Prospective Study of Carotenoids, Tocopherols, and Retinoid Concentrations and the Risk of Breast Cancer[1]

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Abstract

Previous prospective studies have raised the possibility that the antioxidant properties of carotenoids and vitamin E (α-tocopherol) and the role of vitamin A (retinol) in cellular differentiation may be associated with a reduced risk of subsequent breast cancer. To investigate the association between serum and plasma concentrations of retinol, retinyl palmitate, α-carotene, β-carotene, β-cryptoxanthin, lutein, lycopene, total-carotenoids, α-tocopherol, and γ-tocopherol with subsequent development of breast cancer, a nested case control study was conducted among female residents of Washington County, Maryland, who had donated blood for a serum bank in 1974 or 1989. Cases ($n = 295$) and controls ($n = 295$) were matched on age, race, menopausal status, and date of blood donation, and the analyses were stratified by cohort participation. Median concentrations of β-carotene, lycopene, and total carotene were significantly lower in cases compared with controls in the 1974 cohort (13.1, 12.5, and 7.9% difference; $P = 0.01$, 0.04, and 0.04, respectively) and for lutein in the 1989 cohort (6.7% difference; $P = 0.02$). The risk of developing breast cancer in the highest fifth was approximately half of that of women in the lowest fifth for β-carotene [odds ratio (OR) = 0.41; 95% confidence interval (CI) 0.22–0.79; P trend = 0.007], lycopene (OR = 0.55; 95% CI 0.29–1.06; P trend = 0.04), and total carotene (OR = 0.55; 95% CI 0.29–1.03; P trend = 0.02) in the 1974 cohort. There was generally a protective association for other micronutrients in both cohorts, although none reached statistical significance. The results suggest that carotenoids may protect against the development of breast cancer.

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Introduction

Antioxidant micronutrients, such as carotenoids and vitamin E, have been the focus of numerous studies because they may offer cellular protection against a variety of free radicals that can damage DNA. Nine prospective studies have examined the association between serum micronutrient concentrations and risk of breast cancer; however, the results have been inconsistent (Table 1). β-carotene was the most commonly studied carotenoid with three (1–3) of the six (1–6) studies reporting a nonsignificant inverse association with higher concentrations. β-carotene can also indirectly reduce the risk of breast cancer through conversion to retinol (preformed vitamin A) because retinol and its related compounds are involved in the regulation of cell growth and differentiation. Only one (7) of seven prospective serum studies (1–4, 6–8) found an inverse association with retinol. More recently, two studies (3, 6) evaluated additional carotenoids, such as β-cryptoxanthin, lutein, and lycopene. There was a significant dose response of reduced risk of breast cancer with higher lutein and β-cryptoxanthin concentrations (3) and a threshold effect for lycopene (6).

With regard to vitamin E, the evidence is also not convincing. Results from six prospective studies (1, 4–6, 8, 9) show that only one found a significant inverse association (1). However, a follow-up study by authors of the one positive study retracted previous significant findings on the basis that serum of cases were subjected to greater degradation than controls (10).

Despite the moderate number of epidemiological studies investigating the relation between serum micronutrients and risk of breast cancer, small sample size ($\leq$105 cases) and suboptimal storage conditions (-20°C) hampered many previous studies. Given the inconsistent findings and continued interest in identifying modifiable risk factors for breast cancer, we conducted a population-based nested case control study among women who had donated blood up to 20 years before breast cancer development. This larger and more comprehensive prospective study provides an additional layer of data by examining serum micronutrients not considered earlier, retinyl palmitate and γ-tocopherol, in addition to micronutrients examined previously.

Materials and Methods

Study Population. A nested case control study was conducted among female residents of Washington County, Maryland, who participated in a blood collection campaign in 1974, 1989, or both years. The blood collection campaigns were designed to collect blood samples from as many adult residents as possible for a serum bank. In 1974 and 1989, 23,850 and 25,080 Washington County adult residents, respectively, donated blood. Details regarding the Washington County serum bank have been published elsewhere (11, 12). At the time of blood donation, participants were asked to complete a brief questionnaire. This included questions on demographics, smoking status, date

Table 1 Published prospective (nested case control) studies on the association between serum micronutrients and breast cancer

Study	Published year (place)	No. cases/controls	Follow-up years	Micronutrients	(lowest) Comparison RR/OR (highest)					*P* trend
					1	2	3	4	5	
Wald (1)	1984 (Guernsey, U.K.)	39/78	≤14 yr	Retinol	1.00	1.00	0.81	2.29	0.94	N/S[a]
				β-carotene	1.00	0.63	0.65	0.7	0.27	N/S
				Vitamin E	1.00	0.37	0.18	0.18	0.15	<0.01
Willett (4)	1984 (U.S.)	14/31	5 yr	Retinol	Case control difference = 5.4 ± 6.6 μg/dl					N/S
				Carotenoids	Case control difference = 8.9 ± 17.2 μg/dl					N/S
				Vitamin E	Case control difference = −0.16 ± 0.17 mg/dl					N/S
Coates (7)	1988 (Washington)	21/39	≤12 yr	Retinol	1.0	0.9	0.3			0.29
Knekt (9)[b]	1988 (Finland)	67/123	mean 8 yr	α-tocopherol	1.00	1.19	0.62	1.42	0.61	0.92
Russell (8)	1988 (Guernsey, U.K.)	30/288	8 yr	Retinol	Case control difference = −0.7%					N/S
				α-tocopherol	Case control difference = 4.8%					N/S
Knekt (2)	1990 (Finland)	52/93[c]	mean 8 yr	Retinol	Lowest *vs.* higher fifths 1.2					
				β-carotene	Lowest *vs.* higher fifths 0.3					
Comstock (5)	1991 (Maryland)	30/59	14 yr	β-carotene	1.0	0.7	1.1			0.43
				Lycopene	1.0	0.9	0.7			0.29
				Vitamin E	1.0	2.3	0.7	1.7		0.28
Dorgan (6)[d]	1998 (Missouri)	105/203	9.5 yr	Retinol	1.0	1.6	1.6	0.9		0.99
				α-carotene[d]	1.0	1.5	1.8	1.8		0.11
				β-carotene[d]	1.0	0.6	1.1	1.1		0.97
				β-cryptoxanthin	1.0	0.7	0.7	0.6		0.41
				Lutein	1.0	0.8	0.9	0.9		0.11
				Lycopene	1.0	1.2	1.1	0.5		0.02
				α-tocopherol	1.0	1.3	0.9	1.2		0.72
Toniolo (3)[e]	2001 (New York)	270/270	≤9 yr	Retinol	0.78	0.98	1.01	1.00		0.50
				α-carotene	1.99	1.41	1.28	1.00		<0.01
				β-carotene	2.21	1.49	1.29	1.00		0.01
				Total carotenoids	2.31	1.57	1.92	1.00		<0.01
				β-cryptoxanthin	1.68	1.39	1.08	1.00		0.05
				Lutein	2.08	1.22	1.43	1.00		0.01
				Lycopene	1.50	1.62	1.75	1.00		0.15

[a] N/S, not significant.
[b] Adjusted for smoking.
[c] Excludes first 2 years of follow-up.
[d] Adjusted for total serum cholesterol, smoking, and body mass index.
[e] Adjusted for age at first birth, family history of breast cancer, history of benign breast disease, and total cholesterol.

of last menstrual cycle, and hours since last meal. Information on vitamin supplement use was also collected. Blood specimens were stored at −70°C until the time of assay.

Follow-up for incident breast cancer has been established by record linkage with the Washington County cancer registry. The registry identifies all cancer cases in the county from discharge records of the county's only hospital, Washington County hospital, and from death certificates. Breast cancer cases were defined as women for whom breast cancer was the first cancer diagnosis (ICD-9, 174) after blood donation with the exception of nonmelanoma skin cancer and *in situ* cancer of the cervix. Cancer diagnosis had to be after 1989 for women who donated in both years. The present study was restricted to incident breast cancer diagnosed between January 1, 1975 and July 1, 1994. During this period, 346 women developed breast cancer of whom 295 donated in 1974, 115 donated in 1989, and 64 donated in both years. Women who donated blood each time were included in both the 1974 and 1989 cohort-specific analyses. A comparison of breast cancer cases reported to Washington County Cancer Registry with the Maryland Cancer Registry (13) showed that case ascertainment was virtually the same (12, 14).

Controls were selected among female residents of Washington County who had also donated blood to either blood collection campaigns and were not known to be deceased and not diagnosed with invasive cancer at the time the cases were diagnosed. One control was matched to a case based on age (within 1 year), race, menopausal status, and month and year of blood donation. Premenopausal women were also matched on date of last menstrual cycle. All cases and controls were Caucasian, reflecting the racial composition of Washington County. Among the 295 matched sets from the 1974 cohort, 51 sets were excluded because of insufficient sera for the micronutrient assays. Thus, the present study included 244 matched sets for the 1974 cohort and 115 matched sets for the 1989 cohort.

In 1995, a questionnaire, specifically designed to study environmental risk factors for breast cancer, was mailed to cases and controls or their next of kin. This questionnaire was used to assess the association with known risk factors, including reproductive history, behavioral risk factors, hormone use, and family history of breast cancer. These potential confounders were not obtained at baseline. Responses were higher for cases (88.7%) than for controls (75.9%). Among the returned questionnaires, cases had a higher percentage of surrogate responders (10.6%) than controls (5.3%).

Laboratory Assays. After blood was drawn, serum (1974 cohort) or plasma (1989 cohort) aliquots were prepared within 24 h for storage at −70°C until the time of assay. For the purpose of this paper, the term "serum" will be used to refer to both serum and plasma. Samples were thawed in ice water under dim yellow light and sent under dry ice to an independent laboratory. Concentrations of serum micronutrients were determined by reverse phase high performance liquid chromatogra-

Table 2 Median concentrations of micronutrients by case control status and cohort participation, Washington County, MD, 1975–1994

Micronutrients	1974 cohort			1989 cohort		
	Median (range)		% Difference[a]	Median (range)		% Difference[a]
	Cases (n = 244)	Controls (n = 244)		Cases (n = 115)	Controls (n = 115)	
Retinol (μg/dl)	57.4 (30.4, 142.9)	57.9 (28.5, 182.8)	−1.0	61.6 (15.9, 109.7)	61.6 (25.2, 121.9)	0.0
Retinyl palmitate (μg/dl)	5.2 (1.0, 86.5)	5.6 (1.0, 98.5)	−7.1	5.0 (2.1, 32.3)	4.7 (1.8, 24.8)	6.4
α-carotene (μg/dl)	1.6 (0.1, 12.2)	1.7 (0.2, 45.6)	−5.9	2.5 (0.4, 15.5)	2.5 (0.5, 15.8)	0.0
β-carotene (μg/dl)	11.3 (0.7, 82.9)	13.0 (1.6, 75.7)	−13.1[b]	11.2 (1.6, 52.6)	12.3 (1.8, 92.6)	−8.9
β-cryptoxanthin (μg/dl)	8.8 (1.4, 48.4)	8.4 (1.8, 76.5)	4.8	10.5 (1.5, 68.6)	11.2 (2.9, 37.3)	−6.3
Lutein (μg/dl)	19.3 (4.6, 74.8)	21.6 (5.0, 79.6)	−10.4	19.7 (7.0, 64.8)	21.1 (9.4, 56.6)	−6.7[b]
Lycopene (μg/dl)	28.6 (3.1, 93.7)	32.7 (5.5, 88.0)	−12.5[b]	32.6 (6.2, 87.9)	35.5 (4.4, 69.4)	−8.2
Total carotenoids (μg/dl)	72.2 (13.5, 213.4)	78.4 (22.2, 295.7)	−7.9[b]	83.1 (27.5, 256.5)	91.6 (37.3, 208.1)	−9.3
α-tocopherol (mg/dl)	1.10 (0.48, 3.24)	1.12 (0.38, 3.74)	−1.8	1.20 (0.60, 3.24)	1.19 (0.56, 3.08)	1.0
γ-tocopherol (mg/dl)	0.22 (0.04, 0.53)	0.23 (0.03, 0.77)	−4.3	0.22 (0.02, 0.53)	0.23 (0.03, 0.66)	−4.3

[a] Percentage difference = (median case − median control)/median control × 100.
[b] $0.05 > p > 0.01$ by Wilcoxon signed-rank test.

phy using methods outlined by Sowell *et al.* (15). Serum aliquots were grouped by the matched sets. The laboratory personnel were unaware of the case control status and instructed to assay each matched set on the same day using the same batch of reagents. Quality control samples (62), prepared from pooled sera, were interspersed randomly throughout the batch. On the basis of these quality control samples, the intrapair coefficients of variation were <5% for retinol, γ-tocopherol, α-tocopherol, and lutein; 5–10% for lycopene, β-carotene, and total carotene; and 11.9 and 10.4%, respectively, for retinyl palmitate and α-carotene.

Statistical Analyses. The Wilcoxon signed rank test was used to compare micronutrient concentrations between cases and controls. Conditional logistic regression was used to analyze the association between micronutrient concentrations and risk of breast cancer. Nutrients were categorized into fifths according to the distribution in the controls. Trend tests were performed by assigning the median value of each category among the controls to both cases and control in that specific category and using it as the independent variable in the conditional logistic regression modeling. The analyses were performed separately for the 1974 and 1989 cohort for two reasons: (*a*) the 1974 samples were serum, and the 1989 samples were plasma; and (*b*) there was marked difference between the two cohorts in the period of time the specimens had been stored.

Additionally, stratified analyses by menopausal status at the time of diagnosis and tumor estrogen receptor status were performed to evaluate whether risk of breast cancer differed by these factors. Menopausal status was derived from information obtained in the 1995 breast cancer questionnaire by date of last menstrual period and history of hysterectomy and oophorectomy. We also examined the group who participated in both 1974 and 1989 separately to evaluate whether changes in micronutrient concentration over 15 years altered risk of breast cancer. Because the number of women who donated in both years was small (64 matched sets), participants were divided into low or high micronutrient concentration based on the median of the control distribution. Family history of breast cancer, age at first birth, age at menarche, intake of alcohol, smoking status, body mass index, duration of lactation, education, time since last meal, and cholesterol were considered as potential confounders. Exposure to alcohol intake and hormone replacement therapy use were truncated at the date of diagnosis of the case for both cases and controls. Because adjustment for the above factors did not change inferences, the results presented here were adjusted for the matching factors only. STATA version 6.0 was used for the analyses (16).

Results

Selected baseline characteristics for this study population have been published previously (12). Cases and controls were closely matched on age. The mean ages at donation for cases and controls were 51.3 and 51.1 years old, respectively, for the 1974 cohort. For the 1989 cohort, the mean ages at donation for cases and controls were 60.4 and 60.2 years old, respectively. Age at first birth and smoking status at donation were also similar between cases and controls in both cohorts. Cases had earlier age of menarche, were less likely to have lactated, had fewer years of education (1989 cohort only), and were more likely to be regular drinkers compared with controls. None of these differences reached statistical significance. Family history of breast cancer was the only characteristic to show a statistically significant difference between cases and controls, with cases having a higher proportion of women with a family history ($P < 0.01$). The median time to diagnosis because blood draw was 14 years (range 1–20 years) for the 1974 cohort and 3 years (range 1–5 years) for the 1989 cohort. The majority of the women was postmenopausal at diagnosis with the mean ages at diagnosis being 64 and 63 for the 1974 and 1989 cohorts, respectively.

The median micronutrient concentrations are shown in Table 2. In general, the controls had slightly higher median concentrations than cases in both cohorts. Controls had significantly higher median concentrations of β-carotene, lycopene, and total carotenoids (percentage of differences in median values were 13.1, 12.5, and 7.9, respectively) compared with cases in the 1974 cohort. Lutein was the only micronutrient with significantly higher concentrations in controls than in cases (percentage of difference in median of 6.7) in the 1989 cohort. There were essentially no differences in retinol and α-tocopherol concentrations in both cohorts. For retinyl palmitate and α-carotene, controls had higher median concentrations than cases in 1974 cohort, but the opposite (retinyl palmitate) or no difference (α-carotene) was found in the 1989 cohort. Cases had higher median concentrations of β-cryptoxanthin in the 1974 cohort but an opposite direction in the 1989 cohort. The percentage of difference in median values was similar for β-carotene, lutein, lycopene, total-carotenoids, and γ-tocopherol in both cohorts.

The association of micronutrients by categorical fifths and subsequent risk of breast cancer is displayed in Table 3 for the 1974 cohort and Table 4 for the 1989 cohort. There were some differences in the association between the two cohorts. In the 1974 cohort, a statistically significant dose response trend was observed for β-carotene ($P = 0.007$), lycopene ($P = 0.04$), and total carotenoids ($P = 0.02$). Women in the highest fifth for β-carotene, lycopene, or total carotenoids had approximately half of the risk of developing breast cancer compared with women in the lowest fifth. The other micronutrients did not show a significant dose response association.

For the 1989 cohort (Table 4), the highest fifth of lutein was associated with a significantly reduced risk of breast cancer (OR[3] = 0.40, 95% CI 0.17–0.98). Although there was no significant linear dose response trend ($P = 0.11$), the other fifths of lutein had similar reduction in risk compared with the lowest fifth, suggesting a threshold effect. Consistent with the 1974 cohort, higher total carotenoids and lycopene concentrations were associated with a reduced risk, although the CIs were wide, and the trend test was not significant. There was no trend in the association for β-carotene in the 1989 cohort. Conversely, in the 1974 cohort, there was no significant dose response association for lutein.

Because of the 20 year follow-up in the 1974 cohort, the analysis was stratified by the year of diagnosis to examine whether the association differed by the length of time from blood donation to the time of diagnosis. The results for such analyses are presented in Table 5 for the three micronutrients, β-carotene, lycopene, and total carotenoids, which showed significant protective associations. Because few women were diagnosed within 5 years after blood donation (56 cases), a 10-year interval was chosen for the first strata, and 5-year intervals thereafter were chosen to allow adequate numbers. There was a significant reduction in risk of developing breast cancer with higher β-carotene concentrations up to 15 years after blood donation. However, the reduced risk was not observed for women diagnosed 16–20 years after blood donation. Higher lycopene concentrations showed inverse associations among women who were diagnosed throughout the follow-up, although none of the strata reached significance. In contrast, a significant inverse association with total carotenoids was observed only among women who were diagnosed 10–15 years after blood donation. No demonstrable associations were found for the first 10 years, and only weak dose response association was found for women diagnosed 16–20 years after donation. The analyses restricted to breast cancer cases diagnosed >2 years after blood donation in the 1974 cohort were consistent with the overall findings. Because all cases were diagnosed within 5 years in the 1989 cohort, an evaluation of this issue was not possible.

Results from the analyses stratified by menopausal status did not appreciably differ from the unstratified analyses (data not shown). Tumor estrogen receptor status was available for 85% of the 1989 cohort and 47% of the 1974 cohort. The associations were in the same protective direction for β-carotene, lutein, lycopene, and total carotene regardless of tumor estrogen receptor status (data not shown). Additionally, we excluded 78 women (34%) from the 1989 cohort and 89 women (18%) from the 1974 cohort who indicated regular use of supplements from a food frequency questionnaire given in 1989 or used multivitamin or single entity supplement 48 h before

Table 3 The association of micronutrients and subsequent risk of breast cancer (1975–1994) in the 1974 cohort, Washington County, MD

	No. cases/controls	OR	95% CI
Retinol (μg/dl)			
<46.2	51/48	1.00	
46.2–53.4	43/49	0.82	0.46–1.44
53.5–62.6	61/49	1.13	0.65–1.95
62.7–72.9	39/49	0.70	0.37–1.32
≥73.0	50/49	0.97	0.53–1.80
		($P_{trend} = 0.83$)	
Retinyl palmitate (μg/dl)			
<3.4	53/46	1.00	
3.4–4.7	49/45	1.09	0.59–2.02
4.8–6.9	35/48	0.69	0.38–1.26
7.0–12.2	46/46	0.84	0.45–1.55
≥12.3	37/47	0.78	0.40–1.51
		($P_{trend} = 0.39$)	
α-carotene (μg/dl)			
<0.9	46/44	1.00	
0.9–1.4	57/49	1.12	0.62–2.00
1.5–2.2	53/47	1.13	0.62–2.05
2.3–3.4	38/49	0.72	0.39–1.34
≥3.5	37/46	0.69	0.36–1.34
		($P_{trend} = 0.09$)	
β-carotene (μg/dl)			
<7.3	67/50	1.00	
7.3–11.2	54/48	0.79	0.46–1.38
11.3–15.5	44/48	0.67	0.38–1.19
15.6–22.1	47/49	0.63	0.34–1.14
≥22.2	31/49	0.41	0.22–0.79
		($P_{trend} = 0.007$)	
β-cryptoxanthin (μg/dl)			
<4.5	56/49	1.00	
4.5–6.8	34/49	0.62	0.35–1.10
6.9–10.5	53/48	0.98	0.56–1.70
10.6–15.7	48/49	0.90	0.52–1.55
≥15.8	53/49	0.98	0.55–1.75
		($P_{trend} = 0.67$)	
Lutein (μg/dl)			
<13.7	61/49	1.00	
13.7–18.6	52/48	0.81	0.44–1.50
18.7–24.3	46/49	0.71	0.40–1.26
24.4–32.1	34/49	0.51	0.27–0.96
≥32.2	51/49	0.77	0.43–1.40
		($P_{trend} = 0.41$)	
Lycopene (μg/dl)			
<20.1	67/49	1.00	
20.1–28.7	56/49	0.78	0.45–1.34
28.8–37.6	41/49	0.53	0.29–0.97
37.7–49.2	39/51	0.48	0.26–0.88
≥49.3	41/46	0.55	0.29–1.06
		($P_{trend} = 0.04$)	
Total carotenoids (μg/dl)			
<51.4	57/49	1.00	
51.5–71.7	62/49	1.04	0.62–1.76
71.8–91.6	53/48	0.85	0.47–1.53
91.7–121.5	36/49	0.85	0.30–1.06
≥121.6	36/49	0.55	0.29–1.03
		($P_{trend} = 0.02$)	
α-tocopherol (mg/dl)			
<0.91	52/48	1.00	
0.91–1.05	56/50	1.02	0.59–1.74
1.06–1.19	50/50	0.87	0.48–1.58
1.20–1.39	34/48	0.62	0.33–1.17
≥1.40	52/48	0.94	0.52–1.73
		($P_{trend} = 0.69$)	
γ-tocopherol (mg/dl)			
<0.15	55/42	1.00	
0.15–0.20	58/55	0.78	0.45–1.36
0.21–0.26	61/52	0.91	0.53–1.57
0.27–0.31	25/46	0.43	0.23–0.80
≥0.32	45/49	0.70	0.40–1.23
		($P_{trend} = 0.08$)	

[3] The abbreviations used are: OR, odds ratio; CI, confidence interval.

Table 4 The association of micronutrients and subsequent risk of breast cancer (1990–1994) in the 1989 cohort, Washington County, MD

	No. cases/controls	OR	95% CI
Retinol (μg/dl)			
<50.4	23/23	1.00	
50.4–56.3	20/23	0.78	0.30–2.07
56.4–66.7	32/23	1.29	0.58–2.86
66.8–73.7	16/23	0.66	0.26–1.68
≥73.8	24/23	1.03	0.40–2.64
		(P_{trend} = 0.95)	
Retinyl palmitate (μg/dl)			
<3.4	24/22	1.00	
3.4–4.3	20/25	0.69	0.29–1.65
4.4–5.7	17/22	0.70	0.30–1.66
5.8–11.3	29/22	1.03	0.45–2.38
≥11.4	18/22	0.74	0.32–1.72
		(P_{trend} = 0.84)	
α-carotene (μg/dl)			
<1.5	27/21	1.00	
1.5–1.9	13/25	0.40	0.16–1.01
2.0–3.2	36/22	1.34	0.57–3.16
3.3–4.8	18/23	0.65	0.27–1.53
≥4.9	21/22	0.84	0.34–2.08
		(P_{trend} = 0.59)	
β-carotene (μg/dl)			
<7.2	28/24	1.00	
7.2–10.4	22/21	0.91	0.42–1.99
10.5–15.0	22/24	0.83	0.37–1.85
15.1–22.5	27/23	1.01	0.44–2.32
≥22.6	16/23	0.62	0.27–1.42
		(P_{trend} = 0.26)	
β-cryptoxanthin (μg/dl)			
<6.6	32/23	1.00	
6.6–8.9	13/23	0.39	0.16–0.96
9.0–12.6	28/23	0.80	0.38–1.70
12.7–17.2	18/23	0.53	0.23–1.24
≥17.3	24/23	0.70	0.29–1.73
		(P_{trend} = 0.68)	
Lutein (μg/dl)			
<16.5	37/23	1.00	
16.5–19.6	20/23	0.51	0.22–1.15
19.7–24.2	20/23	0.45	0.18–1.10
24.3–30.8	21/23	0.53	0.23–1.24
≥30.9	17/23	0.40	0.17–0.98
		(P_{trend} = 0.11)	
Lycopene (μg/dl)			
<23.6	35/24	1.00	
23.6–31.1	19/23	0.51	0.22–1.19
31.2–38.6	20/22	0.59	0.26–1.32
38.7–49.0	12/23	0.32	0.12–0.82
≥49.1	29/23	0.80	0.34–1.85
		(P_{trend} = 0.57)	
Total carotenoids (μg/dl)			
<67.1	32/23	1.00	
67.1–82.4	24/23	0.66	0.29–1.52
82.5–97.8	22/23	0.63	0.26–1.53
97.9–123.7	15/23	0.37	0.14–0.99
≥123.8	22/23	0.61	0.26–1.43
		(P_{trend} = 0.25)	
α-tocopherol (mg/dl)			
<0.99	29/24	1.00	
0.99–1.11	14/22	0.53	0.22–1.27
1.12–1.29	25/23	0.96	0.45–2.07
1.30–1.64	29/23	1.09	0.47–2.57
≥1.65	18/23	0.67	0.28–1.62
		(P_{trend} = 0.54)	
γ-tocopherol (mg/dl)			
<0.13	22/22	1.00	
0.13–0.18	23/25	0.90	0.40–2.04
0.19–0.26	32/23	1.30	0.61–2.79
0.27–0.33	18/22	0.80	0.33–1.93
≥0.34	20/23	0.80	0.33–1.93
		(P_{trend} = 0.68)	

blood donation. Results from the remaining 51 matched sets from the 1989 cohort and 162 matched sets from the 1974 cohort indicated results that were not substantially different from those including the supplement users.

Additional analyses examining those who participated in both 1974 and 1989 indicated that participants who consistently had higher β-carotene, lutein, lycopene, and total carotenoids in 1974 and again in 1989 had a reduced risk of breast cancer than participants who had lower concentrations at two time points (OR = 0.84, 0.39, 0.66, and 0.49, respectively). With the exception of lutein, the 95% confidence limits were wide and not statistically significant. Participants who had a higher lutein or total carotenoid concentrations in 1974 but had lower concentrations in 1989 had an attenuated protective association (OR = 0.76 and 0.75, respectively). For the same high-to-low change between 1974 and 1989 for β-carotene and lycopene, there was a slight increase in risk of breast cancer (OR = 1.83 and 1.16, respectively). Again, because of the small sample size, none of the CIs reached significance.

Discussion

This nested case control study is the largest and longest (≤20 years follow-up) prospective study evaluating prediagnostic serum concentrations of antioxidant micronutrients in relation to subsequent risk of breast cancer. We have also evaluated a broader spectrum of micronutrients than has been reported previously. Although previous prospective studies have examined β-carotene, lycopene, vitamin E, and retinol, only two (3, 6) examined individual carotenoids, such as lutein and β-cryptoxanthin. The present study expands previous prospective studies by evaluating two additional micronutrients, retinyl palmitate and γ-tocopherol, in relation to breast cancer risk. This study was also able to examine whether the association differed by menopausal status and estrogen receptor status in a relatively unselected group of women.

The central finding from this study is that total carotenoids were associated with reduced risk of breast cancer, although a significant dose response trend was only achieved for the 1974 cohort. In addition, the individual carotenoids, lycopene, and β-carotene were also significantly associated with reduced risk of breast cancer in the 1974 cohort. In the 1989 cohort, lutein showed a reduced risk of breast cancer for women in the higher four-fifths, but there was no significant trend.

This study found slightly different results between the 1974 and 1989 cohort. In contrast to the 1974 cohort, the 1989 cohort did not show any significant dose response trend for lycopene and total carotenoids despite similar protective directions. For β-carotene in the 1989 cohort, evidence of a protective association was weak. Given these mixed findings, the analysis on the issue of the time between blood donation and diagnosis deserves closer attention. The median time to diagnosis after blood donation was 14 years for the 1974 cohort and 3 years for the 1989 cohort. In the stratified analysis (Table 5), a strong dose response association consistently appeared to be in women who donated 10–15 years before breast cancer development. Although β-carotene and lycopene showed a modest trend among women diagnosed between 1975 and 1984, most of the diagnosis occurred in the latter part of the period, well beyond the follow-up period for the 1989 cohort. This varying timeframe between donation and diagnosis may partially explain the difference in findings between the 1974 and 1989 cohort.

In addition to having two cohorts drawn from the same population with a varying length of follow-up, this study has a

Table 5 The association of selected micronutrients and subsequent risk of breast cancer (1975–1994) in the 1974 cohort stratified by year of diagnosis

Year of diagnosis[a] (*n* for case/control)	Third of micronutrient concentrations			*P* trend
	Lowest	Middle	Highest	
	OR	OR (95% CI)	OR (95% CI)	
β-carotene (μg/dl)				
1975–84 (78/78)	1.00	0.39 (0.18–0.85)	0.37 (0.15–0.93)	0.05
1985–89 (69/69)	1.00	0.51 (0.22–1.18)	0.40 (0.16–0.98)	0.06
1990–94 (96/96)	1.00	1.23 (0.59–2.53)	1.29 (0.55–3.04)	0.56
Lycopene (μg/dl)				
1975–84 (78/78)	1.00	0.56 (0.23–1.35)	0.49 (0.20–1.20)	0.11
1985–89 (69/69)	1.00	0.52 (0.24–1.13)	0.47 (0.19–1.16)	0.11
1990–94 (97/97)	1.00	0.72 (0.34–1.48)	0.67 (0.29–1.55)	0.38
Total carotenoids (μg/dl)				
1975–84 (78/78)	1.00	0.88 (0.41–1.90)	1.06 (0.43–2.62)	0.84
1985–89 (69/69)	1.00	0.42 (0.13–1.35)	0.27 (0.09–0.78)	0.02
1990–94 (97/97)	1.00	0.80 (0.39–1.64)	0.68 (0.30–1.55)	0.37

[a] Controls were assigned the year of diagnosis of the matched case.

unique advantage of being able to examine a limited number of women (64 matched sets) who were included in both cohorts. Women with high concentrations of lutein, lycopene, and total carotenoids in both 1974 and 1989 had protective associations. This is consistent with findings from the individual cohort, although dose response trends were not observed for the 1989 cohort. These results also suggest that if there were two measurements on each woman, measurement error could be reduced, and the association may have been stronger. Because of the few women who participated in 1974 and 1989, the interpretation should be viewed with caution.

We considered the possibility of a pooled analysis and performed the analysis by combining the two cohorts based on the rankings of the individuals in each study. Despite the increased sample size, the magnitude and the strength of the association were largely similar to the 1974 cohort (data not shown). This was expected because the 1974 cohort was more than twice the sample size of the 1989 cohort. Because the results from the 1989 cohort were slightly different and buried under the large numbers from the 1974 cohort, we choose to present the stratified rather than the pooled results. The specimens from the two cohorts were also different in terms of storage length and type of blood samples (serum *versus* plasma).

Among the six (1–6) prospective studies that examined β-carotene concentrations, two (1, 2) found a nonsignificant inverse association. The largest study found a statistically significant 2-fold risk of breast cancer among women in the lowest fourth compared with the highest (3). Although the study in Guernsey, United Kingdom found that only women in the highest fifth showed reduced risk of breast cancer, cases had lower mean β-carotene concentrations (36 μg/liter) compared with controls (50 μg/liter; Ref. 1). A study in Finland found a marginal inverse association (OR = 0.3, 95% CI 0.1–1) after excluding breast cancer cases diagnosed within the first 2 years after blood donation (2). An earlier and much smaller pilot study in Washington County did not demonstrate a reduced risk with increasing serum β-carotene; nonetheless, controls did have a 10% higher β-carotene concentration than cases, which is very similar to the present study (5). Prospective studies on intake of β-carotene and risk of breast cancer have also had mixed findings. Four (17–20) of the seven studies showed modest inverse associations (17–23). Despite the large sample size in all four studies, only the most recent study from the Nurses Health Study had a significant inverse association with β-carotene among women with a family history and high alcohol consumption or who were premenopausal (17).

Three studies examined serum lycopene prospectively (3, 5, 6). A study by Dorgan *et al.* (6) found a threshold effect where women in the highest fourth had half the risk compared with women in the lowest fourth (95% CI = 0.2–1.2). The most recent study by Toniolo *et al.* (3) found no association. Our previous Washington County study showed a case control difference of a similar magnitude (16%) to the present study with controls showing higher concentrations (5). The cases in the previous study were not included in our present study. Given that the current study found similar protective associations, although only significant for the 1974 cohort, future studies should investigate the possibility that lycopene may protect against breast cancer.

No notable associations were observed with regard to retinol and its metabolite, retinyl palmitate. Only one small study, based on 21 breast cancer cases, found decreasing risk with increasing retinol concentrations (7). The remaining five prospective studies have not shown any demonstrable association (1, 2, 4, 6, 8). Prospective dietary studies have generally shown modest inverse association between retinol intake and risk of breast cancer. This discrepancy in association between blood retinol and dietary intake of retinol in relation to breast cancer could be explained by the fact that serum retinol is homeostatically regulated. Thus, unlike retinol intake, which can vary over time, retinol concentrations are more likely to reflect long-term circulating retinol. The Nurses Health Study reported a significant protective dose response association with increasing retinol intake (17, 18), and two other prospective studies have found nonsignificant but small decreases in risk with higher retinol intake (19, 22). There was no association in one study (21), and another study found higher risk with higher intake (23). This study agrees with the null results found in five previous serum prospective studies.

The lack of any consistent association with α-tocopherol and γ-tocopherol is in line with previous research (Table 1). The results from this study largely corroborate findings from our previous study of 30 postmenopausal women (5). The differences in concentrations of α-tocopherol and γ-tocopherol between cases and controls were very small. Results from six large prospective studies on dietary intake of vitamin E mirror similar null findings (17–19, 21–23). Use of vitamin E supplements also did not appear to alter overall risk of breast cancer (17–19, 21). The results from this study, combined with pre-

vious prospective studies in both serum and diet, suggest that vitamin E is unlikely to reduce the risk of breast cancer.

There are several limitations to this study. Possible degradation of nutrient concentrations, even with storage at −70°C over 2 decades, has been a major concern. We recently reexamined long-term storage effects (20 years) and found no significant degradation of nutrient concentrations (24). There is also the question of the validity and reliability of responses obtained from surrogate sources. Although there are no studies that examined the validity or reliability of surrogate's responses to questions pertaining to reproductive history, an analysis excluding questionnaires from surrogates showed results that were not substantially different from those presented here. Adjusting for respondent sources also did not change results. We also need to consider the possibility that the protective association observed for carotenoids may be because of some other nutrient present in carotenoid-rich foods. Fruits and vegetables, which are the main sources for carotenoids, also contain many other beneficial nutrients. A single nutrient may not be responsible for the protective association, but the combination of many nutrients could produce the beneficial effect. And lastly, we cannot exclude the possibility that other health behaviors related to high fruit and vegetable consumption may be associated with reduced risk of breast cancer rather than the diet itself, *e.g.*, individuals with a good diet are more likely to exercise and less likely to smoke and drink excessively.

In summary, higher carotenoid concentrations were generally associated with lower risk of breast cancer in both cohorts. There was a significant inverse association with increasing lycopene, β-carotene, and total carotenoids concentrations in the 1974 cohort. Retinol, retinyl palmitate, α-tocopherol, and γ-tocopherol did not show any consistent associations. These results suggest that carotenoids may potentially reduce the risk of breast cancer. Moreover, the results are consistent with the notion that a diet rich in fruits and vegetables may be beneficial against breast cancer.

References

1. Wald, N. J., Boreham, J., Hayward, J. L., and Bulbrook, R. D. Plasma retinol, β-carotene and vitamin E levels in relation to the future risk of breast cancer. Br. J. Cancer, *49:* 321–324, 1984.

2. Knekt, P., Aromaa, A., Maatela, J., Aaran, R. K., Nikkari, T., Hakama, M., Hakulinen, T., Peto, R., and Teppo, L. Serum vitamin A and subsequent risk of cancer: cancer incidence follow-up of the Finnish Mobile Clinic Health Examination Survey. Am. J. Epidemiol., *132:* 857–870, 1990.

3. Toniolo, P., Van Kappel, A. L., Akhmedkhanov, A., Ferrari, P., Kato, I., Shore, R. E., and Riboli, E. Serum carotenoids and breast cancer. Am. J. Epidemiol., *153:* 1142–1147, 2001.

4. Willett, W. C., Polk, B. F., Underwood, B. A., Stampfer, M. J., Pressel, S., Rosner, B., Taylor, J. O., Schneider, K., and Hames, C. G. Relation of serum vitamins A and E and carotenoids to the risk of cancer. N. Engl. J. Med., *310:* 430–434, 1984.

5. Comstock, G. W., Helzlsouer, K. J., and Bush, T. L. Prediagnostic serum levels of carotenoids and vitamin E as related to subsequent cancer in Washington County, Maryland. Am. J. Clin. Nutr., *53:* 260S–264S, 1991.

6. Dorgan, J. F., Sowell, A., Swanson, C. A., Potischman, N., Miller, R., Schussler, N., and Stephenson, H. E., Jr. Relationships of serum carotenoids, retinol, α-tocopherol, and selenium with breast cancer risk: results from a prospective study in Columbia. Missouri (United States). Cancer Causes Control, *9:* 89–97, 1998.

7. Coates, R. J., Weiss, N. S., Daling, J. R., Morris, J. S., and Labbe, R. F. Serum levels of selenium and retinol and the subsequent risk of cancer. Am. J. Epidemiol., *128:* 515–523, 1988.

8. Russell, M. J., Thomas, B. S., and Bulbrook, R. D. A prospective study of the relationship between serum vitamins A and E and risk of breast cancer. Br. J. Cancer, *57:* 213–215, 1988.

9. Knekt, P. Serum vitamin E level and risk of female cancers. Int. J. Epidemiol., *17:* 281–286, 1988.

10. Wald, N. J., Nicolaides-Bouman, A., and Hudson, G. A. Letter: Plasma retinol, β-carotene and vitamin E levels in relation to the future risk of breast cancer. Br. J. Cancer, *57:* 235, 1988.

11. Comstock, G. W., Alberg, A. J., Huang, H. Y., Wu, K., Burke, A. E., Hoffman, S. C., Norkus, E. P., Gross, M., Cutler, R. G., Morris, J. S., Spate, V. L., and Helzlsouer, K. J. The risk of developing lung cancer associated with antioxidants in the blood: ascorbic acid, carotenoids, α-tocopherol, selenium, and total peroxyl radical absorbing capacity. Cancer Epidemiol. Biomarkers Prev., *6:* 907–916, 1997.

12. Helzlsouer, K. J., Alberg, A. J., Huang, H. Y., Hoffman, S. C., Strickland, P. T., Brock, J. W., Burse, V. W., Needham, L. L., Bell, D. A., Lavigne, J. A., Yager, J. D., and Comstock, G. W. Serum concentrations of organochlorine compounds and the subsequent development of breast cancer. Cancer Epidemiol. Biomarkers Prev., *8:* 525–532, 1999.

13. Maryland. Final Maryland Cancer Registry (MCR) Data Report, Northwest Region. Maryland Cancer Registry. Bel Air, MD: Tri-Analytics, Inc., 1993.

14. Wu, K., Helzlsouer, K. J., Comstock, G. W., Hoffman, S. C., Nadeau, M. R., and Selhub, J. A prospective study on folate. B12, and pyridoxal 5′-phosphate (B6) and breast cancer. Cancer Epidemiol. Biomarkers Prev., *8:* 209–217, 1999.

15. Sowell, A. L., Huff, D. L., Yeager, P. R., Caudill, S. P., and Gunter, E. W. Retinol, α-tocopherol, lutein/zeaxanthin, β-cryptoxanthin, lycopene, α-carotene, *trans*-β-carotene, and four retinyl esters in serum determined simultaneously by reversed-phase HPLC with multiwavelength detection. Clin. Chem., *40:* 411–416, 1994.

16. Stata Statistical Software Version Release 6.0. College Station, TX: Stata Corporation.

17. Zhang, S., Hunter, D. J., Forman, M. R., Rosner, B. A., Speizer, F. E., Colditz, G. A., Manson, J. E., Hankinson, S. E., and Willett, W. C. Dietary carotenoids and vitamins A. C, and E and risk of breast cancer. J. Natl. Cancer Inst. (Bethesda), *91:* 547–556, 1999.

18. Hunter, D. J., Manson, J. E., Colditz, G. A., Stampfer, M. J., Rosner, B., Hennekens, C. H., Speizer, F. E., and Willett, W. C. A prospective study of the intake of vitamins C. E, and A and the risk of breast cancer. N. Engl. J. Med., *329:* 234–240, 1993.

19. Rohan, T. E., Howe, G. R., Friedenreich, C. M., Jain, M., and Miller, A. B. Dietary fiber, vitamins A. C, and E, and risk of breast cancer: a cohort study. Cancer Causes Control, *4:* 29–37, 1993.

20. Shibata, A., Paganini-Hill, A., Ross, R. K., and Henderson, B. E. Intake of vegetables, fruits, β-carotene, vitamin C and vitamin supplements and cancer incidence among the elderly: a prospective study. Br. J. Cancer, *66:* 673–679, 1992.

21. Kushi, L. H., Fee, R. M., Sellers, T. A., Zheng, W., and Folsom, A. R. Intake of vitamins A. C, and E and postmenopausal breast cancer. The Iowa Women's Health Study. Am. J. Epidemiol., *144:* 165–174, 1996.

22. Graham, S., Zielezny, M., Marshall, J., Priore, R., Freudenheim, J., Brasure, J., Haughey, B., Nasca, P., and Zdeb, M. Diet in the epidemiology of postmenopausal breast cancer in the New York State Cohort. Am. J. Epidemiol., *136:* 1327–1337, 1992.

23. Verhoeven, D. T., Assen, N., Goldbohm, R. A., Dorant, E., van 't Veer, P., Sturmans, F., Hermus, R. J., and van den Brandt, P. A. Vitamins C and E, retinol, β-carotene and dietary fibre in relation to breast cancer risk: a prospective cohort study. Br. J. Cancer, *75:* 149–155, 1997.

24. Comstock, G. W., Burke, A. E., Hoffman, S. C., Norkus, E. P., Gross, M., and Helzlsouer, K. J. The repeatability of serum carotenoid, retinoid, and tocopherol concentrations in specimens of blood collected 15 years apart. Cancer Epidemiol. Biomarkers Prev., *10:* 65–68, 2001.