

# Relationship between Urinary 15-F<sub>2t</sub>-Isoprostane and 8-Oxodeoxyguanosine Levels and Breast Cancer Risk

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## Abstract

To evaluate the role of oxidative stress in breast cancer, we measured urinary levels of 15-F<sub>2t</sub>-isoprostane (15-F<sub>2t</sub>-IsoP) and 8-oxodeoxyguanosine (8-oxodG) in 400 cases and 401 controls, participants of the Long Island Breast Cancer Study Project. We also analyzed the effect of different factors that are associated with oxidative stress and might influence 15-F<sub>2t</sub>-IsoP and 8-oxodG levels. We observed a statistically significant trend in breast cancer risk with increasing quartiles of 15-F<sub>2t</sub>-IsoP levels [odds ratio (OR), 1.25; 95% confidence interval (95% CI), 0.81-1.94; OR, 1.53; 95% CI, 0.99-2.35; OR, 1.88; 95% CI, 1.23-2.88, for the 2nd, 3rd, and 4th quartile relative to the lowest quartile, respectively;  $P_{\text{trend}} = 0.002$ ]. Although it is possible that increased levels may reflect the stress associated with recent treatment, the positive association was also observed when the analyses were restricted to case women for whom chemotherapy and radiation therapy had not yet been initiated at the time of the urine collection. The association

with the highest quartile compared with lowest quartile of 15-F<sub>2t</sub>-IsoP was similar across strata of age, physical activity, fruit and vegetable intake, alcohol intake, cigarette smoking, body mass index, and menopausal status. We did not observe any association of breast cancer risk with 8-oxodG levels, but when cases with radiation treatment were removed from the analysis, a significant inverse trend ( $P = 0.04$ ) was observed. Among controls, levels of 15-F<sub>2t</sub>-IsoP were higher among current cigarette smokers but did not differ by the amount of physical activity, fruit and vegetable intake, alcohol intake, body mass index, and menopausal status. Among controls, levels of 8-oxodG were higher among postmenopausal women and current and former cigarette smokers but did not differ by the other factors. In summary, our results suggest that urinary markers of lipid peroxidation and oxidative DNA damage may be associated with breast cancer risk. (Cancer Epidemiol Biomarkers Prev 2006;15(4):639-44)

## Introduction

Excessive generation of reactive oxygen species results in oxidative stress that plays a role in a number of pathologic processes including cancer, cardiovascular and neurodegenerative diseases, as well as aging. Reactive oxygen species include superoxide radicals, hydrogen peroxide, and hydroxyl radicals and can be produced by both endogenous and exogenous sources (1). Endogenous sources include normal physiologic processes, such as oxidative phosphorylation, P450 metabolism, including metabolism of estrogens, peroxisomes, and inflammatory cell activation. Various environmental factors, such as cigarette smoking or diet, represent exogenous sources of reactive oxygen species. In addition, alcohol drinking may cause oxidative DNA damage through its metabolism via cytochrome CYP450 2E1 and resulting generation of reactive oxygen species (1). On the other hand, it is believed that higher fruit and vegetable consumption results in decreased oxidative stress due to the presence of antioxidants (2).

Oxidative stress, mediated by reactive oxygen species, may result in direct DNA damage as well as in lipid peroxidation,

protein modification, membrane disruption, and mitochondrial damage (1). Several markers of lipid peroxidation are currently available. Among them, F<sub>2</sub>-isoprostanes are used extensively as clinical markers of human diseases. F<sub>2</sub>-isoprostanes are compounds derived from arachidonic acid via a free radical-catalyzed mechanism. Several groups of F<sub>2</sub>-isoprostanes have been described. However, most studies have focused on the biological activity of 15-F<sub>2t</sub>-isoprostane (15-F<sub>2t</sub>-IsoP; for a review, see ref. 3). Isoprostanes are initially generated from cell membrane-bound arachidonic acid by free radical attack. They are cleaved from the sites of their origin presumably by phospholipases and then circulate in plasma and are excreted in urine (4). F<sub>2</sub>-isoprostanes can be detected in biological fluids, such as urine, blood plasma, bronchoalveolar lavage, or cerebrospinal fluid, as well as in tissues. The main advantage of urinary measurements of F<sub>2</sub>-isoprostanes is that the compounds are very stable and are not formed *ex vivo* (5). On the other hand, in blood plasma and tissues, auto-oxidation may occur. Several methods are available for F<sub>2</sub>-isoprostane detection including gas chromatography-mass spectroscopy and liquid chromatography-mass spectroscopy. However, a reliable ELISA technique suitable for analysis of large numbers of samples has recently been developed (6).

8-Oxodeoxyguanosine (8-oxodG) is the most abundant DNA lesion caused by reactive oxygen species. It is highly mutagenic resulting in GC to TA transversions. After cleavage from DNA as a result of DNA repair, 8-oxodG is excreted in urine. Another significant source of extracellular 8-oxodG may be oxidation of the nucleotide pool (7). Urinary 8-oxodG levels are therefore considered a general biomarker of oxidative stress. Because increased levels were observed among patients with small-cell lung cancer, bladder cancer, and prostate cancer, 8-oxodG may be useful as an indicator of cancer risk

Received 7/27/05; revised 1/11/06; accepted 1/30/06.

**Grant support:** Grants U01 CA/ES66572, P30ES09089, P30ES10126, and K07CA90685-02 from the National Cancer Institute and the National Institute of Environmental Health Sciences, awards from the Breast Cancer Research Foundation and Women at Risk Program, and gifts from private citizens.

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doi:10.1158/1055-9965.EPI-05-0554

(reviewed in ref. 8). Methods used for 8-oxodG detection include high-performance liquid chromatography, tandem mass spectrometry, and a recently developed competitive ELISA (9).

The purpose of our study was to examine the role of oxidative stress, measured by urinary 15-F<sub>2t</sub>-IsoP and 8-oxodG levels, in breast cancer risk using data collected as part of the Long Island Breast Cancer Study Project (10). We also explored, among women without breast cancer, whether urinary 15-F<sub>2t</sub>-IsoP and 8-oxodG levels were associated with age of subject, fruit and vegetable intake, alcohol consumption, cigarette smoking, body mass index (BMI), and menopausal status.

## Materials and Methods

**Subjects.** The Long Island Breast Cancer Study Project is a population-based case-control study investigating environmental factors and other known and suspected risk factors for breast cancer. Details of the study design were previously described (10). Briefly, eligible cases were women residing in Nassau or Suffolk counties, who spoke English, and were newly diagnosed with *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 dialing (<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 ~2 hours. 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.<sup>7</sup> Usual dietary intake in the year before the interview was assessed using a modified Block self-administered food frequency questionnaire as previously described (11). Approximately 50% of cases and controls took antioxidant vitamins on a routine basis (11). At the time of urine collection, a self-administered checklist collected information on cruciferous vegetable consumption, smoking, and coffee and alcohol drinking within the prior 48 hours. In addition, information on use of hormone replacement therapy and tamoxifen in the prior 48 hours was collected. We also collected information on cancer treatment undergone before urine collection, including whether a subject had radiation and/or chemotherapy.

Of the eligible subjects who completed the main questionnaire (1,508 cases and 1,556 controls), 93% of cases and 83% of controls donated single, spot urine samples at the time of the interview. The samples were donated on average within 3 months after breast cancer diagnosis before the onset of chemotherapy for most case subjects, but after surgery. For controls, the time lag between the identification and the interview was ~5.5 months. Urines were shipped on chill packs for overnight delivery to Columbia University for processing and storage. The samples were aliquoted and frozen at -80°C within 24 hours of collection. For the present study, 400 cases and 401 controls were randomly selected for the analyses. Study subjects for whom the data on 15-F<sub>2t</sub>-IsoP and 8-oxodG were available (*n* = 801) did not differ from the entire group of study participants with regard to known risk factors of breast cancer, except for being younger (57 ± 13 versus 58 ± 13 years; *P* = 0.01).

**15-F<sub>2t</sub>-IsoP Immunoassay.** Urinary 15-F<sub>2t</sub>-IsoP levels were analyzed using immunoassay kits from Oxford Biomedical Research (Oxford, MI). Urine samples without vitamin C added were thawed to room temperature and diluted 2× using the dilution buffer provided with the kit. Analysis was done according to the recommendations of the manufacturer. Each sample was analyzed in duplicate; the interassay coefficient of variability for the repeat analysis (*n* = 21) of a quality control sample was 8.5%. Urinary levels are dependent on the hydration status of the subject. Thus, to normalize 15-F<sub>2t</sub>-IsoP levels, urinary creatinine was assayed using a creatinine kit from Sigma (St. Louis, MO). 15-F<sub>2t</sub>-IsoP concentration was then expressed as nmol 15-F<sub>2t</sub>-IsoP/mmol creatinine.

**8-OxodG ELISA.** Urinary 8-oxodG levels were analyzed by competitive ELISA essentially as previously described (12). Wells were coated with 5 ng of 8-oxoG conjugated with bovine serum albumin (total volume, 50 μL/well) by drying the plates overnight at 37°C. They were washed with PBS/Tween (0.05% Tween 20 in PBS) and blocked with 200 μL/well of blocking buffer (1% FCS in PBS/Tween) for 1 hour at 37°C. After blocking, 50 μL of 8-oxodG standards (concentration range, 5-80 ng/mL) and urine samples (diluted 1:1 with PBS) were added followed by 50 μL of primary antibody 1F7 (ref. 12; diluted 1:100 in blocking buffer). This antibody recognizes both 8-oxodG and 8-oxoG with similar sensitivities. Because the standard curve is generated using 8-oxodG, values are expressed as equivalents of 8-oxodG that would cause a similar inhibition in the assay. After incubation for 1.5 hours at 37°C and washing, 50 μL of secondary antibody conjugated with alkaline phosphatase were added. Another 1.5-hour incubation at 37°C was followed by washing with PBS/Tween and with 0.01% diethanolamine in water. The color was developed by adding 100 μL of *p*-nitrophenyl phosphate substrate (1 mg/mL of 1 mol/L diethanolamine) per well and incubating the plates for 30 minutes at 37°C. The absorbance was measured with a microplate reader at 405 nm. Urinary 8-oxodG concentration was expressed as nmol 8-oxodG/mmol creatinine. Any samples with inhibition <20% or >80% were repeat analyzed either without dilution or with further dilution, respectively. Each sample was analyzed in duplicate; the interassay coefficient of variability for the repeat analysis (*n* = 23) of a quality control sample was 25%.

**Statistical Analysis.** We first compared the differences between 400 cases and 401 controls with regard to the levels of 15-F<sub>2t</sub>-IsoP and 8-oxodG (nmol/mmol creatinine). The ANOVA test (13) was used for comparing case-control differences as continuous variables and the  $\chi^2$  test (14) was used for comparing case-control differences as categorical variables. Categories for 15-F<sub>2t</sub>-IsoP and 8-oxodG levels were created based on the quartiles among controls. We also examined case-control differences using unconditional logistic regression (15) adjusting for reference age (date of diagnosis for cases and identification for controls) and other factors that have been associated with breast cancer risk and may also influence oxidative stress pathways (average physical activity level, fruit and vegetable intake, average lifetime alcohol intake, active cigarette smoking status, BMI, and menopausal status). We stratified the association between urinary levels and breast cancer by age, fruit and vegetable intake, average lifetime alcohol intake, cigarette smoking, and BMI to examine effect modification by these factors using definitions that have been previously published (11, 16-18). We also examined the associations with the cases restricted to those women for whom chemotherapy or radiation therapy had not yet been initiated by the time of the urine collection.

We then assessed whether 15-F<sub>2t</sub>-IsoP and 8-oxodG levels differed by age, average physical activity level, fruit and vegetable intake, average lifetime alcohol intake, and active smoking status among 401 controls first by using the ANOVA

<sup>7</sup> <http://epi.grants.cancer.gov/LIBCSP/projects/Questionnaire.html>.

**Table 1. Adjusted ORs and 95% CIs for breast cancer associated with 15-F<sub>2t</sub>-IsoP and 8-oxodG levels in urine**

Risk factor	Cases (%)	Controls (%)	Age-adjusted OR* (95% CI)	Multivariable-adjusted OR <sup>†</sup> (95% CI)
<b>15-F<sub>2t</sub>-IsoP (nmol/mmol creatinine)</b>				
<0.45	68 (17)	100 (25)	1.00	1.00
0.45-0.64	88 (22)	100 (25)	1.26 (0.82-1.92)	1.25 (0.81-1.94)
0.64-0.99	118 (30)	100 (25)	1.59 (1.05-2.41)	1.53 (0.99-2.35)
≥0.99	126 (31)	101 (25)	1.69 (1.12-2.55)	1.88 (1.23-2.88)
			<i>P</i> = 0.007	<i>P</i> = 0.002
<b>8-OxodG (nmol/mmol creatinine)</b>				
<18.85	107 (27)	99 (25)	1.00	1.00
18.85-27.92	111 (28)	100 (25)	1.01 (0.69-1.49)	0.99 (0.64-1.51)
27.92-38.28	92 (23)	100 (25)	0.80 (0.54-1.19)	0.75 (0.48-1.16)
≥38.28	90 (22)	100 (25)	0.79 (0.53-1.18)	0.79 (0.51-1.22)
			<i>P</i> = 0.15	<i>P</i> = 0.16

\*Adjusted for reference age.

<sup>†</sup> For 15-F<sub>2t</sub>-IsoP, the model was adjusted for age, average physical activity level and BMI; for 8-oxodG, the model was adjusted for age, average physical activity level, fruit and vegetable intake, average lifetime alcohol intake, active smoking status, menopausal status, and BMI.

test and second by using linear regression methods (14). We used log transformation for the 15-F<sub>2t</sub>-IsoP or 8-oxodG levels in the linear regression model because this transformation resulted in a more normal, less skewed distribution of the dependent variable. We did not do this transformation when 15-F<sub>2t</sub>-IsoP or 8-oxodG was used as predictor (Tables 1 and 2) because such an assumption is not needed for independent variables. The SAS statistical software (version 9) was used to analyze these data.

## Results

We observed a statistically significant trend in the risk of breast cancer in relation to increasing quartile of 15-F<sub>2t</sub>-IsoP levels [multivariable-adjusted odds ratio (OR), 1.25; 95% confidence interval (95% CI), 0.81-1.94; OR, 1.53; 95% CI, 0.99-2.35; OR, 1.88; 95% CI, 1.23-2.88, for the 2nd, 3rd, and 4th quartile relative to the lowest, respectively; *P*<sub>trend</sub> = 0.002; Table 1]. Results were similar when the 66 women with *in situ* disease were removed from the analysis. To determine whether increased 15-F<sub>2t</sub>-IsoP levels among women with breast cancer are the result of

chemotherapy, we restricted the analysis to the 318 case women who had not received chemotherapy treatment. The results did not materially change, indicating that increased 15-F<sub>2t</sub>-IsoP levels were not chemotherapy related (OR, 1.29; 95% CI, 0.78-2.14; OR, 1.69; 95% CI, 1.03-2.78; OR, 1.84; 95% CI, 1.12-3.04, for the 2nd, 3rd, and 4th quartile relative to the lowest, respectively; *P*<sub>trend</sub> = 0.01). Radiation treatment had been received by 77 cases at some point before providing biospecimens. These women have significantly higher 15-F<sub>2t</sub>-IsoP levels than cases without radiation treatment (mean ± SD, 1.33 ± 1.97 and 0.98 ± 0.85, respectively; *P* = 0.02). When these women were removed from the analysis, the significant trend in risk in relation to increasing quartile of 15-F<sub>2t</sub>-IsoP levels remained (multivariable-adjusted OR, 1.27; 95% CI, 0.78-2.05; OR, 1.60; 95% CI, 0.99-2.58; OR, 1.80; 95% CI, 1.11-2.92, for the 2nd, 3rd, and 4th quartile relative to the lowest, respectively; *P*<sub>trend</sub> = 0.01).

The association of 15-F<sub>2t</sub>-IsoP levels with breast cancer was seen in both younger women (<50 years; OR, 1.57; 95% CI, 0.77-2.05, for the highest quartile relative to the lowest), as well as older women (≥50 years; OR, 2.35; 95% CI, 1.36-4.07, for the highest quartile relative to the lowest), although this association

**Table 2. Multivariable-adjusted Odds Ratios and 95% Confidence Intervals for breast cancer risk associated with urinary levels of 15-F<sub>2t</sub>-IsoP stratified by age, average physical activity level, fruit and vegetable intake, average lifetime alcohol intake, cigarette smoking status, BMI, and menopausal status**

	Multivariable-adjusted* ORs (95% CIs), by quartile of 15-F <sub>2t</sub> -IsoP (nmol/mmol creatinine)			
	<0.45	0.45-0.64	0.64-0.99	≥0.99
<b>Age (y)</b>				
<50	1.00	1.17 (0.60-2.26)	0.98 (0.47-2.0)	1.57 (0.77-2.05)
>50	1.00	1.38 (0.76-2.50)	2.06 (1.19-3.56)	2.35 (1.36-4.07)
<b>Average physical activity level (h/wk)</b>				
<0.7	1.00	1.25 (0.67-2.30)	1.48 (0.82-2.68)	1.55 (0.84-2.85)
>0.7	1.00	1.11 (0.59-2.11)	1.51 (0.80-2.84)	2.19 (1.20-3.99)
<b>Fruit and vegetable intake (no. 1/2 cup serving/wk)</b>				
<27	1.00	1.07 (0.56-2.06)	0.88 (0.46-1.68)	1.87 (0.98-3.55)
>27	1.00	1.40 (0.76-2.58)	2.52 (1.37-4.63)	2.04 (1.13-3.69)
<b>Average lifetime alcohol intake (g/d)</b>				
Nondrinkers	1.00	0.92 (0.45-1.87)	1.61 (0.80-3.22)	1.48 (0.76-2.86)
<15	1.00	1.49 (0.79-2.80)	1.35 (0.72-2.52)	2.13 (1.11-4.06)
>15	1.00	1.72 (0.44-6.77)	1.99 (0.52-7.61)	2.06 (0.54-7.90)
<b>Active cigarette smoking status</b>				
Never	1.00	1.49 (0.81-2.75)	1.59 (0.85-2.97)	2.29 (1.22-4.29)
Current	1.00	1.08 (0.27-4.29)	1.82 (0.53-6.28)	2.66 (0.78-9.11)
Former	1.00	1.08 (0.51-2.30)	1.48 (0.70-3.14)	1.27 (0.60-2.70)
<b>BMI (kg/m<sup>2</sup>)</b>				
<25	1.00	0.99 (0.51-1.93)	1.99 (1.02-3.89)	2.04 (1.11-3.74)
≥25	1.00	1.30 (0.71-2.38)	1.30 (0.73-2.31)	1.79 (0.97-3.29)
<b>Menopausal status</b>				
Premenopausal	1.00	1.13 (0.57-2.20)	1.16 (0.56-2.41)	1.85 (0.91-3.76)
Postmenopausal	1.00	1.40 (0.75-2.60)	1.93 (1.08-3.45)	2.06 (1.15-3.69)

\*Adjusted for all other factors in the table.

**Table 3. Multivariate estimates of the association in controls among age, average physical activity level, fruit and vegetable intake, average lifetime alcohol intake, cigarette smoking status, BMI, and menopausal status and 15-F<sub>2t</sub>-IsoP and 8-oxodG levels in urine**

Risk factor	15-F <sub>2t</sub> -IsoP			8-oxodG		
	$\beta$ (95% CI)*	$\beta$ (95% CI) <sup>†</sup>	$\beta$ (95% CI) <sup>‡</sup>	$\beta$ (95% CI)*	$\beta$ (95% CI) <sup>†</sup>	$\beta$ (95% CI) <sup>‡</sup>
Age (y)						
20-39	Reference	Reference		Reference	Reference	
40-49	0.20 (-0.06-0.45)	0.16 (-0.11-0.44)		0.08 (-0.12-0.28)	0.07 (-0.14-0.28)	
50-59	0.27 (0.02-0.52)	0.08 (-0.29-0.45)		0.19 (-0.01-0.39)	-0.05 (-0.33-0.23)	
60+	0.44 (0.20-0.68)	0.22 (-0.17-0.61)		0.19 (0.004-0.38)	-0.09 (-0.39-0.20)	
Average physical activity level (h/wk)						
0	Reference	Reference		Reference	Reference	
<0.69	-0.01 (-0.22-0.19)	-0.02 (-0.23-0.20)		0.04 (-0.12-0.20)	0.05 (-0.11-0.22)	
0.70-2.6	-0.04 (-0.25-0.16)	-0.02 (-0.24-0.20)		0.06 (-0.10-0.22)	0.10 (-0.07-0.27)	
2.7+	-0.004 (-0.21-0.20)	-0.05 (-0.27-0.17)		0.02 (-0.14-0.18)	0.03 (-0.14-0.20)	
Fruit and vegetable intake (no. 1/2 cup serving/wk)						
0-18	Reference	Reference		Reference	Reference	
19-26	0.05 (-0.16-0.26)	0.15 (-0.08-0.38)		0.07 (-0.10-0.24)	0.13 (-0.05-0.31)	
27-34	0.08 (-0.15-0.31)	0.14 (-0.11-0.39)		0.03 (-0.15-0.20)	0.08 (-0.11-0.27)	
35-46	0.16 (-0.07-0.39)	0.26 (0.002-0.51)		0.01 (-0.16-0.19)	0.002 (-0.19-0.20)	
47+	0.08 (-0.16-0.31)	0.15 (-0.11-0.40)		0.01 (-0.17-0.19)	0.06 (-0.13-0.26)	
Average lifetime alcohol intake (g/d)						
Nondrinkers	Reference	Reference		Reference	Reference	
<15	-0.02 (-0.18-0.13)	-0.07 (-0.24-0.10)		0.07 (-0.06-0.19)	0.06 (-0.07-0.19)	
15-30	0.09 (-0.19-0.38)	0.03 (-0.30-0.37)		0.13 (-0.09-0.35)	-0.02 (-0.28-0.24)	
30+	0.06 (-0.22-0.34)	0.04 (-0.29-0.37)		0.004 (-0.21-0.22)	0.002 (-0.25-0.26)	
Active cigarette smoking status						
Never	Reference	Reference	Reference	Reference	Reference	Reference
Current	0.30 (0.11-0.50)	0.32 (0.10-0.54)	0.31 (0.10-0.52)	0.23 (0.09-0.38)	0.24 (0.07-0.41)	0.21 (0.05-0.37)
Former	0.07 (-0.09-0.23)	0.05 (-0.13-0.24)	0.07 (0.10-0.24)	0.17 (0.05-0.30)	0.15 (0.01-0.29)	0.15 (0.02-0.28)
BMI (kg/m <sup>2</sup> )						
<25	Reference	Reference		Reference	Reference	
≥25	-0.09 (-0.23-0.05)	-0.05 (-0.21-0.11)		0.03 (-0.09-0.14)	0.02 (-0.10-0.14)	
Menopausal status						
Premenopausal	Reference	Reference		Reference	Reference	Reference
Postmenopausal	0.17 (-0.06-0.40)	0.11 (-0.14-0.36)		0.21 (0.03-0.39)	0.24 (0.04-0.43)	0.22 (0.04-0.42)

NOTE: Levels of 15-F<sub>2t</sub>-IsoP and 8-oxodG are log transformed (natural log).

\*Adjusted for age.

† Adjusted for age, average physical activity level, fruit and vegetable intake, average lifetime alcohol intake, active smoking status, BMI and menopausal status.

‡ Best-fitting model.

was statistically significant only in the latter (Table 2). A similar trend was observed in premenopausal women (OR, 1.85; 95% CI, 0.91-3.76, for the highest quartile relative to the lowest) and postmenopausal women (OR, 2.06; 95% CI, 1.15-3.69, for the highest quartile relative to the lowest); however, it was statistically significant only in the latter (Table 2). Similar associations with breast cancer risk were observed for the highest quartile of 15-F<sub>2t</sub>-IsoP levels compared with the lowest quartile irrespective of physical activity level, fruit and vegetable intake, alcohol consumption, cigarette smoking status, and BMI (Table 2).

In contrast, there was no increase in risk for those in the higher quartiles of urinary levels of 8-oxodG (OR, 0.99; 95% CI, 0.64-1.51; OR, 0.75; 95% CI, 0.48-1.16; OR, 0.79; 95% CI, 0.51-1.22, for the 2nd, 3rd, and 4th quartile relative to the lowest, respectively;  $P_{\text{trend}} = 0.16$ ; Table 1). Results were similar when the 66 women with *in situ* cancer or 82 receiving chemotherapy were removed from the analysis. Radiation treatment was also found to significantly affect 8-oxodG levels with mean  $\pm$  SD of  $33.5 \pm 17.4$  and  $28.8 \pm 16.6$  in those with or without treatment, respectively. When these women were removed from the analysis, there was a statistically significant trend of decreased risks with increasing quartiles of urinary levels of 8-oxodG (OR, 0.96; 95% CI, 0.62-1.49; OR, 0.60; 95% CI, 0.38-0.95; OR, 0.70; 95% CI, 0.44-1.11, for the 2nd, 3rd, and 4th quartile relative to the lowest, respectively;  $P_{\text{trend}} = 0.04$ ).

Among controls, 15-F<sub>2t</sub>-IsoP levels increased with age and were significantly higher in postmenopausal women ( $P = 0.02$ ;

data not shown). No significant associations were found between 15-F<sub>2t</sub>-IsoP levels and average physical activity level, fruit and vegetable intake, average lifetime alcohol intake, cigarette smoking status, and BMI. Mean 8-oxodG levels increased with age ( $P = 0.04$ ) and were higher among current active cigarette smokers ( $P = 0.03$ ) and postmenopausal women ( $P < 0.001$ ).

Table 3 reports the findings from a multivariate linear regression model examining the association between breast cancer risk factors and 15-F<sub>2t</sub>-IsoP and 8-oxodG levels among controls. After simultaneously adjusting for age, average physical activity level, fruit and vegetable intake, average lifetime alcohol intake, active smoking status, BMI, and menopausal status, current cigarette smoking status was significantly related to 15-F<sub>2t</sub>-IsoP levels. Former and current cigarette smoking status and menopausal status were significantly related to 8-oxodG levels.

## Discussion

In the present study, we compared levels of oxidative stress using urinary levels of 15-F<sub>2t</sub>-IsoP and 8-oxodG among breast cancer cases and controls. We found significantly elevated ORs for breast cancer risk when 15-F<sub>2t</sub>-IsoP levels were categorized based on quartiles in controls (Table 1). This relationship was found even when those women who had received radiation therapy, which was found to



significantly increase 15-F<sub>2t</sub>-IsoP levels, were removed from the analysis. Radiation treatment has been shown previously to result in increased levels of urinary 8-oxodG (19, 20). There was no significant relationship with 8-oxodG levels when all cases were included in the analysis. However, removal of women with radiation therapy from the analysis resulted in a significant trend of decreased risk with higher levels of urinary 8-oxodG. Although the assay variability was 25%, we still had excellent power to detect case control differences of an OR  $\leq 0.6$  or  $> 1.8$ .

Several studies have provided evidence that oxidative DNA damage and lipid peroxidation play a role in breast cancer. The levels of malondialdehyde, another marker of lipid peroxidation, were elevated in plasma of breast cancer patients as compared with healthy controls (21, 22). The DNA adducts of malondialdehyde have also been measured in breast tissues using immunohistochemical methods; levels were higher in breast cancer cases than in controls (23). Although F<sub>2</sub>-isoprostanes can be assessed in various biological fluids, we used urine because of its ready availability and the high stability of F<sub>2</sub>-isoprostanes in this medium. Unlike plasma, F<sub>2</sub>-isoprostanes are not formed *ex vivo* by auto-oxidation in urine, even if the samples are stored for several days at room temperature (5). To the best of our knowledge, there are no prior studies using this marker in cancer cases and controls. Our results, as well as the observations of others who used malondialdehyde as a marker, suggest a role for lipid peroxidation in breast cancer risk.

Increased urinary 8-oxodG levels have been observed previously among patients with various cancers. Erhola et al. (19) found significantly higher urinary 8-oxodG levels among 14 patients with small-cell lung carcinoma when compared with a control group and non-small-cell lung carcinoma patients. However, there was no difference between all cancer patients and controls. In another small study (9), prostate and bladder cancer patients had significantly elevated 8-oxodG levels than control subjects. Elevated levels of 8-oxodG in the urine of 82 prostate cancer patients compared with 33 healthy men have also been reported (24). Although there are no reported studies on urinary 8-oxodG among breast cancer patients, evidence that oxidative stress may play a role in breast cancer risk has been presented in the study of Li et al. (25), where significantly higher levels of 8-oxodG were observed in normal breast tissue of breast cancer patients than of control subjects. In addition, investigators of a small pilot study (26) reported significantly increased levels of 8-oxodG in lymphocyte DNA from breast cancer cases compared with controls. We found a significant trend for ~30% lower breast cancer risk with increased 8-oxodG excretion. Although the nucleotide pool may be a significant source (7), recent data suggest that 8-oxodG in urine is the result of DNA repair and does not result from diet or cell death (27). Thus, our data indicating lower risk at higher levels of urinary 8-oxodG may be related to higher DNA repair activity.

Among control women, 15-F<sub>2t</sub>-IsoP levels were not associated with age (Table 3). Data on the correlation between age of the subjects and 15-F<sub>2t</sub>-IsoP levels are conflicting. Whereas a positive association with age was observed in one small study (28), no effect of age was found in another (29). Data on the effect of age on 8-oxodG levels are also conflicting with both no effect (30) and positive correlations being reported (31). Miyake et al. (24) reported significantly higher 8-oxodG levels among prostate cancer patients older than 70 years. We found higher levels of 8-oxodG among postmenopausal women but no independent effect of age after considering menopausal status. Others have reported increased levels of 15-F<sub>2t</sub>-IsoP and 8-oxodG in postmenopausal women (32, 33). It has been suggested (32) that antioxidant properties of estrogens may be responsible for lower 15-F<sub>2t</sub>-IsoP levels in premenopausal women but the results were not adjusted for age. The

multivariate regression analysis in our study, with larger numbers of subjects than prior studies, showed that menopausal status had no effect on 15-F<sub>2t</sub>-IsoP levels after adjusting for age. Nakano et al. (33) reported significantly higher 8-oxodG urinary levels among postmenopausal women, but again, results were not age adjusted. Our data indicate significantly higher 8-oxodG levels among postmenopausal women even after adjusting for age.

Although obesity is associated with oxidative stress and an association between BMI and 15-F<sub>2t</sub>-IsoP levels was previously observed (34, 35), we were unable to reproduce these results. Consistent with our results, urinary 8-oxodG levels do not seem to be associated with BMI (30, 36).

Fruit and vegetable consumption should result in lower oxidative stress due to the presence of antioxidants. However, we did not see any association between fruit and vegetable consumption and 15-F<sub>2t</sub>-IsoP or 8-oxodG levels. The results of other studies are conflicting. Whereas increased intake of fruits and vegetables resulted in decreased 15-F<sub>2t</sub>-IsoP and 8-oxodG levels in the study of Thompson et al. (37), no effect was detected in two other studies (38, 39). In the positive study (37), subjects were supplemented with 12 servings of fruits and vegetables a day throughout the intervention. Thus, fruit and vegetable intake in subjects in our study, although comparable to those residing in the Mediterranean, may not have been sufficiently high to affect the markers (11). However, we were able to show that high intake of fruits and vegetables decreases a woman's risk of breast cancer in this study sample (11).

Alcohol intake may result in increased oxidative stress due to reactive oxygen species generation through induction of CYP450 2E1. Increased urinary 15-F<sub>2t</sub>-IsoP levels after alcohol administration were observed in a small study (40). In addition, 8-oxodG levels may be elevated by alcohol consumption (41) although others reported no effect of alcohol intake on urinary (42) or leukocyte DNA (43) 8-oxodG levels. In our study, lifetime alcohol consumption was not associated with either 15-F<sub>2t</sub>-IsoP or 8-oxodG levels.

The effect of cigarette smoking on 15-F<sub>2t</sub>-IsoP levels was shown in several studies (44-47). Quitting smoking led to decreased 15-F<sub>2t</sub>-IsoP levels (46, 48); restarting smoking resulted in increased levels (49). 8-oxodG levels were also elevated in smokers (30, 43, 50, 51). In agreement with these results, significantly higher 15-F<sub>2t</sub>-IsoP levels in current smokers than in never smokers were observed; 8-oxodG levels were significantly elevated in current smokers, as well as in former smokers (Table 3).

A limitation of our study is that samples were collected after breast cancer diagnosis, before the onset of chemotherapy and radiation therapy for most case subjects, but after surgery (10). However, when we restricted our analyses to case subjects who had not received treatment before urine collection, our results did not change. It is still possible that measurement of oxidative stress markers at some critical period during breast cancer development may be more appropriate. A prospective study using samples collected before diagnosis may be more useful for determining if elevated levels of oxidative stress markers in urine predict breast cancer risk, if the critical time period is within the time frame in which the prediagnostic samples were obtained.

A major strength of our study is the large number of subjects, which helps to ensure that the study had sufficient power to detect ORs of at least 1.7 between the highest quartile and the lowest quartile. In addition, the population-based study design ensured that cases and controls arose from the same source population.

In summary, we found modest positive associations between urinary 15-F<sub>2t</sub>-IsoP, but not 8-oxodG levels, and breast cancer risk in this population-based, case-control study. Among control subjects, levels of 15-F<sub>2t</sub>-IsoP were related to current cigarette smoking status. Levels of 8-oxodG were influenced by current or former cigarette smoking status and menopausal status.

## Acknowledgments

We thank the following people for their valuable contributions to the Long Island Breast Cancer Study Project: members of the Long Island Breast Cancer Network; the 31 participating institutions on Long Island and in New York City, NY; our NIH collaborators, Gwen Colman, Ph.D., National Institutes of Environmental Health Sciences; G. Iris O'Brans, M.D., Ph.D., formerly of the National Cancer Institute; members of the External Advisory Committee to the population-based case-control study: Leslie Bernstein, Ph.D. (Committee Chair); Gerald Akland, M.S.; Barbara Balaban, M.S.W.; Blake Cady, M.D.; Dale Sandler, Ph.D.; Roy Shore, Ph.D.; and Gerald Wogan, Ph.D.; as well as other collaborators who assisted with various aspects of our data collection efforts including Gail Garbowski, M.P.H., Julie Britton, Ph.D., Mary S. Wolff, Ph.D., Steve Stellman, Ph.D., Maureen Hatch, Ph.D., Geoff Kabat Ph.D., Bruce Levin, Ph.D., H. Leon Bradlow, Ph.D.; David Camann, B.S.; Martin Trent, B.S.; Ruby Senie, Ph.D.; Carla Maffeo, Ph.D.; Pat Montalvan; Gertrud Berkowitz, Ph.D.; Margaret Kemeny, M.D.; Mark Citron, M.D.; Freya Schnabel, M.D.; Allen Schuss, M.D.; Steven Hajdu, M.D.; and Vincent Vinciguerra, M.D.

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## Relationship between Urinary 15-F<sub>2t</sub>-Isoprostane and 8-Oxodeoxyguanosine Levels and Breast Cancer Risk

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*Cancer Epidemiol Biomarkers Prev* 2006;15:639-644.

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