Longitudinal Biological Exposure to Carotenoids Is Associated with Breast Cancer–Free Survival in the Women's Healthy Eating and Living Study

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Abstract

In some cohort studies, a high-vegetable diet has been associated with greater likelihood of recurrence-free survival in women diagnosed with breast cancer. Carotenoids are obtained primarily from vegetables and fruit and they exhibit biological activities that may specifically reduce the progression of mammary carcinogenesis. The present analysis examines the relationship between plasma carotenoids at enrollment and 1, 2 or 3, 4, and 6 years and breast cancer–free survival in the Women's Healthy Eating and Living Study participants (N = 3,043), who had been diagnosed with early-stage breast cancer. The primary end point was time to a second breast cancer event (a recurrence or new primary breast cancer). An average carotenoid concentration over time was estimated for each participant as the average area under the plasma carotenoid curve formed by the plasma carotenoid concentrations at scheduled clinic visits. Multiple regression Cox proportional hazards analysis with adjustment for prognostic and other factors was used to examine the association between carotenoids and breast cancer–free survival. A total of 508 (16.7%) breast cancer events occurred over a median 7.12 years follow-up. Compared with the lowest tertile, the hazard ratio for the medium/high plasma carotenoid tertiles was 0.67 (95% confidence interval, 0.54-0.83) after adjustment. The interaction between the study group and tertile of average carotenoid concentration over time was not significant (P = 0.23). Higher biological exposure to carotenoids, when assessed over the time frame of the study, was associated with greater likelihood of breast cancer–free survival regardless of study group assignment. (Cancer Epidemiol Biomarkers Prev 2009;18(2):486–94)

Introduction

Carotenoids are bioactive food components provided primarily by vegetables and fruit in the diet. These hydrocarbons function as precursors of vitamin A compounds, including retinoic acid, and other biologically active metabolites that are the products of the eccentric cleavage pathway of carotenoids and oxidation (1-3). Plasma carotenoids are a dietary biomarker for vegetable and fruit intake but these compounds also exhibit several biological activities that may specifically affect the progression of mammary carcinogenesis. Carotenoids exhibit antioxidant activity *in vitro*. However, laboratory evidence strongly suggests that the retinoid-like activities of the carotenoid metabolites are the more important mechanisms by which they may inhibit the progression of carcinogenesis in the human biological system (4, 5). Similar to the effects of retinoids, carotenoids influence cell growth regulation, including the inhibition of growth and malignant transformation and the promotion of apoptosis in transformed cells (6-10). Several carotenoids (β-carotene, canthaxanthin, and lycopene) and eccentric cleavage products of β-carotene have been shown to inhibit both estrogen receptor (ER)–positive and ER-negative breast tumor cell growth *in vitro* (8, 9). Carotenoids also inhibit estrogen signaling of 17β-estradiol and thus inhibit estrogen-induced cell proliferation (10). Estrogenic stimulation is believed to play a causal role in the pathogenesis of breast cancer, and higher serum estrogen concentration has been observed to contribute to risk for recurrence in women who have been diagnosed and treated for early-stage breast cancer (11).

Breast cancer remains the most commonly diagnosed invasive cancer in women, accounting for 26% of new cancer cases and 15% of cancer deaths in women in the United States (12). Current 5-year survival rates for women diagnosed with early-stage breast cancer are

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high but women who have been diagnosed and completed initial treatments remain at increased risk for a new primary cancer, recurrence, or other comorbidities. In some previous cohort studies, a high vegetable diet was associated with increased likelihood of recurrence-free survival in women diagnosed with early-stage breast cancer (13-17).

The Women's Healthy Eating and Living (WHEL) Study, a randomized controlled trial, tested the effect of a diet very high in vegetables, fruit, and fiber, and low in fat, on risk for recurrence and likelihood of survival in women diagnosed and treated for early-stage breast cancer. In an earlier observational analysis of WHEL Study data, total plasma carotenoid concentration at enrollment was observed to be directly related to recurrence-free survival in women assigned to the comparison group, whose diets did not change markedly after enrollment or as a result of participation, controlled for prognostic and other possible influencing factors (18). Women assigned to the WHEL Study intervention group substantially increased vegetable and fruit intakes (19, 20), which was reflected in increased average total plasma carotenoid concentration. However, this was not associated with reduced risk for additional breast cancer events or mortality during a median 7.3-year follow-up period (21).

The purpose of the present analysis was to examine the relationship between total plasma carotenoid concentrations measured at multiple time points during the overall course of the trial and breast cancer–free survival in the total cohort of WHEL Study participants. The effect of study group assignment was considered and included in the analysis.

Materials and Methods

Study Participants. The WHEL Study enrolled 3,088 breast cancer survivors. Of that number, baseline plasma carotenoid data were not available for 45 participants; thus, this analysis was conducted on 3,043 participants (98.5% of the total WHEL Study population). At 1 and 4 y, respectively, plasma carotenoid data were available on 83% (2,472 of 2,967 participants) and 78% (2,108 of 2,696 participants) of the WHEL Study. The WHEL Study was a multisite randomized trial that tested the effect of an intensive dietary intervention on new breast cancer events and survival in a cohort of women previously diagnosed with early-stage breast cancer who were recruited between 1995 and 2000. Details of the study design, protocol, and overall effect of the dietary intervention on outcome during the median 7.3-y follow-up period have been previously reported (21, 22). Eligibility criteria included evidence from the medical record of a diagnosis within the past 4 y of primary operable invasive breast carcinoma categorized using the American Joint Committee on Cancer criteria as stage I tumor ($\geq$1 cm), stage II, stage IIIA, or stage IIIC; ages 18 to 70 y at diagnosis (23); treated with axillary dissection and total mastectomy or lumpectomy followed by primary breast radiation; not scheduled for or currently undergoing chemotherapy; no evidence of recurrent disease or new breast cancer since completion of initial local treatment; and no other invasive cancer in the past 10 y. Participants were recruited from seven clinical sites in the Western United States. The institutional review boards of all the participating institutions approved procedures for this study, and written informed consent was obtained from all study participants before their enrollment.

Data Collection and Study Procedures. Data on the original breast cancer diagnosis (date of diagnosis, tumor stage and grade, tumor ER status) were obtained and confirmed via medical record. Further, medical records for each reported breast cancer recurrence or new primary breast cancer diagnosed after study enrollment were reviewed and confirmed by two oncologists, as previously reported (21, 22). Recurrences were further classified as local/regional or distant metastasis, and carcinoma *in situ* was not counted as a study outcome. The breast cancer event-free interval was defined as the time from the date of enrollment to the development of an additional breast cancer event. Follow-up time was censored at the time of the woman's death (if not from breast cancer), at the last documented staff contact date, or at the study completion date (June 1, 2006). Any report of a breast cancer event or death triggered a confirmation interview and collection of medical records and/or death certificates for review by study pathologists.

The WHEL Study protocol involved a clinic visit at enrollment and specified intervals [1, 2 or 3 (randomly determined), 4, and 6 y], at which a fasting blood sample was collected. Blood samples collected at the clinic visit were immediately placed on ice, protected from light, and separated within 1 h following collection, using centrifugation at 2,300 $\times$ g at 4°C for 10 min. Plasma aliquots were stored at -80°C in cryogenic tubes until analysis. Height and weight were measured using standard procedures and body mass index [BMI, weight (kg)/height (m^2)] was computed. Additional data collected included demographic and tumor characteristics, self-reported menopausal status, history of bilateral oophorectomy, initial treatments, and antiestrogen use. As described in the WHEL Study protocol (22), participants were randomly assigned to one of two study arms (comparison or intensive intervention), stratified by stage of disease, age at study entry, and clinic site.

The intensive intervention was delivered primarily by telephone counseling, supplemented with cooking classes in the 1st year, and monthly newsletters throughout the study. The dietary goals for the intervention group were a daily intake of 5 vegetable servings, 16 oz of vegetable juice or vegetable equivalents, 3 fruit servings, 30 g fiber, and 15% to 20% energy intake from fat. Women randomized to the comparison group were provided with print materials describing dietary recommendations to achieve a daily intake of 5 servings of vegetables and fruit, >20 g fiber, and <30% energy intake from fat and also were provided newsletters and invited to cooking classes during the 1st year. As reported previously, women assigned to the WHEL Study intervention group substantially increased vegetable and fruit intakes, and plasma carotenoid concentrations increased accordingly (19, 20). As previously reported, the mean total plasma carotenoid concentration was 66% higher in the intervention group than the comparison group at 1 y and 40% higher at 4 y.

Laboratory Analysis. Plasma carotenoids, including α-carotene, β-carotene, lutein, lycopene, and β-cryptoxanthin, were separated and quantified using a high-performance liquid chromatography method that has been previously described (24). Briefly, the high-performance liquid chromatography analysis was conducted with a Varian Star 9010, 9050 system with variable wavelength UV/visible light detector (Varian Analytical Instruments) with wavelength set at 450 nm. The mobile phase was acetonitrile/methanol/methylene chloride (70:10:30, v/v/v), with triethylamine (0.13 mL/L acetonitrile) and ammonium acetate (0.1 g/L methanol) modifiers used to enhance recovery. The column was a Supelco Supelcosil LC-18 (25 cm × 4.6 mm × 5 μm). Zeaxanthin and lutein elute together with this analytic method, which quantifies >90% of the carotenoids present in the circulation in humans. The variable described as plasma total carotenoids in the present study is the summed total of the quantified carotenoids. Accuracy was assessed by periodic analysis of National Institute of Standards and Technology standard reference material, and a pooled plasma sample was analyzed with batches of study samples to monitor analytic precision, with a day-to-day coefficient of variation of ~7%. Also, the laboratory participates in the National Institute of Standards and Technology round robin quality assurance program to monitor precision and reliability of these carotenoid measurements.

Total plasma cholesterol concentration was determined with the Kodak Ektachem Analyzer system (Johnson and Johnson Clinical Diagnostics; ref. 25) and used in the interpretation of plasma carotenoid data. Carotenoids are transported in the plasma nonspecifically by cholesterol-rich lipoproteins; thus, the size of the pool in which these compounds exist in the circulation is a nondietary influencing factor that is considered in the analysis of the relationship between plasma carotenoid concentration and breast cancer–free survival (1). Standard reference materials from the manufacturer were used to validate analytic precision of these procedures. The laboratory also participates in the American College of Pathologists quality assurance program to monitor precision and reliability for these lipid measures.

Statistical Analysis. Descriptive statistics were calculated for the study sample. Kruskal-Wallis tests were used to compare among groups for continuous variables and Pearson's χ^2 tests were used for categorical variables. The primary study end point was time to a second breast cancer event, defined as the combined outcome of invasive breast cancer recurrence or new primary breast cancer.

For each participant, with follow-up through t years, an average carotenoid concentration over time was estimated as follows. The area under the plasma carotenoid curve (AUC) was computed by summing the areas of trapezoids formed by the plasma carotenoid concentration at the regularly scheduled clinic visits before t. For example, if a participant had clinic visits at baseline, 1 y, and 2 y, and had an event or was lost to follow-up at t = 2.5 y, then her total plasma carotenoid concentration over time would be the sum of the areas of two trapezoids, one trapezoid formed by her baseline and 1-y values, and the second trapezoid formed by her 1- and 2-y values. To obtain her average carotenoid concentration over time, this total area would be divided by 2 (because she had clinic visits up to 2 y). If this participant had missing carotenoid data at 1 y, then her average carotenoid concentration was calculated as the area formed by the trapezoid defined by her baseline and 2-y plasma concentration divided by 2. Similarly, if this participant had missing 2-y carotenoid data, then her average carotenoid concentration over time (AUC/y) was estimated as the area of the trapezoid formed by her baseline and 1-y plasma values. Thus, the AUC/y represents a summary measure of cumulative plasma carotenoid levels during follow-up.

The focus on total (rather than individual) carotenoids in this analysis is based on the following rationale. Plasma carotenoids are highly correlated, due in part to their co-occurrence in various vegetables and fruit (e.g., carrots are a good source of both α-carotene and β-carotene), which limits the ability to confidently identify associations with specific compounds in an observational analysis. In relevant cell culture studies examining biological activities, carotenoids are generally not examined in a comprehensive and systematic manner, and both provitamin A and nonprovitamin A compounds have been observed to have similar effects.

Associations between average carotenoid concentration over time (as an indicator of biological exposure) and breast cancer events were assessed using a multiple regression Cox proportional hazards model with adjustment for factors associated with outcome (e.g., age, tumor stage, grade, and hormone receptor status). Also, study group, tumor type, tamoxifen use, smoking status, oophorectomy, clinic site, average plasma cholesterol concentration over time (also estimated as AUC/y), and BMI at baseline were used as adjustment variables. Average carotenoid concentration over time (estimated by AUC/y), reflecting biological exposure to carotenoids, was categorized into tertiles based on comparison group 33% and 67% quantiles. These quantiles were used to define three average carotenoid exposure groups (low, medium, and high carotenoid concentration over time). A group effect and interactions between study group assignment and average carotenoid concentration over time were formally tested with likelihood ratio tests. The results are presented as hazard ratios and 95% confidence intervals (95% CI).

The fit of the multiple regression model was assessed using diagnostic plots and residuals. The Schoenfeld's test was applied to test for violation of the proportional hazards assumption. The final model stratified on covariates that did not satisfy the proportional hazards assumption. Kaplan-Meier plots of breast cancer event-free survival were also generated.

Additionally, the analysis was stratified by tumor ER status to examine whether this variable modified the association between carotenoid level and breast cancer outcomes. Because the WHEL Study population included women across a range of cancer stage, we tested an interaction term between stage and plasma AUC/y in the Cox proportional hazards model and also stratified by this variable.

Results

The sample examined in this analysis included 1,506 participants in the intervention group and 1,537 participants

in the comparison group. Key characteristics of these participants and the distribution of these characteristics across the tertiles of average plasma carotenoid concentration over time are shown in Table 1. Tertiles of plasma carotenoid concentration were 0.404 to 1.656 μmol/L (minimum-maximum) for the low tertile, 1.657 to 2.452 μmol/L for the medium tertile, and 2.453 to 10.031 μmol/L for the highest tertile. Several of the associations with plasma carotenoid concentration in univariate analysis have been previously observed in the general population (1); for example, women in the higher tertiles of plasma carotenoid concentration over time were more likely to have a lower BMI and be nonsmokers, compared with the lowest tertile. Race/ethnicity, cancer stage, and tamoxifen use were also associated with differential distribution across the plasma carotenoid concentration tertiles.

In the comparison group, the concordance between baseline tertiles and average carotenoid concentration categories over time was 82%, 67%, and 81%, respectively, for the low, medium, and high carotenoid concentration groups (Table 2), with changes consistent with a regression to the mean effect. For the intervention group, there was a shift in distribution during the clinical trial (illustrated in Fig. 1), with 37% in the low group at baseline moving into medium/high average carotenoid concentration categories and 52% in the medium group at baseline moving into the high average carotenoid

Table 1. Characteristics of participants at study entry (*N* = 3,043)

	Total (*N* = 3,043)	Carotenoid exposure tertile group			*P**
		Low AUC/y† (≤1.656 μmol/L; *n* = 876)	Medium AUC/y† (1.657-2.452 μmol/L; *n* = 856)	High AUC/y† (>2.452 μmol/L; *n* = 1,311)	
Age at diagnosis					
Mean (SD)	51.3 (8.8)	51.73 (8.84)	50.7 (9.23)	51.3 (8.51)	0.074
Median	50.6	51.1	50.05	50.7	
Age at randomization					0.077
Mean (SD)	53.2 (8.9)	53.76 (8.91)	52.73 (9.38)	53.23 (8.65)	
Median	52.7	53.2	52.3	52.6	
BMI at baseline					<0.001
Mean (SD)	27.3 (6.0)	30.95 (6.98)	27.21 (5.48)	24.86 (4.23)	
Median	25.97	29.83	26.18	24.03	
Race/ethnicity [*n* (%)]					0.003
African American	114 (3.7)	39 (4.5)	42 (4.9)	33 (2.5)	
Asian American	95 (3.1)	14 (1.6)	30 (3.5)	51 (3.9)	
Hispanic	163 (5.4)	54 (6.2)	44 (5.1)	65 ([5])	
Non-Hispanic white	2,598 (85.4)	752 (85.8)	722 (84.3)	1,124 (85.7)	
Other	73 (2.4)	17 (1.9)	18 (2.1)	38 (2.9)	
Smoking status [*n* (%)]‡					<0.001
Current smoker	134 (4.4)	71 (8.2)	37 (4.4)	26 ([2])	
Former smoker	1,256 (41.7)	369 (42.5)	354 (41.8)	533 (41.1)	
Never smoker	1,623 (53.9)	429 (49.4)	455 (53.8)	739 (56.9)	
Tumor stage [*n* (%)]					0.006
I	1,176 (38.6)	318 (36.3)	327 (38.2)	531 (40.5)	
IIA	1,011 (33.2)	288 (32.9)	299 (34.9)	424 (32.3)	
IIB	375 (12.3)	107 (12.2)	108 (12.6)	160 (12.2)	
IIIA	368 (12.1)	120 (13.7)	83 (9.7)	165 (12.6)	
IIIC	113 (3.7)	43 (4.9)	39 (4.6)	31 (2.4)	
Tumor grade [*n* (%)]					0.617
Poorly differentiated	1,087 (35.7)	317 (36.2)	306 (35.7)	464 (35.4%)	
Moderately differentiated	1,227 (40.3)	362 (41.3)	352 (41.1)	513 (39.1)	
Well differentiated	478 (15.7)	129 (14.7)	124 (14.5)	225 (17.2)	
Unspecified	251 (8.2)	68 (7.8)	74 (8.6)	109 (8.3)	
Tamoxifen use [*n* (%)]					<0.001
No	1,229 (40.4)	277 (31.6)	346 (40.4)	606 (46.2)	
Yes	1,814 (59.6)	599 (68.4)	510 (59.6)	705 (53.8)	
Tumor ER status [*n* (%)]‡					0.273
Negative	745 (24.8)	197 (22.9)	218 (26)	330 (25.4)	
Positive	2,253 (75.2)	664 (77.1)	621 (74)	968 (74.6)	
Chemotherapy during initial treatment [*n* (%)]‡					0.293
No	917 (30.2)	246 (28.1)	267 (31.2)	404 (30.8)	
Yes	2,124 (69.8)	629 (71.9)	589 (68.8)	906 (69.2)	
Radiation therapy during initial treatment [*n* (%)]‡					0.801
No	1,168 (38.4)	332 (38)	337 (39.4)	499 (38.1)	
Yes	1,871 (61.6)	542 (62)	519 (60.6)	810 (61.9)	

*P values based on Kruskal-Wallis or χ^2 tests comparing participant characteristics across tertiles.
†Average carotenoid concentration over time was estimated as the area under the AUC divided by number of years of follow-up, computed by summing the areas of trapezoids formed by plasma carotenoid concentrations at regularly scheduled clinic visits (described as AUC/y).
‡Numbers do not sum to 3,043 due to missing data.

Table 2. Baseline carotenoid concentration tertile group versus average plasma carotenoid concentration over time, based on plasma carotenoid concentration in the comparison group

Baseline plasma carotenoid concentration tertile	Low AUC/y* (≤1.656 μmol/L)	Medium AUC/y* (1.657-2.452 μmol/L)	High AUC/y* (>2.452 μmol/L)	Total
Comparison group				
Low baseline	421 (82)	81 (16)	11 (2)	513
Medium baseline	84 (16)	344 (67)	84 (16)	512
High baseline	8 (2)	87 (17)	417 (81)	512
Total	513	512	512	1,537
Intervention group				
Low baseline	304 (63)	107 (22)	70 (15)	481
Medium baseline	55 (10)	212 (38)	287 (52)	554
High baseline	4 (1)	25 (5)	442 (94)	471
Total	363	344	799	1,506

NOTE: Each cell contains frequency (row percent).
*Average carotenoid concentration over time was estimated as the area under the AUC divided by number of years of follow-up, computed by summing the areas of trapezoids formed by plasma carotenoid concentrations at regularly scheduled clinic visits (described as AUC/y).

concentration. The majority of the participants who were in the highest tertile of average carotenoid concentration at baseline were also in the highest tertile over time (81% of the comparison group and 94% of the intervention group; Table 2).

The WHEL study recruitment goal was 3,000 breast cancer survivors and was designed to have 82% power to detect a 19% reduction in breast cancer event rate in the intervention group assuming a 24% event rate in the comparison group (21). Assuming a lower overall event rate of 17% as seen at the end of the WHEL Study, there is 81% power to detect a 21% reduction in events in the intervention arm, assuming a comparison group event rate of 18.6%.

A total of 508 (16.7%) breast cancer events occurred over a median 7.12 years follow-up. The highest two tertiles of average plasma carotenoid concentration over time were associated with the best outcome in univariate analyses ($P = 0.003$; Table 3) with event rates of 16.1% in the middle tertile and 15.2% in the high tertile versus 19.5% in the low tertile. Due to the similar event rates in the top two tertiles, these tertiles were combined as one category in subsequent analysis to ease interpretation and reduce the number of parameters to be estimated. The protective effect of being in the top two carotenoid tertiles remained apparent in the multiple regression model after adjustment for key covariates. The likelihood ratio test for group effect or group × carotenoid tertile interaction was not significant ($P = 0.23$). Compared with the lowest tertile, the hazard ratio for the medium/high tertiles was 0.67 (95% CI, 0.54-0.83) after adjustment (see Table 4; Fig. 2).

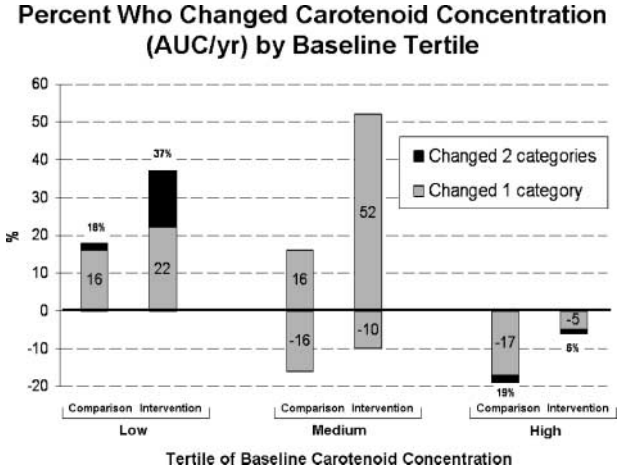

Figure 1. Percentage of participants in the two study groups who changed across the tertiles during the course of the study.

Among the 2,253 women with ER-positive tumors, the hazard ratio comparing the upper plasma carotenoid AUC tertiles with the bottom was 0.7 (95% CI, 0.54-0.91; $P = 0.007$); among 745 women with ER-negative tumors, the hazard ratio was 0.7 (95% CI, 0.46-1.08; $P = 0.11$). The identical hazard ratios between both tumor ER groups suggest that the lack of significant effect of plasma carotenoids among ER-negative women is due to smaller sample size. In fact, in the multiple regression model, the interaction of tumor ER status and plasma AUC/y was not significant ($P = 0.67$), confirming the lack of differential effects across ER groups. An interaction term between stage and plasma AUC/y was not significant ($P = 0.66$). When we stratified by stage, the hazard ratios for the plasma carotenoid effect were similar across stage categories (stage I, 0.64; 95% CI, 0.39-1.04; stage IIA, 0.60; 95% CI, 0.40-0.88; stage IIB/III, 0.72; 95% CI, 0.53-0.98), suggesting that the protective effect of being in the upper tertiles of plasma carotenoid AUC/y was not modified by stage of the original tumor.

Discussion

In this cohort of women with a history of early-stage breast cancer, higher biological exposure to carotenoids, measured over time during a trial testing the effect of a very high vegetable diet, was associated with an estimated 33% greater likelihood of breast cancer–free survival when compared with the lowest tertile of carotenoid exposure, regardless of study group assignment. This effect confirms our previously published findings from the comparison group using only the total plasma carotenoid concentration measured at baseline (18). Over the WHEL Study intervention period, diet intervention reduced the proportion of women in the lowest tertile in that group by 26% but did not improve prognosis.

Table 3. Breast cancer events

	Low AUC/y* (≤1.656 μmol/L)	Medium AUC/y* (1.657-2.452 μmol/L)	High AUC/y* (>2.452 μmol/L)
Local/regional recurrence	22 (2.5)	24 (2.8)	35 (2.7)
Distant recurrence	123 (14.0)	93 (10.9)	133 (10.1)
New primary breast cancer	26 (3.0)	21 (2.5)	31 (2.4)
No evidence of recurrence	705 (80.5)	718 (83.9)	1,112 (84.8)
Total	876 (100.0)	856 (100.0)	1,311 (100.0)

NOTE: Each cell contains frequency (column percent). The *P* value testing a zero association between time to breast cancer–free survival and average plasma carotenoid concentration tertile group (the lowest versus the rest) is 0.003.
*Average carotenoid concentration over time was estimated as the area under the AUC divided by number of years of follow-up, computed by summing the areas of trapezoids formed by plasma carotenoid concentrations at regularly scheduled clinic visits (described as AUC/y).

There are several possible explanations for these results. The first is that carotenoids or their biologically active metabolites reduce the risk for recurrence or new primary breast cancer. Long-term exposure to these compounds may affect cancer progression, and even a dramatic change following breast cancer is not sufficient to modify this risk. Another bioactive food component provided over the long term by a dietary pattern that is associated with higher plasma carotenoids also may be the active ingredient. Another possible explanation is that plasma carotenoids are an indicator of some other lifestyle factor or unmeasured biological characteristic that exerts a protective effect on breast cancer progression.

Although the intervention group exhibited an increase in the average plasma carotenoid concentration during the time frame of the study, the total exposure during the clinical trial was largely influenced by baseline concentration in both the intervention and comparison group study arms. Most women who were in the higher tertiles of total plasma carotenoid concentration at enrollment remained in those higher tertiles throughout the study in both study groups. Despite a substantial increase in average total plasma carotenoids across all strata of baseline levels in the intervention group, the majority of women in the lowest baseline tertile had the lowest biological exposure to carotenoids over the study period in both study groups (82% of the comparison and 63% of the intervention group).

Effect modification by baseline nutrient status has been suggested to explain results from several previous nutrient-based chemoprevention trials (26). Relevant to the present findings, β-carotene supplementation in the Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study did not reduce lung cancer incidence or mortality in the active treatment arm, but when the clinical trial participants were analyzed as a cohort, serum β-carotene concentration at baseline was associated with a lower risk for lung cancer (relative risk, 0.81; 95% CI, 0.69-0.95; for highest versus lowest quintile; ref. 27). The eating pattern or exposures to beneficial dietary constituents over a longer time frame, rather than the relatively short-term exposures and changes in intakes that are instituted in clinical trials, may constitute the more important environmental influence on the molecular and cellular characteristics that affect risk for new cancer or progression of an invasive cancer.

The total plasma carotenoid concentrations observed in this sample of early-stage breast cancer survivors can be compared with those in the general population and in studies of carotenoids and incident breast cancer risk. A mean of 1.676 μmol/L (and a median of 1.446 μmol/L) were observed in the National Health and Nutrition Examination III survey, 1988 to 1994, in women ages 51 to 70 years, indicating that approximately one half of this subgroup of the general population would fall in the lowest tertile in the present analysis (28). Total

Table 4. Cox proportional hazards model for time to breast cancer event

Variable	Hazard ratio (95% CI)
Tumor stage I (reference)	
Stage IIA	2.00 (1.52-2.63)
Stage IIB	3.05 (2.20-4.21)
Stage IIIA	3.96 (2.90-5.42)
Stage IIIC	8.91 (6.11-12.98)
Tumor grade, poorly differentiated (reference)	
Moderately differentiated	0.88 (0.71-1.09)
Well differentiated	0.59 (0.41-0.85)
Unspecified	0.99 (0.68-1.44)
No tamoxifen use (reference)	
Any tamoxifen use	0.75 (0.60-0.94)
Comparison group (reference)	
Intervention group	1.06 (0.89-1.27)
Plasma total carotenoids AUC/y, lowest tertile (≤1.656 μmol/L; reference)*	
Medium/high AUC/y (>1.656 μmol/L)*	0.67 (0.54-0.83)

NOTE: Stratified on categorized years from diagnosis to randomization (0-1, 1-2, 2-3, and >3 y) because this variable violated the proportional hazards assumption. Controlled for age at randomization, clinic site, tumor hormone receptor status, tumor type, chemotherapy, oophorectomy, smoking status, plasma cholesterol, and BMI. The likelihood ratio test *P* value testing the interaction effect of plasma carotenoid concentration tertile group and intervention group is 0.23.
*Average carotenoid concentration over time was estimated as the area under the AUC divided by number of years of follow-up, computed by summing the areas of trapezoids formed by plasma carotenoid concentrations at regularly scheduled clinic visits (described as AUC/y).

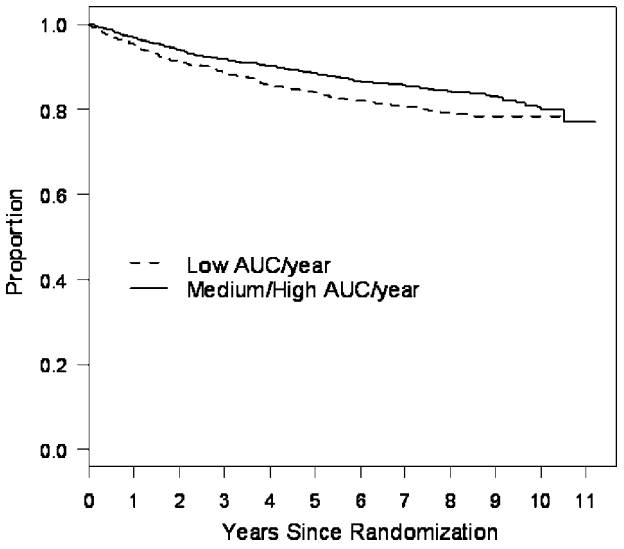

Figure 2. Kaplan-Meier estimates of breast cancer–free survival by tertile of average plasma carotenoid concentration over time. Average carotenoid concentration over time was estimated as the AUC, computed by summing the areas of trapezoids formed by plasma carotenoid concentrations at regularly scheduled clinic visits (described as AUC/y).

plasma carotenoids were inversely related to risk for incident breast cancer (odds ratio, 0.73; 95% CI, 0.55-1.05; P_{trend} = 0.05; highest versus lowest quintile) in women with a median total plasma carotenoid concentration of 1.85 μmol/L in the Nurses' Health Study (29). Cases and controls had mean serum total carotenoids of 2.342 and 2.632 μmol/L, respectively, in the New York University Women's Health Study, in which serum carotenoid concentration was associated with reduced risk for breast cancer (odds ratio, 2.31; 95% CI, 1.35-3.96; lowest versus highest quartile; ref. 30). Average total carotenoid concentration in the latter two studies would fall in the middle tertile in the present analysis.

Although negative findings from the β-carotene supplement trials have dampened the enthusiasm for the potential role of carotenoids in chemoprevention (26, 31, 32), laboratory evidence relating to the possibility of a specific role for dietary carotenoids in reducing the risk and progression of breast cancer continues to be very supportive. Absorption and tissue uptake of carotenoids is unregulated, although influenced by several other dietary and nondietary factors; thus, serum and peripheral tissue concentrations are primarily determined by dietary intakes. Serum and breast tissue concentrations have been observed to be highly correlated in women with benign breast disease or breast cancer (33); thus, activities of carotenoids and metabolites studied in cell culture are relevant to the human biological system. Retinoids, carotenoids, and eccentric cleavage products of β-carotene that are endogenously present in human tissues have been shown to inhibit the proliferation of both ER-positive and ER-negative breast cancer cells at physiologic concentrations (8, 9). The β-carotene metabolite apo-14′-carotenoic acid activates retinoic acid receptor and retinoid X receptor–mediated transcriptional pathways, but laboratory studies suggest that retinoic acid receptor expression is not the complete explanation for the growth inhibition of breast cancer cells and that other mechanistic pathways are possible (8). More recently, β-carotene and lycopene have been shown to inhibit proliferation induced by either estradiol or genistein and to inhibit estrogen signaling of both of these compounds in human mammary cancer cells (10).

Findings from a randomized phase III trial of fenretinide, a synthetic derivative of *all-trans* retinoic acid, in 2,867 women who had been diagnosed with breast cancer support the possibility of a specific effect of carotenoids and/or their metabolites on risk and progression of breast cancer (34). Similar to the carotenoids (and unlike preformed vitamin A), administration of fenretinide can selectively accumulate in human breast tissue and has a favorable toxicity profile. After 8 years, the main results of that trial showed no difference in contralateral or ipsilateral breast cancer (the primary study outcomes), but post hoc analysis revealed a substantial benefit for premenopausal women that has persisted several years after treatment cessation (35).

An alternative explanation for the observed association between total plasma carotenoid concentration and risk for an additional breast cancer event is that higher carotenoid concentration may be an indicator of unmeasured lifestyle or other factors that affect risk and progression of mammary carcinogenesis. In the WHEL Study population, total plasma carotenoid concentration was positively associated with higher intake of vegetables and fruit, lower BMI, and nonsmoking status—all factors known to be associated with reduced risk for cancer. This explanation was previously suggested when the negative results of the β-carotene supplement trials in lung cancer prevention were in marked contrast to the findings from observational studies, in which serum and dietary β-carotene and lung cancer risk were consistently inversely related (36).

It is also possible that the present findings may be due to genetic or other biochemical characteristics that promote a higher total plasma carotenoid concentration and also have an unrelated and independent effect on mammary carcinogenesis. Interindividual variability in plasma carotenoid concentration in response to a given level of intake is commonly observed (1) and this variability is likely to be determined in part by genetic differences in metabolizing enzymes. Enzymes responsible for both central cleavage (producing retinal) and eccentric cleavage (producing apo-carotenals) of β-carotene have been well characterized (2, 3), but much remains to be learned about other enzymes involved in carotenoid metabolism and degradation. For example, oxidative metabolites of β-carotene have been shown to induce P450 enzymes (37, 38) and variability in the genetic and epigenetic characteristics of these and other metabolizing enzymes could influence both plasma carotenoids and independently affect risk and progression of breast cancer.

This study has some strengths and limitations. One strength is that plasma carotenoids were measured at several time points during the follow-up period, whereas most studies of the associations between dietary factors, such as carotenoids and cancer outcomes, have relied on a single measurement. This study involved a fairly large

number of breast cancer survivors, but subsample size still limits the statistical power to detect associations in those subsets. Although adjustments for numerous potentially confounding factors were conducted in this analysis, the possibility of unmeasured confounding must be recognized when observing associations of any type.

In summary, higher biological exposure to carotenoids, indicated by higher average total plasma carotenoid concentration measured at multiple time points during a 7-year follow-up period, is associated with greater likelihood of breast cancer–free survival in women who have been diagnosed and treated for early-stage breast cancer. These results suggest that longer-term exposure to a high vegetable and fruit dietary pattern that promotes higher plasma carotenoid concentration may improve prognosis and survival. As biologically active food components with demonstrated effects on human breast cells and mammary carcinogenesis in laboratory studies, a specific effect of carotenoids on breast cancer progression is possible. Alternatively, other lifestyle factors or biological characteristics that are associated with increased plasma carotenoids may be responsible for the beneficial effects. More research is needed to understand the biology of breast cancer and how risk and progression may be influenced by dietary factors such as the carotenoids.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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