.31–1.55)
Body mass index (kg/m^2)[e]						
≤22.00	23	15	1.00	21	17	1.00
22.01–26.00	36	30	0.86 (0.38–1.93)	30	31	0.81 (0.38–1.71)
≥26	51	47	0.75 (0.32–1.77)	46	48	0.77 (0.35–1.70)
Not stated	23	41	0.36 (0.15–0.84)	13	14	0.77 (0.29–2.03)
Multivitamin use						
Never	48	43	1.00	40	33	1.00
Ever	59	46	1.25 (0.67–2.36)	57	61	0.77 (0.42–1.43)
Not stated	26	44	0.47 (0.23–0.95)	13	16	0.70 (0.30–1.62)
B-vitamin use						
Never	86	72	1.00	78	68	1.00
Ever	18	14	1.06 (0.47–2.38)	18	25	0.57 (0.27–1.21)
Not stated	29	47	0.46 (0.24–0.86)	14	17	0.68 (0.30–1.50)

[a] A total of 48 sets who donated in both programs (1974 and 1989) were included in both parts (1974 and 1989) of the table.
[b] The category "not stated" included missing information as well as all nonrespondents to the 1995 questionnaire.
[c] Only included females in whom hormone replacement was started at least 1 yr prior to diagnosis of the case; females who reported hormone replacement <1 year prior to or after diagnosis of the case were included in the "none" category.
[d] Information on education obtained from baseline questionnaire (1974 or 1989); for all other variables, information was obtained from 1995 questionnaire.
[e] Information on body mass index was calculated from self-reported current weight and height [weight/(height)^2].

centrations were divided into thirds. The highest analyte concentration was defined as the reference category. The conditional logistic regression model was used to calculate trend tests with median values of each fifth or third control value as exposure scores.

We considered the following risk factors for breast cancer as potential confounders: family history of breast cancer, bilateral ovariectomy, age at menarche, age at menopause, age at first birth, number of pregnancies, months of breast feeding, oral contraceptive use, hormone replacement therapy, education and marital status at time of blood donation, body mass index, and regular physical exercise. Only those variables that were associated with both breast cancer risk, using an arbitrary significance level of $P < 0.15$, and analyte concentration, using an arbitrary significance level of $P < 0.10$, were considered for inclusion into the conditional logistic regression model. A less restrictive significance level to assess the associations between potentially confounding factors and breast cancer was chosen to enhance the probability that risk factors for breast cancer that might potentially confound our results were identified. Because matched ORs and ORs adjusted for potential confounders did not differ substantially, only matched

Table 3 Median analyte concentrations for cases and controls by year of blood donation and menopausal status

	1974 cohort				1989 cohort			
	Cases	Controls	% differences[a]	P^b (paired *t* test)	Cases	Controls	% differences[a]	P^b (paired *t* test)
Total population								
n	133	133			110	110		
Folate[c] (ng/ml)	3.5	3.6	−3.1	0.55	8.6	8.0	+6.9	0.51
B12 (pg/ml)	407.3	459.3	−11.3	0.03	421.1	459.9	−8.4	0.02
PLP (pmol/ml)	27.4	26.5	+3.4	0.63	45.7	41.0	+11.5	0.77
Homocysteine (nmol/ml)	9.6	10.3	−7.5	0.52	8.6	8.5	+1.1	0.97
Premenopausal at donation-postmenopausal at diagnosis group								
n	57	57			22	22		
Folate[c] (ng/ml)	3.3	3.5	−5.5	0.61	7.4	8.0	−8.5	0.99
B12 (pg/ml)	407.3	429.1	−5.1	0.53	461.4	501.4	−8.0	0.70
PLP (pmol/ml)	25.8	24.4	+5.8	0.14	43.2	61.9	−30.2	0.12
Homocysteine (nmol/ml)	9.3	10.2	−8.6	0.35	6.2	7.3	−15.6	0.10
Postmenopausal at donation-postmenopausal at diagnosis group								
n	63	63			85	85		
Folate (ng/ml)	4.2	3.8	+8.9	0.57	8.6	8.0	+7.6	0.63
B12 (pg/ml)	412.6	481.9	−14.4	0.03	405.7	452.1	−10.3	0.02
PLP (pmol/ml)	28.8	26.8	+7.6	0.90	48.2	40.8	+18.1	0.71
Homocysteine (nmol/ml)	10.2	11.6	−12.4	0.68	9.1	9.2	−0.5	0.52

[a] Percentage difference was calculated as: (median case − median control/median control × 100.
[b] Paired *t* test calculated to assess differences in log mean concentrations of each case-control set.
[c] One missing value for folate in total 1989 cohort and pre-pre group, one control; paired *t* tests performed excluding the respective case-control set.

ORs on the association between analytes and breast cancer risk are presented. Effect modification was assessed by including product terms in the conditional regression model. A two-tailed P of <0.05 was used to define a significant association.

Results

Table 1 compares baseline characteristics for the study participants. The 48 sets who donated in both programs were included only once, using their baseline information in 1974. All but one case-control set was white. Cases and controls were closely matched on age, menopausal status, and year of blood donation. In the 1974 cohort, all case-control sets were matched within 1 month of blood donation; in the 1989 cohort, all but one case-control set, which had donated 104 days apart, were matched within 3 weeks of blood donation. For the 1989 cohort, information was also collected on time of blood donation. Of all sets in this cohort, 69.1% were matched within 2 h of blood donation. Cases and controls were similar with regard to smoking status at time of donation as well as hours since last meal. Cases were better educated than controls. The majority of cases (85.6%) were diagnosed >2 years after blood donation.

Table 2 shows matched ORs and their 95% CIs (95% CI) for selected risk factors for breast cancer stratified by year of blood donation. The information for the 48 case-control sets who donated in both programs was included in each program. Family history was associated with an ~2-fold increase in the risk of breast cancer and ≤12 years of education was associated with a decreased risk of breast cancer. Higher levels of alcohol intake were associated with increased risk, but this was not statistically significant. Age at menarche and age at first birth were not significantly associated with breast cancer risk, but the 1974 and the 1989 cohort differed with regard to direction of association, suggesting that there was no demonstrable association. All other risk factors investigated, including B-vitamin supplementation (folate, B12, B6, and B-vitamin complex), were not significantly associated with breast cancer risk.

Median concentrations of the measured analytes according to year of blood donation and menopausal status are shown in Table 3. Median concentrations among controls differed by year of blood donation. Median concentrations of folate were 124% and PLP concentrations were 55% higher in the 1989 cohort compared to the 1974 cohort. On the other hand, median homocysteine concentrations were 18% lower in the 1989 cohort. Because of these considerable differences in analyte concentrations between the two cohorts, analysis was stratified by year of blood donation. In both cohorts, folate, PLP, and homocysteine concentrations were not significantly different between cases and controls. B12 concentrations were significantly lower in cases compared to controls in both cohorts. The association between B12 concentrations and breast cancer persisted in all menopausal subgroups and reached statistical significance for the post-post group (postmenopausal at donation and postmenopausal at diagnosis) in both cohorts.

Tables 4 and 5 show matched ORs and their respective 95% CIs for the association between fifths (or thirds) analyte concentrations of controls and breast cancer by year of blood donation and menopausal subgroups. The calculated ORs were not consistent with a monotonic trend for any measured analyte. In the total 1974 population, women in the lowest fifth of B12 concentration had a significantly increased risk of breast cancer compared to those in the highest fifth B12 concentration (OR = 2.54, 95% CI, 1.11–5.80; median B12 concentration in the lowest fifth group = 280 pg/ml). The observed increased risk in the lowest fifth B12 concentration was primarily due to increased risk of breast cancer among women in the lowest fifth of B12 who were postmenopausal at donation. Results were similar for the 1989 cohort but did not reach statistical significance (OR = 2.10, 95% CI, 0.87–5.06; median B12 concentration in the lowest fifth group = 312 pg/ml).

In the total 1974 population, an increased risk of breast cancer was observed for the lowest two-fifths of homocysteine concentrations. After stratification by menopausal subgroups, this association persisted only for women who were premenopausal at donation and postmenopausal at the time of diagnosis.

Table 4 Matched ORs and 95% CIs by fifths of analyte level of controls and menopausal status: 1974 cohort

Fifths	Matched ORs (95% CIs)		
	Total population (133 cases, 133 controls)	Pre-post group[a] (57 cases, 57 controls)	Post-post group[a] (63 cases, 63 controls)
Folate			
5 (highest)	1.00	1.00	1.00
4	1.13 (0.51–2.48)	0.40 (0.11–1.51)	2.62 (0.74–9.28)
3	0.59 (0.23–1.50)	0.67 (0.18–2.45)	0.87 (0.23–3.38)
2	1.22 (0.56–2.69)	0.63 (0.20–1.96)	2.36 (0.70–7.95)
1 (lowest)	1.08 (0.50–2.37)	1.57 (0.49–4.96)	0.66 (0.17–2.60)
	$P_{trend} = 0.73$	$P_{trend} = 0.71$	$P_{trend} = 0.79$
B12			
5 (highest)	1.00	1.00	1.00
4	1.14 (0.49–2.66)	1.08 (0.30–3.89)	1.55 (0.45–5.34)
3	0.94 (0.39–2.23)	0.43 (0.11–1.77)	1.14 (0.29–4.43)
2	0.96 (0.43–2.13)	1.28 (0.40–4.04)	1.18 (0.34–4.13)
1 (lowest)	2.54 (1.11–5.80)	1.10 (0.34–3.51)	4.00 (1.05–15.20)
	$P_{trend} = 0.05$	$P_{trend} = 0.73$	$P_{trend} = 0.08$
PLP			
5 (highest)	1.00	1.00	1.00
4	0.71 (0.35–1.43)	0.65 (0.23–1.86)	0.58 (0.19–1.74)
3	0.64 (0.29–1.40)	0.65 (0.22–1.94)	0.70 (0.21–2.29)
2	0.53 (0.24–1.14)	0.34 (0.09–1.33)	0.40 (0.12–1.37)
1 (lowest)	0.92 (0.41–2.04)	0.64 (0.17–2.38)	0.86 (0.26–2.79)
	$P_{trend} = 0.26$	$P_{trend} = 0.25$	$P_{trend} = 0.34$
Homocysteine			
5 (highest)	1.00	1.00	1.00
4	0.56 (0.25–1.25)	0.65 (0.16–2.63)	0.33 (0.09–1.19)
3	0.94 (0.42–2.09)	0.51 (0.11–2.36)	0.90 (0.29–2.82)
2	1.82 (0.84–3.95)	2.39 (0.65–8.80)	1.07 (0.36–3.23)
1 (lowest)	2.08 (0.91–4.78)	2.55 (0.72–9.06)	1.35 (0.42–4.31)
	$P_{trend} = 0.02$	$P_{trend} = 0.05$	$P_{trend} = 0.25$

[a] Pre-post group; premenopausal at donation-postmenopausal at diagnosis group; post-post group, postmenopausal at donation-postmenopausal at diagnosis group.

However, these observed associations do not suggest either a linear association or a threshold effect.

To assess a possible effect of including persons with latent preclinical cancer at blood donation, we analyzed the data using cases diagnosed >2 years after blood donation. Because there were only two cases in the 1974 cohort, who were diagnosed within 2 years of donation and who also had serum available for the B-vitamin assays, we examined only the 1989 cohort after excluding the 54 sets in which the cases were diagnosed within 2 years after blood donation. Exclusion of those early diagnosed cases did not change our results for any of the measured analytes. The matched OR for the lowest fifth B12 compared to the highest fifth among those diagnosed >2 years after blood donation was 2.80 (95% CI, 0.79–9.84).

Smoking, vitamin supplementation, or number of drinks might affect dietary intake or metabolism of vitamins and, thus, might be either part of the hypothesized causal pathway as well as potential confounders. In any case, adjustment for smoking, vitamin supplementation, or number of drinks per week did not alter the overall results.

Where possible, effect modification between fifth concentrations of the measured analytes and alcohol consumption and smoking status at time of donation was investigated. There was no indication for any conclusive effect modification between fifths concentrations of the measured analytes and smoking status at time of donation and alcohol consumption for either total cohorts.

Discussion

In this prospective study, no evidence for a protective association between higher concentrations of folate and PLP or lower concentrations of homocysteine and subsequent breast cancer risk was found. To the best of our knowledge, this is the first prospective study on these associations.

Two case-control studies in western New York investigated the association between dietary folate intake and breast cancer risk among postmenopausal and premenopausal women. When the highest fourth of intake was compared to the lowest, a 30% reduction in postmenopausal breast cancer risk was found (OR = 0.70, 95% CI, 0.48–1.02). The association between folate intake and breast cancer risk was attenuated after adjustment for either carotene, vitamin C, or α-tocopherol intake (28). In the study among premenopausal women, a higher intake of vegetables appeared to be protective against breast cancer, but results did not suggest an independent protective effect of folic acid on breast cancer risk (29).

In our study, an increased risk of breast cancer was observed among women in the lowest fifth of the distribution of vitamin B12 as compared to women in the other four higher fifths, suggesting a threshold effect for B12. In all menopausal subgroups, cases tended to have lower concentrations of B12 as compared to controls, although the observed threshold effect was limited to women postmenopausal at donation. The threshold effect was observed among concentrations not considered to be deficient. Only 8% of cases and 3% of controls in the 1974 cohort and 5% of cases and no control in the 1989 cohort had serum B12 concentrations below 200 pg/ml (normal range, 200–900 pg/ml; Ref. 30).

The mechanisms underlying our observed association on B12 and breast cancer might be explained by the role of B12 as a cosubstrate in the synthesis of methionine, for which a methyl group is transferred from methyltetrahydrofolate to homocys-

Table 5 Matched ORs and 95% CIs by fifths or thirds of analyte level of controls and menopausal status: 1989 cohort

Fifths or thirds (pre-pre group)	Matched ORs (95% CIs)		
	Total population (110 cases, 110 controls)	Pre-pre group[a] (22 cases, 22 controls)	Post-post group[a] (85 cases, 85 controls)
Folate[b]			
5 (highest)	1.00	NA[c]	1.00
4	0.89 (0.37–2.13)	NA	0.80 (0.32–2.02)
3	0.68 (0.31–1.50)	1.00	0.69 (0.28–1.67)
2	0.81 (0.35–1.86)	1.43 (0.31–6.57)	0.80 (0.34–1.89)
1 (lowest)	0.79 (0.33–1.90)	0.89 (0.10–7.70)	0.67 (0.26–1.72)
	$P_{trend} = 0.41$	$P_{trend} = 0.86$	$P_{trend} = 0.36$
B12			
5 (highest)	1.00	NA	1.00
4	1.53 (0.64–3.65)	NA	1.13 (0.41–3.11)
3	1.05 (0.42–2.60)	1.00	0.66 (0.21–2.01)
2	1.57 (0.61–4.07)	3.19 (0.63–16.14)	1.00 (0.34–2.91)
1 (lowest)	2.10 (0.87–5.06)	2.03 (0.25–16.13)	2.25 (0.86–5.91)
	$P_{trend} = 0.15$	$P_{trend} = 0.30$	$P_{trend} = 0.20$
PLP			
5 (highest)	1.00	NA	1.00
4	0.78 (0.33–1.84)	NA	1.08 (0.44–2.66)
3	1.22 (0.55–2.70)	1.00	1.17 (0.48–2.82)
2	0.84 (0.35–2.01)	2.17 (0.40–11.68)	0.50 (0.17–1.46)
1 (lowest)	0.64 (0.25–1.68)	3.73 (0.52–26.91)	0.63 (0.23–1.76)
	$P_{trend} = 0.80$	$P_{trend} = 0.20$	$P_{trend} = 0.44$
Homocysteine			
5 (highest)	1.00	NA	1.00
4	0.87 (0.37–2.05)	NA	0.72 (0.27–1.91)
3	0.93 (0.39–2.24)	1.00	0.96 (0.37–2.50)
2	0.75 (0.32–1.76)	1.00 (0.19–5.24)	0.82 (0.32–2.12)
1 (lowest)	1.33 (0.54–3.25)	4.00 (0.69–23.18)	0.69 (0.26–1.80)
	$P_{trend} = 0.84$	$P_{trend} = 0.17$	$P_{trend} = 0.59$

[a] Pre-pre group, premenopausal at donation-premenopausal at diagnosis group; post-post group, postmenopausal at donation-postmenopausal at diagnosis group.
[b] One missing value for folate in total 1989 cohort and pre-pre group, one control.
[c] NA, not applicable because third levels were used for the pre-pre group.

teine (Fig. 2). Two crucial implications derive from this reaction: (*a*) it is the only means to provide *de novo* methyl groups for many methylation processes that are mediated by S-adenosylmethionine; and (*b*) it is also the only reaction to regenerate unsubstituted tetrahydrofolate from 5-methyltetrahydrofolate (31). Thus, lower concentrations of B12 might result in reduced synthesis of *de novo* methyl groups, leading to DNA hypomethylation, which might play a role in carcinogenesis (7, 9, 10, 13, 32–39). Through diminished availability of unsubstituted tetrahydrofolate, which is involved in reactions generating thymidylate and purines, lower B12 concentrations might also lead to reduced DNA synthesis and, thus, impaired DNA repair mechanisms (7).

A major advantage of our study, besides being community based, is its long follow-up period, especially for the 1974 cohort, with over 49% of cases being diagnosed >10 years after blood donation. However, there are some limitations inherent in our study design. (*a*) Only nonfasting blood samples were collected. However, time since last meal was not associated with breast cancer risk, so any misclassification should have been nondifferential, thus biasing our results toward the null (40). Furthermore, adjustment for time since last meal did not alter the observed associations. (*b*) Samples donated from controls in 1989 exhibited considerably higher folate and PLP concentrations and lower homocysteine concentrations than samples donated in 1974. Vitamin B12 concentrations on the other hand were only slightly higher among the 1989 controls compared to the 1974 controls. The same trend was observed among 50 individuals without cancer, who donated in both programs, *i.e.*, in 1974 and 1989. Dietary changes as well as possible storage effect over time might account for the differences in analyte concentrations between 1974 and 1989. However, because case and control samples have been collected during the same time period and were stored, handled, and assayed in the same manner, storage effect should not have affected relative case-control differences. In addition, all analyses were conducted stratified by year of blood donation. Comparison of log mean differences between cases and controls of the two cohorts provided further evidence that observed relative case-control differences are valid. Log mean differences between cases and controls in the total 1974 cohort ($n = 133$) did not differ substantially from log mean differences between cases and controls in the 1989 only cohort ($n = 62$; folate, $P = 0.52$; B12, $P = 0.47$; PLP, $P = 0.15$; homocysteine, $P = 0.67$).

In conclusion, our findings suggest a threshold effect for B12 with an increased risk of breast cancer among postmenopausal women in the lowest fifth of the serum B12 concentration compared to the higher four-fifths. However, the possibility cannot be excluded that an unidentified protective factor for breast cancer associated with higher B12 concentrations might have led to the observed protective association between vitamin B12 and breast cancer. We did not find any evidence for a protective association between higher concentrations of folate and PLP or lower concentrations of homocysteine and subsequent breast cancer risk. Because, to our knowledge, this is the first prospective study to examine the association between these B-vitamins and subsequent breast cancer risk, its findings need

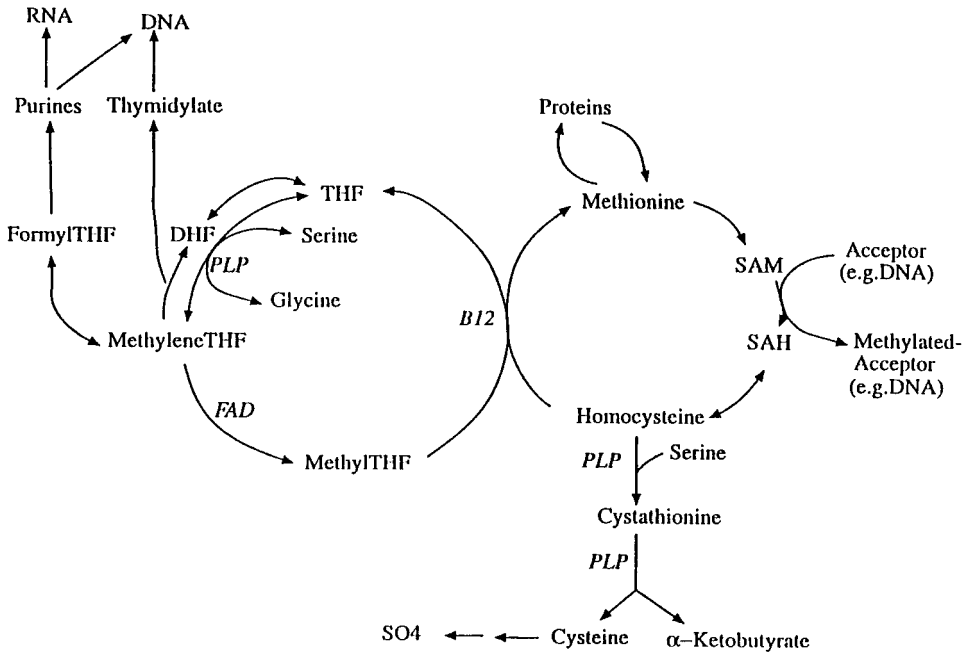

Fig. 2. Folate-dependent metabolism. *DHF*, dihydrofolate; *THF*, tetrahydrofolate; *FAD*, flavin adenine dinucleotide; *SAM*, *S*-adenosylmethionine; *SAH*, *S*-adenosylhomocysteine.

to be replicated before they can be considered as more than tentative. In view of the fact that most accepted risk factors for breast cancer are very difficult or even impossible to alter, there is a continued need to investigate potentially modifiable risk factors such as intake of these B-vitamins and their serum concentrations.

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