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Plasma fluorescent oxidation products and risk of estrogen receptor-negative breast cancer in the Nurses' Health Study and Nurses' Health Study II

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Abstract

Purpose—Findings from epidemiologic studies of oxidative stress biomarkers and breast cancer have been mixed; although no studies have focused on estrogen receptor-negative (ER–) tumors, which may be more strongly associated with oxidative stress. We examined pre-diagnostic plasma fluorescent oxidation products (FIOP), a global biomarker of oxidative stress, and risk of ER– breast cancer in a nested case-control study in the Nurses' Health Study (NHS) and NHSII.

Methods—ER– breast cancer cases (n=355) were matched to 355 controls on age, month/time of day of blood collection, fasting status, menopausal status and menopausal hormone use. Conditional logistic regression models were used to examine associations of plasma FIOP at three emission wavelengths (FIOP_360, FIOP_320, and FIOP_400) and risk of ER– breast cancer.

Results—We did not observe any significant associations between FIOP measures and risk of ER– breast cancer overall; the RR_{Q4vsQ1} (95%CI) =0.70 (0.43-1.13), $p_{trend}=0.09$ for FIOP_360; 0.91(0.56-1.46), $p_{trend}=0.93$ for FIOP_320; and 0.62 (0.37-1.03), $p_{trend}=0.10$ for FIOP_400. Results were similar in models additionally adjusted for total carotenoid levels, and in models stratified by age and by total carotenoids. Although, high (vs. low) levels of FIOP_360 and FIOP_400 were associated with lower risk of ER– breast cancer in lean women (body mass index

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The analysis presented here complies with current laws of the country in which they were performed.

(BMI)<25 kg/m²) but not in overweight/obese women, these differences were not statistically significant ($p_{\text{int}}=0.23$ for FLOP_360; $p_{\text{int}}=0.37$ for FLOP_400).

Conclusions—Our findings suggest that positive associations of plasma FLOP concentrations and ER– breast cancer risk are unlikely.

Keywords

fluorescent oxidation products; oxidative stress; breast cancer; estrogen receptor

Introduction

Oxidative stress, resulting from inadequate clearance of reactive oxygen species (ROS), causes damage to lipids, proteins, and DNA, and may be related to carcinogenesis [1-3]. While several small case-control studies have observed positive associations between lipid peroxidation markers of oxidative stress, including malondialdehyde (MDA) and 15-F₂T-isoprostanes (15-F₂T-IsoP), and overall breast cancer risk [4-12], these findings have not been confirmed in larger prospective studies [13,14]. Plasma fluorescent oxidation products (FLOP) quantify the interaction of oxidative products with proteins, lipids and DNA and thus represent a more global measure of oxidative stress compared to traditional biomarkers of lipid peroxidation [16]. Further, unlike MDA and 15-F₂T-IsoP, FLOP are stable in blood with delayed processing between collection and freezing [15]. In our prior nested case-control studies, we did not observe consistent associations between plasma FLOP and overall breast cancer risk [16,17], although an elevated risk of breast cancer was observed among women with persistently high concentrations of FLOP_320 [16].

Given the role of carotenoids as antioxidants reducing oxidative stress-induced DNA damage [18], and the suggestion from large pooled analyses that carotenoids are inversely associated with estrogen receptor-negative (ER–) breast cancer [19,20], the role of oxidative stress on tumorigenesis may conceivably be more important in the etiology of this subset of breast tumors. However, to our knowledge, no prior study has examined associations of plasma FLOPs with ER– breast cancer specifically, while accounting for carotenoid measures. Thus, we used a nested case-control design within the Nurses' Health Study (NHS) and Nurses' Health Study II (NHSII) to examine associations between pre-diagnostic plasma FLOP concentrations and ER– breast cancer and evaluated whether associations were modified by total plasma carotenoids. Our 2 prior investigations included 45 [16] and 150 [15] ER– cases, and here we include those cases along with an additional 160 ER– cases (total=355) for a more thorough examination of oxidative stress in this aggressive tumor subtype.

Methods

Study population

The NHS began in 1976 when 121,700 female registered nurses, ages 30-55 years, completed a mailed questionnaire. The NHSII was established in 1989 among 116,430 female registered nurses, ages 25-42 years. Women in both ongoing cohorts continue to be followed via biennial questionnaire to assess lifestyle factors and disease diagnoses.

Between 1989 and 1990, 32,826 NHS participants, ages 43-70 years, provided a blood sample. NHSII participants, ages 32-54 years, provided blood samples between 1996 and 1999. Further details of the blood collection procedure for NHS [21,22] and NHSII [23] have been described previously. Briefly, participants had blood drawn and shipped the sample on ice to our laboratory via overnight courier. All samples were processed in our laboratory into plasma, white blood cell, and red blood cell components and have been stored at -130 degrees Celsius in continuously monitored liquid nitrogen freezers. The stability of plasma FLOP concentrations using these procedures has been previously demonstrated [15].

Case and control selection

A total of 710 NHS and NHSII participants who contributed blood samples and were cancer-free at the time of the blood collection were included in this nested case-control study. A total of 355 ER- invasive breast cancer cases were diagnosed after the blood collection and before June 1, 2006 (NHS) and June 1, 2007(NHSII); all cases were confirmed via medical record review. Information on ER, progesterone receptor (PR) status, and tumor characteristics were abstracted from pathology reports.

A single control was matched to each case on factors at blood collection; age (within 1 year), month (+/- 1 month) and time of day (+/- 2 hours) of blood collection, fasting status (≥ 10 hours since last meal vs. < 10 hours or unknown), and menopausal status and menopausal hormone therapy (HT) use (premenopausal, postmenopausal/no HT use, postmenopausal/HT use, unknown menopausal status). Cases and controls were also matched on menopausal status at the time of the case diagnosis. This study was approved by the Committee on the Use of Human Subjects in Research at the Brigham and Women's Hospital (Boston, MA).

Laboratory assays

Laboratory personnel, blinded to case-control status, assayed case-control sets together in random order. Details of the laboratory procedures used to quantify plasma FLOP concentrations have been described previously [24]. Briefly, plasma samples were mixed with ethanol/ether and with supernatant added for spectrofluorometric readings in the laboratory of coauthor (TW) at the University of Cincinnati. Fluorescence was determined as relative fluorescent intensity per milliliter of plasma (FI/mL). FLOP measures were assayed at three excitation levels: FLOP_360 (excitation 360 nm, emission 420nm), FLOP_320 (excitation 320 nm, emission 420 nm), and FLOP_400 (excitation 400nm, emission 475 nm). FLOP_360 characterizes the interaction between lipid oxidation products and protein, DNA and carbohydrates [24]. FLOP_320 represents the interaction between lipid oxidative products, particularly lineolate, with DNA and metals [25,26]. FLOP_400 represents the interaction between MDA, proteins, and phospholipids [26,27]. Intraclass correlation coefficients (ICC) for a single FLOP measure over 3 years in NHS women ranged from 0.44 (FLOP_360) to 0.70 (FLOP_400); ICCs over 10 years ranged from 0.14 (FLOP_320) to 0.30 (FLOP_360) [28]. The three FLOP measures were moderately to strongly correlated with Spearman correlations $r=0.69$ between FLOP_360 and FLOP_320, $r=0.45$ between FLOP_320

and FLOP_400, and $r=0.73$ between FLOP_360 and FLOP_400. In prior epidemiologic research, FLOP_360 has been the most commonly used FLOP measure [29,15].

Plasma carotenoids may be associated with FLOP measures and breast cancer, and could confound or modify associations. Therefore, total plasma carotenoid concentrations were considered as potential confounders and/or effect modifiers in this analysis. For this analysis, we included 643 (91%) of women for which total carotenoid information was available. Carotenoids were assayed using reverse-phase high performance liquid chromatography [30] at the Micronutrient Analysis Laboratory in the Department of Nutrition at the Harvard School of Public Health. Total carotenoids were calculated as the sum of α -carotene, β -carotene, β -cryptoxanthin, lycopene, and lutein/zeaxanthin. Total carotenoids were weakly correlated with FLOP measures (Spearman correlations $r=0.15$ between FLOP_360 and total carotenoids, $r=-0.12$ between FLOP_320 and total carotenoids, and $r=-0.06$ between FLOP_400 and total carotenoids) [16].

We observed batch variation for FLOPs in the 10% percent of samples included as masked quality control samples. Thus, we adjusted for this batch variation using methods described by Rosner et al [31] as we have used previously [32]. After recalibration, overall CVs for each FLOP were $<20\%$.

Statistical methods

Statistical outliers in FLOP concentrations were identified using the generalized extreme Studentized deviate (ESD) many-outlier detection approach [33]; $N=13$ (FLOP_400), $n=15$ (FLOP_360), and $n=44$ (FLOP_320) extreme values were identified and excluded in sensitivity analyses. Quartiles were created based on the distribution of FLOP concentrations among controls. Wald tests for trend were conducted modeling the median across quartiles of FLOP concentrations as a continuous variable.

NHS and NHSII data was pooled and we used conditional logistic regression models to estimate relative risks (RR) and 95% confidence intervals (CI) adjusting for matching factors and covariates. Information on potential covariates, including family history of breast cancer (yes/no), history of benign breast disease (yes/no), age at menarche (<12 , 12, 13, 14+ years), age at first birth and parity (nulliparous, 1-2 children <25 years, 3+ children <25 years, 1-2 children >25 years, 3+ children >25 years), alcohol intake (0, 1 to <5 , 5 to <15 , 15 grams/day), smoking status (never, past, current), physical activity (<3 , 3 to <9 , 9 to <18 , 18 to <27 , and 27+ MET-hrs/week) and body mass index (BMI; <25 , 25 to <30 and ≥ 30 kg/m²), was collected on the biennial questionnaires or the questionnaire returned with the blood samples. We additionally examined models including measured total carotenoid concentrations as a covariate. We evaluated whether the association between FLOP and ER+ breast cancer risk varied by BMI (<25 vs. ≥ 25 kg/m²), age at blood collection (<50 vs. ≥ 50 years), and total carotenoids (low vs. high, dichotomized at the median) using unconditional logistic regression, controlling for matched factors and covariates. We additionally examined models restricted to fasting samples (8+ hours) and models stratified by lag time from blood collection to breast cancer diagnosis (dichotomized at median) to assess whether blood fasting status or lag time influenced results. All statistical tests were two-sided and p values

were considered statistically significant at <0.05 ; analyses were conducted in SAS v. 9.3 (SAS Institute, Cary, NC).

Results

Cases and controls were similar with regard to BMI, smoking status, alcohol consumption, age at menarche, age at first birth and plasma FLOP concentrations, although cases were more likely than controls to report a family history of breast cancer and a prior diagnosis of benign breast disease, and were less physically active (Table 1).

We did not observe any significant associations between plasma FLOP levels and risk of ER– breast cancer overall (Table 2). For example, the RR_{Q4vsQ1} (95%CI) = 0.70 (0.43–1.13), $p_{trend}=0.09$ for FLOP_360; 0.91(0.56–1.46), $p_{trend}=0.93$ for FLOP_320; and 0.62(0.37–1.03), $p_{trend}=0.10$ for FLOP_400. Results were similar when we additionally adjusted for total carotenoid levels; RR_{Q4vsQ1} (95%CI) = 0.77 (0.45–1.33), $p_{trend}=0.26$ for FLOP_360; 0.95(0.57–1.60) =, $p_{trend}=0.57$ for FLOP_320; and 0.69(0.40–1.18) =, $p_{trend}=0.30$ for FLOP_400. Plasma FLOP levels also were not significantly associated with ER– breast cancer in models restricted to women fasting at least 8 hours prior to blood collection, in analyses excluding outliers and in analysis stratified by lag time between blood collection and breast cancer diagnosis (data not shown).

We did not observe any heterogeneity in associations in analysis stratified by age (<50 vs. 50 years), or by total carotenoid level (dichotomized at median); and no significant interaction was apparent for any of the FLOPs (all $p_{int} \geq 0.25$) (Table 3). For FLOP_360 and FLOP_400, results did appear to differ according to BMI at blood collection although the interactions were not statistically significant ($p_{int}=0.23$ and $p_{int}=0.37$ respectively). For example, risk of ER– breast cancer was 60% lower among lean women ($BMI < 25 \text{ kg/m}^2$) in the highest vs. lowest quartile of FLOP_360 (RR_{Q4vsQ1} (95%CI)=0.40 (0.20–0.81), $p_{trend}=0.01$) and FLOP_400 (0.42 (0.22–0.82), $p_{trend}=0.02$), while no significant associations were observed among women with $BMI \geq 25 \text{ kg/m}^2$; FLOP_360 (RR_{Q4vsQ1} (95%CI)=1.10 (0.54–2.24), $p_{trend}=0.93$) and FLOP_400 (0.96 (0.46–1.99), $p_{trend}=0.88$).

Discussion

In this large, nested case-control study, we did not observe any significant associations between plasma FLOP levels and ER– breast cancer overall, or in analyses stratified by age and total plasma carotenoid concentrations. However, plasma FLOP_360 and FLOP_400 were associated with a reduced risk of ER– breast cancer among lean women, while no significant associations were observed for women with $BMI \geq 25 \text{ kg/m}^2$, although the interaction was not significant.

While this study is the first to focus primarily on the role of plasma FLOP and ER– breast cancer, our null findings are generally consistent with prior prospective studies of both urinary [13,14] and plasma [16,17] markers of oxidative stress and overall breast cancer risk. We unexpectedly observed significant inverse associations of FLOP_360 and FLOP_400 and risk of ER– breast cancer among women with $BMI < 25 \text{ kg/m}^2$ in this study, however the interactions by BMI were not statistically significant. A reduced risk of overall breast cancer

in relation to a urinary marker of lipid peroxidation was also observed among lean women in a prior prospective study of Chinese women [13]; however, in our prior smaller study, we observed a positive association of FLOP_320 and overall breast cancer risk among lean women and no heterogeneity in associations of FLOP_360 and FLOP_400 by BMI [16].

While associations did not vary by total carotenoids in this study, nor in our prior study of premenopausal FLOP measures and total breast cancer [15], a suggestion of an increased risk of overall breast cancer in relation to FLOP_360 and FLOP_320 among women with low plasma carotenoid levels was observed in our prior study of primarily postmenopausal women [16].

Reactive oxygen species (ROS), generated from both endogenous metabolic processes [34-36] and exogenous exposures including alcohol and radiation [37,38], are generally cleared through enzymatic destruction or exogenous antioxidants. Given that antioxidants, including carotenoids, may neutralize these reactive molecules, thereby reducing the burden of oxidative stress and potentially inhibiting carcinogenesis, the reduced risk of ER- breast cancer in relation to carotenoids [19] may be partially explained through oxidative stress pathways. If carotenoids reduce oxidative stress-induced cell damage, we might expect oxidative stress biomarkers to be more strongly associated with ER- breast cancer. Further, chronic exposure to oxidative stress, through hydrogen peroxide-induced proliferation of intracellular ROS, has been shown to cause loss of ER- α expression and conversion of estrogen-dependent breast cancer cells into estrogen-independent phenotype [39], suggesting the importance of oxidative stress on ER- breast cancer risk. However, despite the biological plausibility of positive associations between oxidative stress and ER- breast cancer, we did not observe any significant positive associations in this study. In fact, inverse associations of plasma FLOP and ER- breast cancer were observed among lean women in this study, although the interaction with BMI was not significant, and the finding may be due to chance. While it is plausible that ROS may induce DNA damage, promoting lipid peroxidation and playing a role in etiology of cancer, ROS might also mediate or trigger protective mechanisms, such as apoptosis, which could have beneficial function in breast cancer [40] and may be more apparent in proliferative subtypes, such as ER- tumors. Future studies are warranted to elucidate the complex biological pathways whereby oxidative stress may promote, or perhaps inhibit breast carcinogenesis.

Strengths of this study include the relatively large sample size of ER- breast cancer cases with measured carotenoid levels, and use of comprehensive plasma measures of oxidative stress. Plasma FLOP concentrations, which are thought to reflect global levels of oxidation, have been associated with exposures related to oxidative stress including cigarette smoking and cholesterol [29]. However, given our lack of findings with breast cancer, other specific markers of oxidative stress may be more important in breast cancer etiology, although urinary markers of lipid peroxidation also were not associated with breast cancer risk in the prospective Shanghai Women's Health Study [13,14]. Due to the uncommon nature of ER- tumors, we were somewhat limited in our ability to conduct stratified analyses as part of this study. While our study relied on a single measurement of FLOP levels, reproducibility over a 2-3 year period (ICCs=0.44-0.70) [28], though a bit lower for 10-year (ICCs=0.14-0.30) [16], suggests that one measure is an adequate reflection of longer-term exposure. However,

persistently high levels of FLOP_320 were associated with a suggested elevated risk of breast cancer in our prior study [16], suggesting that duration of exposure to ROS could be important. It is also possible that different time periods of exposure may have differing effects for carcinogenesis. In fact, timing during the menstrual cycle, pregnancy, and lactation have been suggested as important etiologic windows for the influence of oxidative stress in breast cancer [40]. Thus, future studies are warranted to examine whether the risk of breast cancer in relation to oxidative stress may vary depending upon the timing and duration of the exposure.

In summary, we did not observe significant associations between plasma FLOP measures and risk of ER– breast cancer overall in this large nested case-control study. Our findings suggest that positive associations of plasma FLOP concentrations and ER– breast cancer risk are unlikely. Although a suggested inverse association of plasma FLOP_360 and FLOP_400 and ER– breast cancer was observed among lean women, the interaction was not significant and there is not strong biologic plausibility for this finding. Given the consistent findings of inverse associations of carotenoids and ER-breast cancer, oxidative stress pathways should continue to be investigated as a potential etiologic mechanism for this aggressive breast cancer subtype.

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Table 1

Characteristics of cases and controls at blood collection

	Cases (n=355)	Control (n=355)
	Mean (SD) ^a	Mean (SD) ^a
Age at Blood Draw, years ^b	54.9 (6.0)	55.1 (6.1)
Body Mass Index, kg/m ²	25.6 (4.3)	25.4 (4.3)
Alcohol Consumption, grams/day	4.9 (7.9)	5.0 (7.9)
Total activity, MET-h/week	20.5 (36.4)	30.1 (105)
Age at menarche, years	12.5 (1.4)	12.5 (1.2)
Age at first birth, years ^c	23.7 (6.4)	23.0 (6.8)
Premenopausal, %	42	39
Parous, %	87	88
Current Smoker, %	12	12
Family History of Breast Cancer, %	13	9
Benign Breast Disease, %	43	32
Median (IQR)		
FLOP_360, FI/ml	206 (178-248)	213 (181-263)
FLOP_320, FI/ml	375 (273-578)	376 (281-565)
FLOP_400, FI/ml	63 (55-76)	65 (57-78)

^aValues are means(SD) or percentages and are standardized to the age distribution of the study population.^bValue is not age adjusted^cAmong parous women

Plasma fluorescent oxidative products and risk of ER negative breast cancer: NHS and NHSII

Table 2

		1st Quartile		2nd Quartile		3rd Quartile		4th Quartile		P _{trend}
		OR (95% CI)		OR (95% CI)		OR (95% CI)		OR (95% CI)		
FIOP_360	Cupoints, FI/mL	<181		181-213		213-263		263		
	No. cases	97/87		106/91		81/89		71/88		
	<i>Unadjusted</i>	1.0 (referent)		1.03 (0.69-1.56)		0.81 (0.52-1.26)		0.74 (0.49-1.12)		0.09
	<i>Multivariable</i>	1.0 (referent)		1.04 (0.67-1.61)		0.89 (0.54-1.47)		0.70 (0.43-1.13)		0.09
	<i>Multivariable + carotenoids</i>	1.0 (referent)		1.05 (0.65-1.69)		0.91 (0.53-1.58)		0.77 (0.45-1.33)		0.26
FIOP_320	Cupoints, FI/mL	<281		281-376		376-565		565		
	No. cases	96/88		83/89		83/89		93/89		
	<i>Unadjusted</i>	1.0 (referent)		0.82 (0.52-1.29)		0.82 (0.51-1.31)		0.92 (0.60-1.43)		0.92
	<i>Multivariable</i>	1.0 (referent)		0.80 (0.49-1.30)		0.80 (0.47-1.34)		0.91 (0.56-1.46)		0.93
	<i>Multivariable + carotenoids</i>	1.0 (referent)		0.73 (0.43-1.23)		0.71 (0.40-1.26)		0.95 (0.57-1.60)		0.57
FIOP_400	Cupoints, FI/mL	<57		57-65		65-78		78		
	No. cases	119/89		80/85		75/93		81/88		
	<i>Unadjusted</i>	1.0 (referent)		0.66 (0.42-1.02)		0.56 (0.36-0.87)		0.63 (0.41-0.98)		0.06
	<i>Multivariable</i>	1.0 (referent)		0.61 (0.38-0.99)		0.58 (0.36-0.94)		0.62 (0.37-1.03)		0.10
	<i>Multivariable + carotenoids</i>	1.0 (referent)		0.60 (0.35-1.02)		0.65-0.39-1.10)		0.69 (0.40-1.18)		0.30

Adjusted for: age at menarche, age at first birth/parity, family history of breast cancer, history of benign breast disease, alcohol consumption, smoking status, BMI at blood draw, physical activity

Table 3
Plasma fluorescent oxidative products and risk of ER negative breast cancer: NHS and NHSII, stratified results

	# cases/ controls	1st Quartile OR (95% CI)	2nd Quartile OR (95% CI)	3rd Quartile OR (95% CI)	4th Quartile OR (95% CI)	P _{trend}	P _{het}
FIOP_360							
BMI <25 kg/m ²	197/198	1.0 (referent)	0.72 (0.39-1.30)	0.63 (0.33-1.18)	0.40 (0.20-0.81)	0.01	0.23
BMI ≥25 kg/m ²	158/157	1.0 (referent)	1.62 (0.84-3.13)	0.99 (0.49-2.01)	1.10 (0.54-2.24)	0.93	
Age <50 years	97/99	1.0 (referent)	0.42 (0.15-1.14)	0.85 (0.31-2.27)	0.36 (0.12-1.08)	0.15	0.57
Age ≥50 years	258/256	1.0 (referent)	1.43 (0.87-2.35)	0.82 (0.47-1.41)	0.80 (0.46-1.40)	0.17	
Total carotenoids <111 µg/dL	165/154	1.0 (referent)	0.92 (0.50-1.71)	0.59 (0.29-1.20)	0.80 (0.35-1.82)	0.38	0.71
Total carotenoids ≥111 µg/dL	158/166	1.0 (referent)	1.92 (0.92-4.01)	1.43 (0.68-3.02)	0.99 (0.47-2.06)	0.47	
FIOP_320							
BMI <25 kg/m ²	197/198	1.0 (referent)	0.74 (0.40-1.37)	0.52 (0.28-0.96)	0.79 (0.43-1.48)	0.76	0.69
BMI ≥25 kg/m ²	158/157	1.0 (referent)	0.83 (0.42-1.62)	1.36 (0.67-2.77)	1.02 (0.52-2.00)	0.88	
Age <50 years	97/99	1.0 (referent)	0.26 (0.10-0.67)	0.39 (0.14-1.07)	0.62 (0.24-1.60)	0.95	0.56
Age ≥50 years	258/256	1.0 (referent)	1.12 (0.67-1.89)	0.95 (0.57-1.59)	1.12 (0.66-1.88)	0.72	
Total carotenoids <111 µg/dL	165/154	1.0 (referent)	0.83 (0.41-1.65)	0.77 (0.39-1.53)	1.40 (0.70-2.80)	0.21	0.31
Total carotenoids ≥111 µg/dL	158/166	1.0 (referent)	0.68 (0.34-1.35)	0.63 (0.31-1.26)	0.75 (0.37-1.53)	0.67	
FIOP_400							
BMI <25 kg/m ²	197/198	1.0 (referent)	0.47 (0.25-0.86)	0.40 (0.21-0.77)	0.42 (0.22-0.82)	0.02	0.37
BMI ≥25 kg/m ²	158/157	1.0 (referent)	0.79 (0.39-1.62)	0.74 (0.38-1.46)	0.96 (0.46-1.99)	0.88	
Age <50 years	97/99	1.0 (referent)	0.35 (0.13-0.96)	0.48 (0.18-1.31)	0.32 (0.11-0.98)	0.07	0.25
Age ≥50 years	258/256	1.0 (referent)	0.78 (0.46-1.31)	0.60 (0.36-1.02)	0.77 (0.45-1.32)	0.30	
Total carotenoids <111 µg/dL	165/154	1.0 (referent)	0.52 (0.26-1.05)	0.56 (0.27-1.15)	0.65 (0.30-1.39)	0.29	0.61
Total carotenoids ≥111 µg/dL	158/166	1.0 (referent)	0.81 (0.41-1.57)	0.79 (0.39-1.61)	0.77 (0.39-1.55)	0.49	

Adjusted for: age at blood collection, month/time of day of blood collection, fasting status, menopausal hormone use, menopausal status at diagnosis, age at menarche, age at first birth/parity, family history of breast cancer, history of benign breast disease, alcohol consumption, smoking status, BMI at blood draw, physical activity