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Plasma protein carbonyl levels and breast cancer risk

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Abstract

To study the role of oxidative stress in breast cancer risk, we analysed plasma levels of protein carbonyls in 1050 cases and 1107 controls. We found a statistically significant trend in breast cancer risk in relation to increasing quartiles of plasma protein carbonyl levels (OR=1.2, 95% CI=0.9–1.5; OR=1.5, 95% CI=1.2–2.0; OR=1.6, 95% CI=1.2–2.1, for the 2nd, 3rd and 4th quartile relative to the lowest quartile, respectively, *P* for trend=0.0001). The increase in risk was similar for younger (<50 years) and older women, more pronounced among women with higher physical activity levels (≥0.7 hrs/week for 4th quartile *versus* lowest quartile OR=2.0, 95% CI=1.4–3.0), higher alcohol consumption (≥15 grams/day for 4th quartile *versus* lowest quartile OR=2.3, 95% CI=1.1–4.7), and hormone replacement therapy use (HRT, OR=2.6, 95% CI=1.6–4.4 for 4th quartile *versus* lowest quartile). The multiplicative interaction terms were statistically significant only for physical activity and HRT. The positive association between plasma protein carbonyl levels and breast cancer risk was also observed when the analysis was restricted to women who had not received chemotherapy or radiation therapy prior to blood collection. Among controls, oxidized protein levels significantly increased with cigarette smoking and higher fruit and vegetable consumption, and decreased with alcohol consumption >30 grams per day. Women with higher levels of plasma protein carbonyl and urinary $15F_{2t}$-isoprostane had an 80% increase in breast cancer risk (OR=1.8, 95% CI=1.2–2.6) compared to women with levels below the median for both markers of oxidative stress. In summary, our results suggest that increased plasma protein carbonyl levels may be associated with breast cancer risk.

Keywords: oxidative stress • protein carbonyl • breast cancer

Introduction

Reactive oxygen species (ROS) are generated during normal cellular metabolism, as a result of the influence of various environmental factors, as well as during pathological processes [1]. ROS are responsible for DNA, lipid and protein damage and play an important role in the development and progression of many human diseases, including cancer [2]. Protein oxidation may result in structural changes and loss of function through peptide bond cleavage due to oxidation of the protein backbone, formation of protein–protein

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cross-linked derivatives or oxidation of amino acid side chains [3]. The oxidation of protein side chains occurs mainly on proline, arginine, lysine and threonine residues resulting in carbonyl formation [4]. Carbonyl groups formed during the oxidation are chemically stable which is useful for both in terms of detection and stability during sample storage [4].

Protein carbonyl levels are the most frequently used biomarker of protein oxidation and their accumulation has been observed with aging and in several human diseases, including cancer (reviewed in [5];[6]). Several methods of protein carbonyl groups detection are available [5], including a colorimetric assay based on derivatization of the carbonyl group with 2,4-dinitrophenylhydrazine (DNPH) [7]. A stable 2,4-nitrophenylhydrazone (DNP) product results that can be detected spectrophotometrically. Because this method is relatively time-consuming and requires higher amount of biological samples, Buss *et al.* [8] developed a highly sensitive enzyme-linked immunosorbent assay using anti-DNP antibodies. The assay is reproducible and correlates well with the classical colorimetric assay.

In the present study, we investigated the association of oxidative stress, measured as the levels of protein carbonyl groups in plasma, with breast cancer risk among women who participated in the Long Island Breast Cancer Study Project (LIBCSP). Among controls, we also determined the correlation of plasma protein carbonyl levels with age, average lifetime physical activity, fruit and vegetable intake, alcohol consumption, cigarette smoking, body mass index (BMI) and menopausal status, factors that may affect oxidative stress.

Materials and methods

Participants

The LIBCSP is a population-based study investigating the associations between environmental factors and breast cancer. Details of the case-control study design were described previously [9]. Briefly, eligible cases were women residing in Nassau or Suffolk counties, who spoke English, and were newly diagnosed with a first primary *in situ* or invasive breast cancer between August 1, 1996, and July 31, 1997. There were no age or race restrictions. Population-based controls were identified using random digit dialling (<65 years of age) and Health Care Financing Administration rosters (65+ years of age), and frequency matched to the expected age distribution of the cases by 5-year age group.

Covariate exposure assessment

The main questionnaire was administered by a trained interviewer in the participant's home and lasted approximately 2 hrs. Information was assessed on known and suspected risk factors for breast cancer, including passive and active cigarette smoking, lifetime alcohol use, menstrual and reproductive histories, hormone use, body size by decade of adult life, lifetime participation in recreational activities, prior medical history and family history of breast cancer (http://epi.grants.cancer.gov/LIBCSP/projects/ Questionnaire.html). Usual dietary intake in the year prior to the interview was assessed using a modified Block self-administered food frequency questionnaire, as previously described [10]. We also collected information on cancer treatment undergone prior to blood collection, including whether a participant had radiation and/or chemotherapy.

Of the eligible participants who completed the main questionnaire (1508 cases and 1556 controls), 73% of cases and 73% of controls donated a non-fasting blood samples at the time of the interview. The samples were donated on average within 3 months after breast cancer diagnosis, prior to the onset of chemotherapy for most case participants, but after surgery. For controls, the time lag between the identification and the interview was about 5.5 months. Blood samples, collected in ethylenediaminetetraacetic acid (EDTA)-treated tubes, were shipped at room temperature for overnight delivery to Columbia University for processing and storage. The samples were aliquoted and frozen at −80°C within 24 hrs of collection. In the present study, blood plasma samples from 1050 cases and 1107 controls were analysed.

Plasma protein carbonyl assay

The levels of plasma protein carbonyl groups were assessed using a non-competitive ELISA, essentially as described in [8], with some modifications [11]. Briefly, the oxidized protein standards were prepared by incubation of bovine serum albumin (BSA) (50 mg/ml) with 0.73 M H_2O_2 and 0.42 mM Fe^{2+} for 1 hr at 37°C. The reaction was stopped with 40 μM butylated hydroxytoluene. The carbonyl content of the oxidized BSA standard was measured spectrophotometrically [7]. It was then diluted with native (unoxidized) BSA and phosphate-buffered saline (PBS) to give a final carbonyl content of 2.0 nmol/mg protein and protein concentration of 4 mg/ml. Total protein concentration in the

plasma samples was measured using Bicinchoninic Acid Kit (Sigma) and the samples were diluted with PBS to a final protein concentration of 4 mg/ml. After the derivatization with DNPH, the plate was coated with 200 µl of sample (1 µg) and incubated overnight at 4°C in the dark. The plate was washed with PBS/Tween (0.05% Tween 20 in PBS) and blocked with 0.1% BSA in PBS for 1.5 hr. After another washing step, biotinylated primary anti-DNP antibody (Molecular Probes; diluted 1:1500 with 0.1% BSA, 0.1% Tween 20 in PBS) was added and the plate was incubated at 37°C for 1 hr. Another washing was followed by adding the streptavidin-biotynylated horseradish peroxidase conjugate (Amersham; diluted 1:4000 in 0.1% BSA, 0.1% Tween 20 in PBS) and incubation at room temperature for 1 hr. Colour was developed by adding the tetramethyl benzidine (TMB) liquid substrate system (Sigma) and the reaction was stopped with H_2SO_4 after 15–25 min incubation in the dark. The absorbance was measured with a microplate reader at 450 nm. Plasma protein carbonyl concentration was expressed as nmol carbonyl/ml plasma. Each sample was analysed in duplicate; the interassay coefficient of variability for the repeat analysis (N=53) of a quality control sample was 17%. As a quality control, a pooled plasma sample was repeatedly frozen and thawed before analysis; there was no significant difference in protein carbonyl levels with up to four freeze-thaw cycles.

Statistical analysis

The SAS statistical software (Version 9) was used to analyse these data. We first compared the differences between 1050 cases and 1107 controls with regard to the plasma protein carbonyl levels (nmol carbonyl/ml plasma). The Analysis of Variance Test [12] was used for comparing case-control differences as continuous variables and the chi-square Test [13] was used for comparing case-control differences. Quantiles of plasma protein carbonyl levels were created based on the distribution among controls. We also examined case-control differences using unconditional logistic regression [14] adjusting for reference age (date of diagnosis for cases and date of identification for controls). Other factors that were considered as possible confounders include those that have been associated with breast cancer and may also influence oxidative stress pathways, including average physical activity level, fruit and vegetable intake, average lifetime alcohol intake, active cigarette smoking status, BMI, menopausal status, age at menarche, age at first birth (AFB), parity, oral contraceptive (OC) use and hormone replacement therapy (HRT) use (using definitions that have been previously published [10,15–17]). Only those that changed the effect estimates by more than 10% were included in the final models. We stratified the association between plasma protein carbonyl levels and breast cancer by the same factors to examine effect modification on a multiplicative scale. We also examined the associations with the cases restricted to those women for whom chemotherapy (N=827) or radiation therapy (N=831) had not yet been initiated by the time of the blood collection.

We then assessed whether plasma protein carbonyl levels differed by age, average physical activity level, fruit and vegetable intake, average lifetime alcohol intake, active smoking status, BMI, menopausal status, age at menarche, AFB, parity, OC use and HRT use among 1107 controls first by using the Analysis of Variance Test and second by using linear regression methods [13]. We used log transformation for the plasma protein carbonyl levels in the linear regression model to meet the assumption of normality. We estimated a saturated model with all factors suspected to influence plasma protein carbonyl levels and a parsimonious model with other those variables which were statistically significantly related to the outcome.

Results

We observed a statistically significant trend in breast cancer risk in relation to increasing quartiles of plasma protein carbonyl levels (multivariate-adjusted OR = 1.2, 95% CI = 0.9–1.5; OR = 1.5, 95% CI = 1.2–2.0; OR = 1.6, 95% CI = 1.2-2.1 for the 2nd, 3rd and 4th quartile relative to the lowest quartile, respectively, *P* for trend = 0.0001) (Table 1). Estimates differed little between models with and without adjustment for other breast cancer risk factors. Among cases, there were no differences in plasma protein carbonyl level between those with or without chemotherapy or radiation therapy prior to blood collection. Considering plasma protein carbonyl levels categorized into other quartile groupings (tertiles, quintiles, deciles) yielded similar effect estimates (data not shown). Thus, after removal of the 214 women who had received chemotherapy and the 209 who had received radiation therapy, results did not materially change (OR = 1.1, 95% CI = 0.8–1.5; OR = 1.7, 95% CI =1.2–2.3; OR = 1.7, 95% CI 1.2–2.3 for the 2nd, 3rd and 4th quartile relative to the lowest quartile, respectively, *P* for trend = 0.0001). We also determined whether removal of women currently on HRT impacted results but again they did not materially change (OR = 1.0, 95% CI =0.7–1.4; OR = 1.4, 95% CI =1.0–2.0; OR = 1.3, 95% CI 0.9–1.8 for the 2nd, 3rd and 4th quartile relative to the lowest quartile, *P* for trend = 0.02). Removing those who

Table 1 Adjusted odds ratios and 95% confidence intervals for breast cancer associated with plasma protein carbonyl level

Protein carbonyl (nmol/ml)	Cases (%)	Controls (%)	Odds ratio* (95% CI)	Odds ratio** (95% CI)
<11.5	191 (18)	276 (25)	1.00	1.00
11.5–14.8	240 (23)	277 (25)	1.23 (0.96, 1.59)	1.18 (0.90, 1.55)
>14.8–19.2	304 (29)	276 (25)	1.58 (1.23, 2.02)	1.51 (1.16, 1.97)
>19.2	312 (30)	277 (25)	1.60 (1.25, 2.04)	1.60 (1.23, 2.09)
			P<0.0001	p = 0.0001

* Adjusted for age.
** Adjusted for age, average physical activity level, fruits and vegetables intake, average alcohol intake, active smoking status, menopausal status, BMI, ever use HRT, ever use OC, age at menarche, parity and age at first birth.

received chemotherapy and radiation therapy in addition to those on HRT resulted in ORs of 1.0 (95% CI 0.7–1.4), 1.6 (95%CI 1.1–2.3 and 1.4 (95%CI 0.9–2.0) for the 2nd, 3rd and 4th quartile relative to the lowest quartile (*P* for trend = 0.02).

The increase in breast cancer risk by quartile of plasma protein carbonyl level was similar for younger (<50 years) and older women or by fruits and vegetables consumption (Table 2). The association between plasma protein carbonyl level and breast cancer risk was more pronounced among women with higher physical activity levels (≥ 0.7 hrs/week for 4th quartile *versus* lowest quartile OR = 2.0, 95% CI = 1.4–3.0), with higher average lifetime alcohol consumption (≥ 15 grams/day for 4th quartile *versus* lowest quartile OR = 2.3, 95% CI = 1.1–4.9) and who used HRT (OR = 2.6, 95%CI = 1.6–4.4 for 4th quartile *versus* lowest quartile). But, the multiplicative interaction terms were statistically significant only for physical activity (*P*=0.02) and use of HRT (*P* =0.04).

To assess whether breast cancer risk is related to increased levels of markers of protein oxidation and lipid peroxidation, we compared plasma protein carbonyl levels and urinary 15F_{2t}-isoprostane levels (analysed in a subset of 400 cases and 401 controls in our previous study [18]). Women with higher levels of plasma protein carbonyl (≥ median) and higher levels of 15F_{2t}-isoprostane (≥ median) had a 1.8-fold increase in breast cancer risk (OR = 1.8, 95% CI = 1.2–2.6) compared to women with levels below the median for both markers of oxidative stress (Table 3).

In univariate analyses, among controls, plasma protein carbonyl levels significantly increased with age (*P*=0.02, Table 4), and were significantly higher in post-menopausal compared to pre-menopausal women (*P*=0.01) and in those with age of menarche <13 yrs (*P*<0.01). Contrary to our expectations, levels were also higher with increased fruits and vegetables intake (*P*=0.04). Similar trends were observed when fruits and vegetables were considered separately. No significant associations were found between plasma protein carbonyl levels and average physical activity level, average lifetime alcohol intake, cigarette smoking status, BMI and other reproductive variables.

Table 5 reports the findings from multivariate linear regression models examining the association between selected breast cancer risk factors and plasma protein carbonyl levels among controls. Higher average physical activity, higher consumption of alcohol and later age of menarche were associated with decreased plasma protein carbonyl levels. In the best-fitting model, higher fruit and vegetable intake and post-menopausal status were associated with increased plasma protein carbonyl levels and high alcohol consumption and later age of menarche with decreased levels.

Discussion

Protein oxidation, measured as plasma levels of protein carbonyl groups, was significantly associated with breast cancer risk in our study. Participants with the highest quartile of plasma protein carbonyl levels had a 60% increased risk of developing breast cancer compared to participants with plasma protein carbonyl levels in the lowest quartile. Several previous studies have provided evidence that protein oxidation might be associated with cancer risk. Increased levels of plasma protein carbonyl groups were observed in patients with Hodgkin's lymphoma [19], with bladder cancer [20] and in children with various malig-

Table 2 Breast cancer risk associated with plasma protein carbonyl level stratified by age, average physical activity level, fruits and vegetable intake, alcohol intake, cigarette smoking status, BMI, menopausal status, HRT and OC use, age of menarche and first birth and parity

		OR* (95% CI)			
		<11.5	**11.5–14.8**	**>14.8–19.2**	**>19.2**
		N=191/276[$]	N=240/277	N=304/276	N=312/277
Age (years)	<50	1.00	1.22 (0.74–2.02)	1.42 (0.87–2.33)	1.76 (1.07–2.90)
	≥50	1.00	1.17 (0.84–1.64)	1.56 (1.13–2.15)	1.52 (1.10–2.09)
Average physical activity level (hrs/week)	<0.7	1.00	1.43 (0.97–2.11)	1.41 (0.97–2.05)	1.29 (0.89–1.89)
	≥0.7	1.00	0.94 (0.63–1.39)	1.64 (1.11–2.43)	2.04 (1.39–2.99)
Fruits and vegetables intake (No. of $^1/_2$ cup serving/week)	<27	1.00	1.17 (0.78–1.77)	1.84 (1.22–2.78)	1.73 (1.14–2.64)
	≥27	1.00	1.13 (0.78–1.64)	1.29 (0.90–1.84)	1.50 (1.06–2.13)
Average lifetime alcohol intake (gram/day)	Non-drinkers	1.00	0.98 (0.62–1.55)	1.33 (0.85–2.07)	1.32 (0.85–2.05)
	<15	1.00	1.39 (0.93–2.07)	1.57 (1.06–2.32)	1.74 (1.17–2.57)
	≥15	1.00	1.14 (0.54–2.40)	1.82 (0.88–3.77)	2.30 (1.09–4.85)
Cigarette smoking status	Never	1.00	1.34 (0.90–2.01)	1.62 (1.09–2.39)	2.02 (1.37–2.97)
	Current	1.00	1.08 (0.56–2.07)	1.27 (0.66–2.36)	1.71 (0.86–3.32)
	Former	1.00	1.06 (0.65–1.72)	1.57 (0.98–2.52)	1.20 (0.75–1.91)
BMI (kg/m^2)	<25	1.00	1.05 (0.70–1.57)	1.47 (0.99–2.18)	1.72 (1.16–2.55)
	≥25	1.00	1.37 (0.94–2.01)	1.55 (1.07–2.25)	1.60 (1.10–2.32)
Menopausal status	Pre-menopausal	1.00	1.21 (0.76–1.93)	1.74 (1.09–2.75)	1.87 (1.17–3.00)
	Post-menopausal	1.00	1.14 (0.81–1.61)	1.39 (0.99–1.94)	1.49 (1.07–2.07)
Ever use HRT	Non-users	1.00	1.00 (0.72–1.38)	1.42 (1.03–1.96)	1.30 (0.94–1.78)
	Users	1.00	1.75 (1.02–3.02)	1.56 (0.94–2.58)	2.64 (1.59–4.40)
Ever use OC	Non-users	1.00	1.50 (1.01–2.21)	1.39 (0.95–2.01)	1.79 (1.23–2.60)
	Users	1.00	0.93 (0.63–1.38)	1.69 (1.15–2.50)	1.43 (0.97–2.11)
Age at menarche	<13 yrs	1.00	1.12 (0.76–1.65)	1.32 (0.91–1.89)	1.41 (0.98–2.04)
	≥13 yrs	1.00	1.23 (0.83–1.83)	1.86 (1.24–2.78)	1.86 (1.24–2.79)
Parity	Nulliparous	1.00	1.41 (0.59–3.36)	1.29 (0.54–3.07)	2.31 (1.02–5.21)
	Parous	1.00	1.18 (0.88–1.58)	1.56 (1.18–2.07)	1.55 (1.17–2.06)
Age at first birth	<25 yrs	1.00	1.23 (0.82–1.84)	1.67 (1.13–2.47)	1.21 (0.82–1.79)
	≥25 yrs	1.00	1.15 (0.74–1.78)	1.50 (0.98–2.28)	2.12 (1.37–3.26)

*Adjusted for age, average physical activity level, fruits and vegetables intake, average alcohol intake, active smoking status, menopausal status, BMI, ever use HRT, ever use OC, age at menarche, parity and age at first birth.
[$] N=cases/controls.

Table 3 Breast cancer risk associated with the 15-F_{2t}-isoprostane and plasma protein carbonyl levels

15F_{2t}isoprostane	Carbonyl	Cases (%)	Controls (%)	OR (95% CI)*
<median	<median	73 (18)	92 (23)	1.00
≥median	<median	80 (21)	107 (27)	0.94 (0.62, 1.44)
<median	≥median	88 (22)	98 (25)	1.07 (0.70, 1.63)
≥median	≥median	154 (39)	103 (26)	1.76 (1.19, 2.62)
				P = 0.002

* Adjusted for age.

nancies [21], but not in a small study of lung cancer [22]. To the best of our knowledge, our study is the first analysis of the relationship between protein oxidation and breast cancer risk. Also, our study completes the investigation of a panel of oxidative stress markers among the participants of LIBCSP [18]. According to a recent report [23], it is critical to simultaneously analyse several markers of oxidative stress to better understand the processes leading to the particular pathology being studied.

The process of aging is accompanied by increasing levels of oxidative damage, including protein oxidation (reviewed in [3]). Increased levels of protein carbonyls have been observed in elderly participants as compared with younger participants in some [24], [25] but not all [26] studies. We also found increased plasma protein carbonyl levels in older women (Table 4).

In the multivariate analysis, higher physical activity was associated with decreased plasma protein carbonyl levels (Table 5). In general, physical exercise can produce an imbalance between ROS and antioxidants and thus lead to oxidative stress [27], but the results of different studies are conflicting. Decreased plasma protein carbonyl levels were found in participants after strenuous physical exercise [28] and in sedentary participants as a result of physical exercise [29], but levels increased in participants in a super-marathon race [30]. Thus, the biological meaning of our observation is not clear; however, it is in agreement with other studies.

Fruits and vegetables contain both antioxidant and pro-oxidant factors so their effect on oxidative stress markers may be variable. Decreased levels of various oxidative stress markers have been observed after administration of fruits and vegetables-rich food in several studies [31–33]. Analysis of data from the LIBCSP showed that higher fruits and vegetables consumption was associated with significantly decreased breast cancer risk among post-menopausal women (OR 0.72, 95% CI 0.53–0.99 for the highest quintile compared with the lowest) with weaker associations among pre-menopausal [10]. Contrary to this data, we observed significantly increased plasma protein carbonyl levels among participants with higher fruits and vegetables consumption (Table 4& 5). Similar results were observed by Dragsted *et al.* [34] in an intervention study where participants receiving a diet rich in fruits and vegetables, or a pill containing vitamins and minerals corresponding to those in the fruits and vegetables diet, had higher protein carbonyl formation at lysine residues than those on placebo. They hypothesized that the pro-oxidant activity of vitamin C might be responsible for their findings. Thus fruits and vegetables may protect against breast cancer by multiple pathways.

Alcohol induces oxidative stress due to the metabolism of ethanol. Three pathways of ethanol metabolism have been described and each results in free radical production leading to oxidative damage [35]. Several studies have observed increased protein carbonyl levels among patients with chronic alcoholism [36–38] suggesting that repeated exposure to high alcohol levels may be necessary to affect protein carbonyl levels. Lower levels of oxidative stress markers were reported among moderate alcohol drinkers [39, 40], but these studies concentrated on white and red wine drinkers. It is probable that the presence of antioxidants in wine diminishes the negative effect of ethanol on oxidative stress markers. In the present study, we saw significantly lower plasma protein carbonyl levels with elevated alcohol intake. This may reflect the type of alcoholic beverages consumed by the participants in our study (prevalence of wines rich in antioxidants), as well as relatively low levels of alcohol exposure that were not sufficient to

Table 4 Mean (and SD) of plasma protein carbonyl levels (continuous) within strata of age, average physical activity level, fruits and vegetables intake, average lifetime alcohol intake, cigarette smoking status, body mass index (BMI) and menopausal status, ever use HRT, ever use OC, age at menarche, parity and age at first birth among controls

Risk factor		N	Carbonyl	P value*	Log-Carbonyl	P value*
Age (years)	20–39	102	14.9±7.0		2.58±0.55	
	40–49	266	16.2±12.4		2.66±0.48	
	50–59	315	16.6±6.6		2.73±0.44	
	60+	424	15.7±5.9	0.25	2.68±0.40	0.02
Average physical activity level (hrs/week)	0	276	16.4±6.4		2.72±0.40	
	<0.69	243	16.8±12.8		2.69±0.51	
	0.70–2.6	263	15.5±6.4		2.67±0.39	
	2.7+	257	15.6±6.6	0.26	2.64±0.49	0.27
Fruits and vegetables intake (No. of # cup serving/week)	0–18	246	16.2±12.9		2.63±0.58	
	19–26	220	14.9±5.7		2.63±0.41	
	27–34	197	16.1±6.8		2.70±0.40	
	35–46	220	16.2±6.1		2.72±0.38	
	47+	208	16.6±6.5	0.29	2.73±0.41	0.04
Average lifetime alcohol intake (gram/day)	Non-drinkers	403	16.1±6.2		2.70±0.42	
	<15	545	16.0±9.7		2.67±0.45	
	15–30	84	16.4±7.5		2.70±0.44	
	30+	74	14.6±5.9	0.49	2.57±0.58	0.10
Cigarette smoking status	Never	501	15.7± 6.5		2.65±0.48	
	Current	208	16.6± 13.2		2.69±0.45	
	Former	396	16.2±6.5	0.37	2.71±0.39	0.18
BMI (kg/m^2)	<25	548	16.0±9.7		2.67±0.48	
	≥25	544	16.1±6.4	0.88	2.70±0.42	0.25
Menopausal Status	Pre-menopausal	373	15.5±7.0		2.64±0.49	
	Post-menopausal	688	16.4±8.9	0.10	2.71±0.42	0.01
Ever use HRT	Non-users	799	16.1±8.8		2.69±0.45	
	Users	307	15.7±6.4	0.38	2.66±0.44	0.47
Ever use OC	Non-users	562	15.9±6.0		2.70±0.39	
	Users	544	16.1±10.0	0.68	2.66±0.50	0.24
Age at menarche	<13 yrs	608	16.4±6.4		2.72±0.41	
	≥13 yrs	491	15.6 ± 10.0	0.10	2.63±0.49	<0.01
Parity	Nulliparous	123	16.2±6.4		2.70±0.40	
	Parous	983	16.0±8.4	0.84	2.68±0.45	0.53
Age at first birth	<25 yrs	553	16.0±6.3		2.69±0.43	
	≥25 yrs	430	16.0±10.5	0.90	2.66±0.47	0.26

* Comparing differences between mean values by category.

Table 5 Multivariate estimates of the association among age, average physical activity level, fruit and vegetable intake, average lifetime alcohol intake, cigarette smoking status, body mass index (BMI), menopausal status, ever used HRT, ever used OC, age at menarche, parity, and age at first birth and the log of plasma protein carbonyl levels among controls

Risk factor		β (95% CI)*	β (95% CI)**	β (95% CI)***
Age (years)	20–39	Reference	Reference	
	40–49	0.09 (-0.02, 0.19)	0.09 (-0.02, 0.20)	
	50–59	0.15 (0.05, 0.25)	0.07 (-0.07, 0.20)	
	≥60	0.11 (0.01, 0.20)	0.01 (-0.14, 0.16)	
Average physical activity level (hrs/week)	0	Reference	Reference	
	<0.69	-0.02 (-0.10, 0.05)	-0.02 (-0.10, 0.06)	
	0.70–2.6	-0.04 (-0.12, 0.03)	-0.06 (-0.14, 0.02)	
	≥2.7	-0.07 (-0.15, 0.01)	-0.09 (-0.17, -0.004)	
Fruits and vegetables intake (No. of $^1/_2$ cup serving/week)	0–18	Reference	Reference	Reference
	19–26	-0.003 (-0.08, 0.08)	-0.01 (-0.10, 0.08)	-0.01 (-0.10, 0.07)
	27–34	0.07 (-0.02, 0.15)	0.07 (-0.03, 0.16)	0.06 (-0.03, 0.15)
	35–46	0.08 (-0.002, 0.16)	0.11 (0.02, 0.20)	0.08 (-0.001, 0.17)
	≥47	0.09 (0.01, 0.18)	0.12 (0.02, 0.21)	0.09 (0.005, 0.18)
Average lifetime alcohol intake (gram/day)	Non-drinkers	Reference	Reference	Reference
	<15	-0.02 (-0.08, 0.03)	-0.02 (-0.08, 0.04)	-0.03 (-0.09, 0.03)
	15–30	0.004 (-0.10, 0.11)	0.01 (-0.10, 0.13)	-0.02 (-0.09, 0.13)
	≥30	-0.13 (-0.24, -0.02)	-0.14 (-0.27, -0.01)	-0.12 (-0.24, -0.005)
Cigarette smoking status	Never	Reference	Reference	Reference
	Current	0.05 (-0.03, 0.12)	0.05 (-0.03, 0.14)	0.06 (-0.02, 0.14)
	Former	0.05 (-0.01, 0.11)	0.07 (0.003, 0.14)	0.05 (-0.01, 0.12)
BMI (kg/m^2)	<25	Reference	Reference	
	≥25	0.03 (-0.03, 0.08)	0.01 (-0.05, 0.06)	
Menopausal status	Pre-menopausal	Reference	Reference	Reference
	Post-menopausal	0.10 (0.02, 0.18)	0.09 (-0.01, 0.18)	0.11 (0.02, 0.20)
Ever use HRT	Non-users	Reference	Reference	Reference
	Users	-0.03 (-0.09, 0.03)	-0.06 (-0.12, 0.01)	-0.06 (-0.12, 0.01)
Ever use OC	Non-users	Reference	Reference	
	Users	-0.02 (-0.08, 0.04)	-0.01 (-0.07, 0.06)	
Age at menarche	<13 yrs	Reference	Reference	Reference
	≥13 yrs	-0.09 (-0.14, -0.04)	-0.08 (-0.14, -0.02)	-0.08 (-0.13, -0.02)
Parity	Nulliparous	Reference	Reference	
	Parous	-0.04 (-0.12, 0.05)	-0.05 (-0.14, 0.05)	
Age at first birth	<25 yrs	Reference	Reference	
	≥25 yrs	-0.03 (-0.09, 0.03)	-0.03 (-0.10, 0.03)	

* Adjusted for age.
** Adjusted for age, average physical activity level, fruits and vegetables intake, average lifetime alcohol intake, active smoking status, body mass index, menopausal status, ever use HRT, ever use OC, age at menarche, parity and age at first birth.
*** Best-fitting model.

increase oxidative stress even in the group of women consuming >30 g alcohol/day.

Significantly increased levels of oxidized proteins in smokers than in non-smokers were observed [22] with similar, but non-significant results in another study [11]. We found a significant increase in plasma protein carbonyl levels among former smokers compared with non-smokers (Table 5). We observed no modification by BMI, parity or AFB. Plasma protein carbonyl levels were significantly lower in those with older age at menarche. Shorter life-time exposure to estrogen as a result of late menarche may decrease oxidative stress induced by estrogen metabolism.

In our previous study [18], we observed an association between urinary 15-F_{2t}-isoproprostane, a marker of lipid peroxidation, and breast cancer risk in a subset of women analysed in the present study. The combination of elevated ($\geq$ median) plasma protein carbonyl and 15-F_{2t}-isoproprostane levels resulted in a 1.8-fold increase in breast cancer risk compared to women with levels below the median for both markers of oxidative stress. Analysis of a combination of several oxidative stress markers may provide more relevant information about risk than any one measure.

A limitation of our study is that the blood samples were shipped at room temperature after collection and haemolysis may have contributed to additional oxidative stress. However, all samples were handled the same way and thus, time between collection of samples and their processing cannot account for the differences between cases and controls. Another limitation is that samples were collected after breast cancer diagnosis. Although extensive efforts were undertaken to obtain the samples prior to the onset of chemotherapy and radiation therapy for most case participants, all breast cancer cases had undergone surgery before blood draw [9]. It has been shown that surgery results in significant decrease of ROS production and levels of oxidative stress markers, including protein carbonyl levels in colon [41] and laryngeal cancer patients [42]. Moreover, total plasma antioxidant capacity increases after a tumour removal [43]. In our study, we observed no effect of non-surgical treatment on carbonyl level and when we restricted our analyses to case participants who had not received chemotherapy and radiation therapy prior to blood collection, our results did not change substantially. A prospective study using samples collected before diagnosis may be more useful for determining if elevated levels of protein oxidation predict breast cancer risk. A major strength of our study is the large number of participants, which helped to ensure that the study had sufficient power. In addition, the population-based study design ensured that cases and controls arose from the same source population.

In summary, we found positive associations between plasma protein carbonyl levels and breast cancer risk in this population-based, case-control study. Among controls, oxidized protein levels significantly increased with cigarette smoking and, unexpectedly, with higher fruit and vegetable consumption but decreased with alcohol consumption >30 grams per day. Elevation in two oxidative stress biomarkers, urinary $15F_{2t}$-isoprostanes and oxidized protein was associated with increased breast cancer risk. Our results add additional support to the hypothesis that oxidative stress may play a role in breast cancer etiology.

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