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Oxidative stress-related genotypes, fruit and vegetable consumption and breast cancer risk

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Dietary antioxidants may interact with endogenous sources of pro- and antioxidants to impact breast cancer risk. A nested case–control study of postmenopausal women (505 cases and 502 controls) from the Cancer Prevention Study-II Nutrition Cohort was conducted to examine the interaction between oxidative stress-related genes and level of vegetable and fruit intake on breast cancer risk. Genetic variations in catalase (*CAT*) (*C−262T*), myeloperoxidase (*MPO*) (*G−463A*), endothelial nitric oxide synthase (*NOS3*) (*G894T*) and heme oxygenase-1 (*HO-1*) [$(GT)_n$ dinucleotide length polymorphism] were not associated with breast cancer risk. Women carrying the low-risk *CAT CC* [odds ratio (OR) = 0.75, 95% confidence interval (CI) 0.50–1.11], *NOS3 TT* (OR = 0.54, 95% CI = 0.26–1.12, *P*-trend = 0.10) or *HO-1 S* allele and *MM* genotype (OR = 0.56, 95% CI = 0.37–0.55), however, were found to be at non-significantly reduced breast cancer risk among those with high vegetable and fruit intake (≥median; *P*-interactions = 0.04 for *CAT*, *P* = 0.005 for *NOS3* and *P* = 0.07 for *HO-1*). Furthermore, those with ≥4 putative low-risk alleles in total had significantly reduced risk (OR = 0.53, 95% CI = 0.32–0.88, *P*-interaction = 0.006) compared with those with ≤2 low-risk alleles. In contrast, among women with low vegetable and fruit intake (< median), the low-risk *CAT CC* (OR = 1.33, 95% CI = 0.89–1.99), *NOS3 TT* (OR = 2.93, 95% CI = 1.38–6.22) and *MPO AA* (OR = 2.09, 95% CI = 0.73–5.95) genotypes appeared to be associated with raised breast cancer risk, with significantly increased risks observed in those with ≥4 low-risk alleles compared with participants with ≤2 low-risk alleles (OR = 1.77, 95% CI = 1.05–2.99, *P*-interaction = 0.006). Our results support the hypothesis that there are joint effects of endogenous and exogenous antioxidants.

Introduction

Oxidative stress may play an important role in carcinogenesis. Oxidants are formed in response to both endogenous processes and exogenous exposures and subsequently damage important cellular macromolecules. In response to increased oxidants in mammalian cells, antioxidant defenses, including both enzymatic and non-enzymatic molecules, are activated through several signal transduction pathways to counteract the effects of reactive species (1). Loss of balance between pro- and antioxidant processes has been indicated in the pathogenesis of many chronic diseases including breast cancer (2).

As a rich source of exogenous antioxidants, consumption of vegetables and fruits has been indicated as a means to reduce cancer risk. Epidemiologic data on the association between vegetables and fruits and breast cancer risk remain unclear (3). As reviewed by the 1997 World Cancer Research Fund (4), diets high in vegetables and fruits were reported to reduce breast cancer risk in most case–control studies and some earlier cohort studies, yet some recent large cohort studies did not indicate a relationship between vegetable or fruits intake and breast cancer risk (3). Although it is possible that there is no effect of these dietary components on breast cancer risk, the discrepancy may also be partly due to limitations in diet assessment methodology and study design and the possible variant risk profiles by heterogenous breast cancers jointly defined by tumor characteristics such as hormone receptor status (5,6).

It is also possible that heterogeneity in endogenous oxidative processes may mask relationships between breast cancer risk and a diet high in fruit and vegetables. Redox-sensitive signaling cascades can interfere with expression of downstream oxidative stress-related enzymes (7), which include important antioxidant enzymes such as catalase (CAT) (7) and heme oxygenase-1 (HO-1) (8). On the other hand, myeloperoxidase (MPO) can generate reactive oxygen species (ROS), and other enzymes, such as endothelial nitric oxide synthase (NOS3), may generate reactive nitrogen species or function as an antioxidant, depending upon conditions.

Among primary antioxidant enzymes neutralizing ROS, CAT is the most potent enzyme (9) and inducible by exposure to reactive species, particularly hydrogen peroxide (H_2O_2) (2). Located in the peroxisomes of nearly all cells, CAT is a heme enzyme converting H_2O_2 into H_2O and O_2 to directly reduce the production of HO and lipid hydroperoxides. A $C \rightarrow T$ substitution at nucleotide position −262 in the 5′ region of *CAT* results in reduced enzyme activity (9) and we previously found the low activity *T* allele to be associated with increased breast cancer risk (10), particularly among low consumers of fruits and vegetables.

An alternate pathway to reduction of H_2O_2 by CAT is through generation of hypochlorous acid, a potent oxidizing agent, by MPO. In addition to ROS generation, MPO is also a phase I metabolic enzyme involved in the activation and biotransformation of numerous carcinogens (11). A −463 ($G \rightarrow A$) substitution at the specificity protein 1 (SP1)-binding site in the promoter region of *MPO* results in ∼25 times lower transcription activity, leading to less inflammatory potential (12), and has been associated with decreased risk of lung (13–15) and breast cancer (16).

NOS3 can be upregulated by increased ROS level (17), with the enzyme catalyzing production of low (nanomolar) levels of nitric oxide (NO). NO is a multifunctional short-lived molecule that can have both carcinogenic and anticancer effects, depending upon a number of factors (18–21). A functional polymorphism (894 $G \rightarrow T$) in exon 7 leads to reduced enzyme activity and lower endogenous NO levels (22–24). Relationships between this polymorphism and breast cancer risk have been inconsistent (25–31), and no main effects between this polymorphism were observed with breast cancer risk in this nested case–control population, but potential interactions were noted for smoking (31) and iron intake (26).

HO-1 can be strongly induced by many stress stimuli (8). It is a rate-limiting enzyme in the degradation of heme, a potent genotoxic oxidant, leading to the generation of free iron, biliverdin and carbon

Abbreviations: BMI, body mass index; CAT, catalase; CPS, Cancer Prevention Study; DHEAS, dehydroepiandrosterone sulfate; FFQ, food frequency questionnaire; HO-1, heme oxygenase-1; LIBCSP, Long Island Breast Cancer Study Project; MAP, mitogen-activated protein; MPO, myeloperoxidase; NO, nitric oxide; NOS3, endothelial nitric oxide synthase; PI3K, phosphatidylinositol-3-kinase; ROS, reactive oxygen species; SNP, single nucleotide polymorphism; SP1, specificity protein 1.

monoxide (32). Biliverdin can be further reduced to bilirubin, which is an efficient scavenger of ROS; thus, the induction of HO-1 is an important antioxidant mechanism against oxidative stress. $(GT)_n$ repeats within the 5′-flanking region of the *HO-1* gene regulate the transcription activity of this enzyme, and variable lengths are associated with differential activity (33). Less than 26 *GT* repeats (short group, *S*) are associated with higher transcription rates; the medium length group (*M*), consisting of 27–32 *GT* repeats, has intermediate activity and the longer group (*L*), >33 repeats, is associated with less induction of HO-1 activity under oxidative stress (34). *L* genotypes have been associated with increased risk of cancer (35–37), including breast cancer in this study population (26), and other oxidative stress-mediated chronic diseases (34,38), although not all studies have supported these findings (33,39).

The effects of genetic factors on cancer risk, however, may be dependent on relevant exogenous exposures. We hypothesized that dietary sources of exogenous antioxidants may interact with endogenous sources of pro- and antioxidants to impact breast cancer risk. Such interactions were noted in our previous findings that dietary antioxidants can work in concert with polymorphisms in *MPO* and *CAT* (10,16), in the Long Island Breast Cancer Study Project (LIBCSP, 1996–1997), a population based case–control study.

To further investigate this hypothesis in the context of a prospective study, we analyzed data (502 cases and 505 controls) from a case–control study nested in the Cancer Prevention Study (CPS)-II Nutrition Cohort that was established by the American Cancer Society in 1992. Although the sample size is smaller than that of LIBCSP, dietary information was collected prior to breast cancer diagnosis, which prevents recall bias, and may better reflect the potential causal associations of exposures on cancer risk. In addition, the cohort included participants from 21 states and is more representative of the USA population than participants of the LIBCSP study, which only included residents from two counties on Long Island, NY. With these advantages, we were able to further test our previous findings and investigate variants in additional genes in oxidative stress-related pathways, in relation to fruit and vegetable intake and risk of postmenopausal breast cancer. We have previously evaluated three of these polymorphisms (*MPO*, *NOS3* and *HO-1*) in relation to breast cancer in the same nested case–control study (26,31). Here, we further examined potential effect modifications by intake of vegetable, fruit and antioxidant nutrients.

Methods

Study population

The CPS-II Nutrition Cohort was established by the American Cancer Society in 1992 to investigate the relationship between lifestyle exposures and cancer incidence and mortality. The CPS-II Nutrition Cohort has been described previously (40). Briefly, the Nutrition Cohort was a subset of the larger CPS-II cohort, which was started in 1982 and involved ∼1.2 million participants from 50 USA states. Among them, ∼184 000 USA men and women from 21 states with state cancer registries completed a mailed self-administered questionnaire in 1992 or 1993 and thus comprised the CPS-II Nutrition Cohort. The 10-page self-administered questionnaire included questions on demographic, environmental, medical, reproductive, dietary and behavior factors. Most participants were 50–74 years of age at the time of enrollment. Starting in 1997, follow-up questionnaires were sent to living participants every other year to obtain updated exposure information and to ascertain newly diagnosed cancers. The response rate was ∼90% in both 1997 and 1999 and 91% in 2001 among living cohort members (40). The self-reported incident cancers were verified through medical records, linkage with state cancer registries or death certificates.

From June 1998 through June 2001, blood samples were collected from 39 376 CPS-II Nutrition Cohort members (21 965 postmenopausal women) who provided informed consent. Each blood sample was separated into serum, plasma, red blood cells and buffy coat. The separated samples were then stored in liquid nitrogen vapor phase at −130°C before analysis. Among the women who had a blood sample available and had no previous history of cancer (other than non-melanoma skin cancer), 509 breast cancer cases were identified between 1992 and 2001. Using risk-set matching, one postmenopausal control was selected for each case from female cohort members who were cancer free (except for non-melanoma skin cancer) and had a blood sample available at the time the case was diagnosed with breast cancer (41). Controls were matched to cases on age (±6 months), race/ethnicity (White, African-American, Hispanic, Asian and other/unknown) and date of blood collection (±6 months). Seven of the cases and four of the selected controls were excluded from the final analysis because they were later found to be premenopausal or not to have breast cancer (if a case). Thus, 502 cases and 505 matched controls remained in the analyses to investigate relationships between vegetable and fruit intake, genetic variation and breast cancer risk. All aspects of the CPS-II cohort are approved by the Emory University Institutional Review Board.

Dietary assessment

In the CPS-II Nutrition Cohort, baseline dietary data were obtained by using a semiquantitative 68-item food frequency questionnaire (FFQ), which is a modification of the brief 60-item Health Habits and History Questionnaire developed by Block (42). The FFQ queried on portion size, ranging from 'small' to 'medium' to 'large', which was then converted to standard servings (43). Questions on frequency of consumption ranged from 'never or less than one time per month' to 'two or more per day' for food and to 'six or more per day' for beverages. We included total servings of fruits, but not fruit juice, in the fruit group. Vegetable intake included total servings of vegetable, but not salad or potatoes. In addition, the FFQ queried use of several vitamin supplements during the 12 months period prior to interview. Dietary and total nutrient intakes were estimated using the Diet Analysis System version 3.8a (44). The FFQ was validated using four 24 hours dietary recalls randomly collected over a 1 year period as the comparison measure in a subset of the Nutrition Cohort (n = 441). For food groups, correlation coefficients ranged from 0.52 for vegetables to 0.62 for fruits. The correlations for antioxidant nutrients ranged from 0.27 for dietary vitamin E to 0.65 for dietary vitamin C (45).

Genotyping

Genomic DNA was extracted from buffy coat and genotyped for the *CAT C−262T* (rs 1001179), *MPO G−463A* (rs 2333227), *HO-1* $(GT)_n$ length (rs 3074372) and *NOS3 G894T* (rs 1799983) polymorphisms using Taqman (Applied Biosystem, Foster City, CA). Genotyping was performed by laboratory personnel blinded to case–control status. In order to validate the experimental process, 10% blind duplicates were randomly interspersed with the case–control samples. Concordance rates between quality control duplicates were 100% for *CAT*, *MPO* and *NOS3* and 80% for *HO-1*. The success rate for each genotyped single nucleotide polymorphism (SNP) was at least 95%.

Statistical analysis

Among controls, genotyping distributions for all polymorphisms were tested for Hardy–Weinberg equilibrium using a χ^2 test. To investigate the distribution of epidemiological characteristics among cases and controls, *t*-test and χ^2 test were used for continuous and categorical variables, respectively.

In preliminary analyses, both conditional and unconditional logistic regression were used to examine main associations, with very similar results obtained from both approaches. Odds ratios (ORs) and 95% confidence intervals (CIs) from the unconditional analyses are presented.

To be able to compare results to those from the LIBCSP studies, we computed the risk associated with the low-risk *AA* or *GA* genotypes of *MPO* in reference to the common *GG* genotype and contrasted *CC* genotype of *CAT* that represents higher CAT activity against the combined *TC* and *TT* group. For *NOS3*, the heterozygous or homozygous genotypes with the *T* allele were evaluated using the common genotype *GG* as the referent group. For *HO-1* $(GT)_n$ repeats, we compared the genotypes with higher HO-1-protective antioxidant activity ($SS + SM + SL + MM$) to the genotypes with reduced activity/higher risk ($LL + LM$) that served as the referent group (26).

All multivariate models were adjusted for age, race (Caucasian/other), body mass index (BMI) (log transformed), family history of breast cancer (yes/no), age at menarche (log transformed), age at menopause, smoking status (ever/never), hormone replacement therapy use (ever/never) and parity (yes/no). Total caloric intake (log transformed) was also included in all models to adjust for potential confounding by total energy intake. All covariates had <4% missing values. Date of blood collection, as a matching variable for cases and controls, did not confound associations since genotype is fixed and therefore was not included as a covariate in the models.

Consumption of fruits, vegetables, and specific antioxidants was dichotomized (high versus low intake), based on median intake among controls.

Vegetable intake was dichotomized by 1 serving/day (≤1 versus >1), fruit intake by 1 serving/day (≤1 versus >1), vegetable and fruit intake by 2.2 servings/day (≤2.2 versus >2.2). For specific antioxidants, we first evaluated effect modification by antioxidants derived from food sources only, followed by antioxidant intake from both food and supplement sources. Nutrient components evaluated were the antioxidants vitamin C, vitamin E and β-carotene. Food-derived dietary vitamin C intake was dichotomized at 114.5 mg/day (≤114.5 versus >114.5); dietary and supplement vitamin C was dichotomized at 171 mg/day (≤171 versus >171), dietary vitamin E at 7.2 IU/day (≤7.2 versus >7.2), dietary and supplement vitamin E at 13.01 IU/day (≤13.01 versus >13.01), dietary β-carotene at 1920.8 μg/day (≤1920.8 versus >1920.8) and dietary and supplement β-carotene at 2498.3 μg/day (≤2498.3 versus >2498.3). To examine the possible synergistic or additive effect of genes in oxidative stress signaling pathways, we also considered joint effects by summing 'low-risk' alleles (*C* allele for *CAT*, *A* allele for *MPO*, *T* allele for *NOS3* and genotypes with *'S'* allele and the homozygous *MM* genotype for *HO-1*). Associations with breast cancer risk were examined with respect to the total number of low-risk alleles, categorized as 0–2, 3, and ≥4 low-risk alleles. In addition, we also stratified our findings by supplement use (any supplemental vitamin C, vitamin E, β-carotene or multiple vitamin use), as we did previously in the LIBCSP (10).

To evaluate effect modification between fruit and vegetable intake and genotypes, we created joint categories of genotypes, vegetable and fruit intake and antioxidant intake. In addition, interaction cross-product terms between each polymorphism and fruit and vegetable consumption or dietary antioxidants were constructed. We compared the log-likelihood statistic between models with a multiplicative interaction term and models without an interaction term. The likelihood ratio test with $P < 0.05$ was applied to test statistical interaction. *P*-values for trend were determined by modeling the number of variant alleles for each genotype as a continuous variable in the model. Statistical package SAS 9.0 was used to perform all the analyses. All statistical tests were two sided at the significance level of $P = 0.05$.

Results

In controls, observed frequencies for the variant *CAT C* (78%), *MPO A* (22%) and *NOS T* (30%) alleles were similar to those previously reported in Caucasian populations (10,16,28,30) as was the distribution of *HO-1* (*GT*) repeat variants (34,38). All genotypes were in Hardy–Weinberg equilibrium ($P > 0.05$).

CAT, MPO, NOS3 and HO-1 genotypes and breast cancer risk

Associations between genotypes and breast cancer risk among postmenopausal women are shown in Table I. After adjustment for possible confounders, no significant associations were observed between breast cancer risk and polymorphisms in *CAT (C−262T)*. Similarly, as previously reported in this study population, no associations were observed with *MPO* (*G−463A*) or *NOS3* (*G894T*) (26,31), whereas women with *HO-1* genotypes (*LS + MM + MS + SS*) not including *LL* and *LM* (referent group) had reduced breast cancer risk, of borderline significance, in both the age-adjusted and fully adjusted models (26).

Genotypes, vegetable and fruit consumption and breast cancer risk

As shown in Table II, associations between genotypes and breast cancer risk were examined by level of vegetable, fruit and vegetable and fruit consumption combined. For women carrying the low-risk *CAT CC*, *NOS T* allele and the *HO-1 S* allele and *MM* genotype, breast cancer risks were observed to be non-significantly reduced among those with higher levels of vegetable and fruit consumption (≥ median), with the strongest reductions in risk seen for vegetable and fruit intake combined (*P*-interactions = 0.04 for *CAT*, $P = 0.005$ for *NOS3* and $P = 0.07$ for *HO-1*, respectively), which were unchanged by further adjustments for iron intake and use of vitamin supplements (data not shown).

In contrast, among women with low vegetable and fruit intake (< median), the *CAT CC* genotype was associated with non-significant increases in breast cancer risk with the strongest relationship observed for fruit and vegetable intake combined (OR = 1.33, 95% CI = 0.89–1.99). Similarly, increased risks were also observed for women with the variant *NOS3 TT* genotype, particularly when fruit and vegetable intake were considered together (*NOS TT* OR = 2.93, 95% CI = 1.38–6.22, *P*-trend = 0.02), and for *MPO A* allele carriers, with the strongest association being observed among those with low vegetable intake (*P*-trend = 0.05, *P*-interaction = 0.17).

The relationships between breast cancer risk and genotypes were also assessed by low and high intakes of dietary vitamin C, vitamin E and β-carotene, with and without inclusion of supplement-related intakes. In all instances, relationships with breast cancer risk were either absent or attenuated when compared with those associated with vegetable and fruit intake, and *P*-interactions comparing those with high and low intakes were not statistically significant (data not shown). Similar results were obtained when we additionally controlled for iron intake and ever intake of supplemental vitamins (data not shown).

As shown in Table III, when total number of low-risk alleles or genotypes were summed for *CAT* (*C* allele), *MPO* (*A* allele), *NOS3*

Table I. Breast cancer risk associated with *CAT* (*C262T*), *MPO* (*G−463A*), *NOS3* (*G894T*) and *HO-1* length polymorphisms among postmenopausal women, American Cancer Society, 1992–2001

Polymorphism	Age-adjusted model[a]			Fully adjusted model[b]		
	Case (*n*)	Control (*n*)	OR (95% CI)	Case (*n*)	Control (*n*)	OR (95% CI)
CAT (C−262T)						
CT + TT	202	190	1 (ref)	175	170	1 (ref)
CC	295	303	0.92 (0.71–1.18)	261	259	0.91 (0.74–1.30)
MPO (G−463A)						
GG	282	285	1 (ref)	245	250	1 (ref)
GA	172	162	1.07 (0.82–1.41)	153	140	1.09 (0.81–1.46)
AA	23	15	1.55 (0.79–3.03)	19	13	1.65 (0.79–3.47)
			P-trend = 0.27			*P*-trend = 0.24
NOS3 (G894T)						
GG	242	236	1 (ref)	209	205	1 (ref)
GT	200	209	0.94 (0.72–1.22)	173	183	0.96 (0.72–1.28)
TT	47	40	1.15 (0.73–1.81)	46	34	1.30 (0.80–2.13)
			P-trend = 0.89			*P*-trend = 0.55
HO-1						
LL + LM	233	217	1 (ref)	205	188	1 (ref)
SS + SM + SL + MM	245	275	0.83 (0.65–1.07)	212	241	0.78 (0.59–1.03)

[a]Adjusted for age.
[b]Fully adjusted model controlled for age, log BMI, race (Caucasian/other), family history of breast cancer (yes/no), log age at menarche, age at menopause, smoking status (ever/never), hormone replacement therapy (ever/never), parity (yes/no) and log caloric intake.

Table II. Breast cancer risk associated with low and high intake of vegetables and fruits among postmenopausal women, American Cancer Society, 1992–2001

Genotype	Low consumption			High consumption			*P*-interaction
	Cases (*n*)	Controls (*n*)	OR (95% CI)[a]	Cases (*n*)	Controls (*n*)	OR (95% CI)[a]	
CAT (C−262T)							
Vegetables[b]							
CT + TT	98	107	1 (ref)	74	61	1 (ref)	0.18
CC	153	140	1.19 (0.82–1.71)	106	113	0.85 (0.54–1.34)	
Fruit[b]							
CT + TT	96	95	1 (ref)	77	71	1 (ref)	0.28
CC	163	144	1.10 (0.76–1.59)	96	112	0.83 (0.53–1.29)	
Vegetable + fruit[b]							
CT + TT	81	87	1 (ref)	91	78	1 (ref)	0.04
CC	143	115	1.33 (0.89–1.99)	114	137	0.75 (0.50–1.11)	
MPO (G−463A)							
Vegetable[b]							
GG	142	156	1 (ref)	101	92	1 (ref)	0.17
GA	83	74	1.24 (0.83–1.85)	67	62	0.99 (0.62–1.57)	
AA	14	6	2.67 (0.98–7.25)	5	6	0.92 (0.26–3.30)	
			P-trend = 0.05			*P*-trend = 0.91	
Fruit[b]							
GG	142	147	1 (ref)	100	100	1 (ref)	0.26
GA	94	77	1.26 (0.85–1.87)	58	61	0.90 (0.56–1.44)	
AA	13	7	2.13 (0.81–5.59)	6	5	1.51 (0.43–5.38)	
			P-trend = 0.08			*P*-trend = 0.99	
Vegetable + fruit[b]							
GG	130	128	1 (ref)	111	117	1 (ref)	0.57
GA	75	61	1.21 (0.79–1.87)	75	75	1.03 (0.67–1.57)	
AA	11	6	2.09 (0.73–5.95)	8	6	1.59 (0.52–4.91)	
			P-trend = 0.14			*P*-trend = 0.59	
NOS3 (G894T)							
Vegetable[b]							
GG	114	117	1 (ref)	92	85	1 (ref)	0.36
GT	103	108	1.01 (0.69–1.48)	68	70	0.85 (0.53–1.35)	
TT	27	18	1.55 (0.80–3.01)	19	16	0.93 (0.43–2.00)	
			P-trend = 0.34			*P*-trend = 0.62	
Fruit[b]							
GG	123	117	1 (ref)	84	85	1 (ref)	0.31
GT	95	101	0.91 (0.62–1.35)	76	78	0.93 (0.59–1.46)	
TT	35	20	1.59 (0.86–2.94)	11	14	0.78 (0.33–1.88)	
			P-trend = 0.37			*P*-trend = 0.59	
Vegetable + fruit[b]							
GG	99	103	1 (ref)	106	97	1 (ref)	0.005
GT	88	87	1.10 (0.72–1.68)	82	89	0.84 (0.55–1.27)	
TT	31	11	2.93 (1.38–6.22)	15	23	0.54 (0.26–1.12)	
			P-trend = 0.02			*P*-trend = 0.10	
HO-1							
Vegetable[b]							
LL + LM	121	112	1 (ref)	83	72	1 (ref)	0.67
SS + SM + SL + MM	116	131	0.81 (0.561.17)	92	106	0.65 (0.41–1.02)	
Fruit[b]							
LL + LM	115	107	1 (ref)	89	75	1 (ref)	0.09
SS + SM + SL + MM	130	129	0.93 (0.64–1.35)	80	111	0.59 (0.38–0.92)	
Vegetable + fruit[b]							
LL + LM	101	93	1 (ref)	103	87	1 (ref)	0.07
SS + SM + SL + MM	111	106	0.96 (0.64–1.45)	96	131	0.56 (0.37–0.85)	

[a]Adjusted for age, log BMI, race (Caucasian/other), family history of breast cancer (yes/no), log age at menarche, age at menopause, smoking status (ever/never), hormone replacement therapy (ever/never), parity (yes/no) and log caloric intake.
[b]Low and high consumption are based on median values of control group: vegetable, ≤1 versus >1 serving/day; fruit, ≤1 versus >1 serving/day; vegetable + fruit, ≤2.2 versus >2.2 servings/day.

(*T* allele) and *HO-1* (*S* allele carriers and *MM* genotype), those with ≥4 low-risk alleles benefited most from higher vegetable and fruit intake with the greatest risk reductions observed for fruit and vegetable intake combined (OR = 0.53, 95% CI = 0.32–0.88, *P*-trend = 0.01, *P*-interaction = 0.006). Similar to the patterns observed with individual genotypes, those with lower fruit and vegetable consumption and a greater number of low-risk alleles showed modest increases in breast cancer risk. Compared with participants with ≤2 low-risk alleles of the four oxidative stress-related genes, those with ≥4 low-risk alleles and low vegetable and fruit intake had the highest breast cancer risk (OR = 1.77, 95% CI = 1.05–2.99, *P*-trend = 0.03, *P*-interaction = 0.006).

Discussion

In this study, we did not observe a trend toward decreased risk of breast cancer with the *CAT CC* genotype, as was observed in postmenopausal women in the LIBCSP (OR = 0.83, 95% CI = 0.66–1.04),

Table III. Breast cancer risk associated with the number of low-risk alleles (*CAT C, MPO A, NOS3 T, HO-1 S* and *M*) by low and high intake of vegetables and fruits among postmenopausal women, American Cancer Society, 1992–2001

Number of low risk alleles	Low consumption			High consumption			*P*-interaction
	Cases (*n*)	Controls (*n*)	OR (95% CI)[a]	Cases (*n*)	Controls (*n*)	OR (95% CI)[a]	
Vegetable[b]							
≤2	64	74	1 (ref)	50	44	1 (ref)	0.18
3	73	69	1.25 (0.77–2.02)	54	43	1.21 (0.66–2.21)	
≥4	84	82	1.17 (0.73–1.87)	59	70	0.77 (0.44–1.36)	
			P-trend = 0.53			*P*-trend = 0.30	
Fruit[b]							
≤2	59	68	1 (ref)	55	48	1 (ref)	0.04
3	76	70	1.37 (0.83–2.25)	52	43	1.06 (0.60–1.89)	
≥4	93	85	1.31 (0.81–2.11)	51	69	0.67 (0.39–1.16)	
			P-trend = 0.31			*P*-trend = 0.14	
Vegetable + fruit[b]							
≤2	48	61	1 (ref)	66	54	1 (ref)	0.006
3	67	64	1.44 (0.85–2.45)	60	48	1.04 (0.61–1.78)	
≥4	84	63	1.77 (1.05–2.99)	58	89	0.53 (0.32–0.88)	
			P-trend = 0.03			*P*-trend = 0.01	

[a]Adjusted for age, log BMI, race (Caucasian/other), family history of breast cancer (yes/no), log age at menarche, age at menopause, smoking status (ever/never), hormone replacement therapy (ever/never), parity (yes/no) and log caloric intake.
[b]Low and high consumption are based on median values of control group: vegetable, ≤1 versus >1 serving/day; fruit, ≤1 versus >1 serving/day; vegetable + fruit, ≤2.2 versus >2.2 servings/day.

whereas associations between *MPO AA* genotype and postmenopausal breast cancer risk were similar between the two studies. In addition, we observed borderline significant interactions between vegetable and fruit intake and genotypes, in the same direction as our previous findings in the LIBCSP study (10,16), with *CAT CC* genotype being inversely associated with breast cancer risk only among women with higher consumption of fruits and vegetables. Similarly, the low-risk *NOS T* allele and the *HO-1 S* allele and *MM* genotype were also found to be protective among women with high fruit and/or vegetable intake, particularly when the total number of low-risk alleles was jointly considered. In contrast to findings from the LIBCSP study, we observed non-significant elevations in breast cancer risk among *CAT CC* homozygotes, particularly among those with low fruit and vegetable intake. There were indications, however, that breast cancer risk was also elevated in carriers of the low-risk *MPO A* allele, in accordance with previous findings in postmenopausal women (16), as well as the low-risk *NOS T* allele; a clear dose-dependent increase in risk was observed for low vegetable and fruit consumers when all low-risk alleles were summed and jointly considered. These findings support our previous hypothesis that genotypes resulting in better neutralization of ROS are associated with reduced breast cancer risk and that these protective effects are enhanced by high fruit and vegetable intake. Not expected, however, was the finding that these same genotypes may potentially be associated with increased postmenopausal breast cancer risk, if accompanied by low fruit and vegetable intake.

To our knowledge, very few studies have investigated the *CAT* (*−262C → T*) and *MPO* (*−463G → A*) polymorphisms in relation to breast cancer risk (10,16,46,47). In the LIBCSP case–control study, the high-activity *CAT CC* genotype was associated with reduced breast cancer risk of borderline significance among pre- and postmenopausal women (10), with risk of disease being lowest among those consuming higher levels of fruits and vegetables (10). The weak replication of the association between *CAT* genotype and breast cancer risk may be due, in part, to the smaller sample size of postmenopausal women in the current study, providing less power to detect an association if one exists, and/or to differences in the age and menopausal distribution of study participants between the two studies, with the LIBCSP study including both pre- and postmenopausal participants over age 20, whereas the present study was confined to postmenopausal women. Moreover, findings from our current study suggests that among postmenopausal women, breast cancer risk may actually be raised among those consuming low levels of vegetables and fruits and carrying the low-risk *CAT CC* genotype, with similar relationships noted for the low-risk *MPO* and *NOS3* genotypes. CAT activity is known to decline with age, which may be associated with age-associated declines in antioxidant capacity (48–50). Thus, the older population in this study may, on average, have lower CAT activity compared with the LIBCSP population. Thus, inclusion of both pre- and postmenopausal women into one group in the LIBCSP study might have attenuated strata-specific relationships.

Menopause-related differences in sex hormone levels may also, in part, account for differences observed between the American Cancer Society and the LIBCSP studies since sex hormones can regulate CAT and other oxidative stress pathways through complex mechanisms. Estradiol and certain phytoestrogens can regulate antioxidant enzymes through interaction with estrogen receptors and can activate mitogen-activated protein (MAP) kinase and nuclear factor-κB pathways (51), and some *in vitro* and *in vivo* studies have observed lower CAT concentrations in the presence of estradiol and progesterone (52–54). Other evidence also indicates that steroid hormones can regulate the expression of *MPO* (12,55,56), and estradiol, and potentially other steroid hormones such as dehydroepiandrosterone sulfate (DHEAS), can stimulate *NOS3* expression in breast cancer cell lines through both estrogen receptor-dependent and receptor-independent phosphorylation by the phosphatidylinositol-3-kinase (PI3K)–Akt pathway (57–60).

In our previous case–control study (the LIBCSP), the *MPO AA* genotype was associated with a statistically borderline lower breast cancer risk only among premenopausal women, with no associations observed with postmenopausal breast cancer (16). The *MPO* polymorphism has been widely studied in relation to lung cancer and some other cancers, with most (13–15,61–63), but not all, (64,65) studies indicating a reduced risk with the low-activity *A* allele. In the Nurses Health Study (46), women with *AA* genotypes and higher consumption of vegetable and fruit were also at non-significant decreased risk of breast cancer (OR = 0.58, 95% CI = 0.30–1.12), similar to our earlier findings among premenopausal women (16). However, both our prior (16) and current study observed a non-significant increased risk among postmenopausal *AA* carriers with lower consumption of vegetable and fruit, with attenuated or non-significant inverse associations among those with high consumption of fruits and vegetables. The underlying mechanism accounting for the raised breast cancer risk observed among postmenopausal women with the *MPO AA* genotype is unclear and could be due, in part, to hormonal influences.

There is some evidence to suggest that estrogen can differentially regulate the expression of *MPO* by genotype (56), and estrogens have been shown to induce MPO (66). Compared with premenopausal women, postmenopausal women have lower levels of MPO activity (55), which can be increased by use of hormone replacement therapy (55). It is possible that in older postmenopausal women, against a low antioxidant background of low fruit and vegetable intake, low plasma levels of MPO may contribute to increased levels of oxidative stress since MPO would have some antioxidant effects due to decreased superoxide anion generation (55).

The role of another oxidative stress-related enzyme, NOS3, on breast cancer risk is largely dependent on its product NO. NO plays a dual role in cancer, and its ultimate effect depends on the activity and localization of NOS isoforms, overall levels of NO, the surrounding microenvironment, the cellular susceptibility to NO and other related proteins such as p53 (18–21). The complicated function of NO may underlie previous inconsistent findings across studies regarding its relationship with breast cancer risk (25–31). This is the first study to assess vegetable and fruit intake as a potential modifier of the relationship between *NOS3* genotype and breast cancer risk. The low-activity *NOS T* allele was found to be inversely associated with breast cancer risk among women with a high consumption of vegetables and fruits, but was associated with borderline increased risk among low vegetable and fruit consumers. *In vivo* studies have suggested that some components of vegetable and fruit, such as flavonoids and carotenoids, may moderately increase the activity of endothelial nitric oxide synthase, probably by protecting its essential cofactor tetrahydrobiopterin, resulting in the steady production of NO and the downstream cyclic guanosine-3,5-monophosphate level that mediates its cytoprotective function, especially for vascular protection (67–69). However, increased NO level could be harmful because of its capability to break DNA strands and impair the function of p53 (70,71); thus, the much lower concentrations of NO resulting from the *NOS3 T* variant may protect endothelial cells from ROS exposures (72,73). In addition, a large number of polyphenols from plant-based food can act as efficient scavengers of the derivatives of NO, peroxynitrite and peroxynitrite-derived radicals, to reduce mutagenesis (74–77). The plausible protective function of lower basal NO produced by *NOS3 G894T* variants may be attenuated, and even become deleterious, among those with lower vegetable and fruit consumption, where basal NO level may be diminished. However, our results could also be a result of chance only, and further investigation is needed.

Short and medium *HO-1* genotypes reduced breast cancer risk, primarily among women with higher vegetable and fruit intake, with a *P*-interaction that was borderline significant. HO-1 is highly induced by oxidative stress, as well as a wide range of dietary antioxidants, such as α-lipoic acid found in broccoli and spinach, resveratrol found in grapes and isothiocyanates from brassica vegetables, according to *in vitro* and *in vivo* experiments (78–82). The higher exposure to exogenous antioxidants from higher intake of vegetables and fruits could increase the expression and activity of HO-1, particularly among participants who have the wild-type shorter promoters, thereby increasing its protective function in various tissues.

No previous studies have examined the potential interaction between *HO-1* promoter length polymorphism and dietary antioxidant intake on breast cancer risk. However, our findings are inconsistent with a recent study in which the *HO-1* long allele was associated with increased lung function decline among subjects with high serum β-carotene levels (83). These disparate findings may be due to differences in disease site, study population and measurement of vegetable and fruit intake. Although serum β-carotene levels as a biomarker of fruit and vegetable intake has the advantage of objectivity compared with questionnaire data, it assumes that a one-time β-carotene measurement can represent long-term and average levels of β-carotene (83). In contrast, dietary habits measured by questionnaire have been shown to be stable for long periods of time among middle-aged and older people (84). Our findings with *HO-1* are consistent with our findings with *CAT* and *MPO*, i.e. that genotypes related to reduced oxidative stress are associated with lower breast cancer risk primarily among women who consume higher amounts of fruits, vegetables and other dietary antioxidants (10–16).

The inverse associations with breast cancer risk for potentially higher antioxidant (*CAT CC* genotype, *HO-1 SS* + *SM* + *SL* + *MM* genotypes) and lower pro-oxidant enzyme activity (*MPO GA* + *AA* genotypes and *NOS3 GT* + *TT* genotypes) among higher consumers of vegetables and fruits are consistent with the hypothesis that ROS are better neutralized endogenously in an environment high in exogenous antioxidants. Vegetables and fruits contain numerous anticarcinogenic substances and antioxidants that could contribute to enhance the protective effects of high-activity variants of antioxidant enzymes. The complex anticarcinogenic and antioxidant components of vegetable and fruits may work together to interact with antioxidant enzymes. Thus, effect modification with individual antioxidant vitamins may not be observable and may explain why associations with dietary vitamin E and β-carotene consumptions were less stable in comparison. Among individuals consuming low levels of vegetables and fruits, breast cancer risk was raised with the high antioxidant/low pro-oxidant variants. The reasons for this are unclear, but may, in part, be due to hormonal influences since this study population was entirely composed of postmenopausal women and sex hormones are known to influence oxidative stress pathways.

Correlated with each other in the metabolic pathway for ROS, it is plausible that *CAT*, *MPO*, *NOS3* and *HO-1* genotypes combine to lower ROS levels and may have a cumulative protective impact on breast cancer etiology. We evaluated possible joint effects by summing low-risk alleles. The number of low-risk alleles significantly interacted with fruit and fruit and vegetable intake combined to impact breast cancer risk. Although the simplified multigenic approach was based on the assumption that all the low-risk alleles contribute equally to reduced breast cancer risk, it did show that genes related to the same pathway have a cumulative effect on overall disease risk. And with the increased statistical significance of the effect modification observed, the multigenic approach may be a more sensitive method for detecting a true association compared with a single gene approach.

A main limitation of this study was the relatively small sample size, particularly when assessing stratification by vegetable and fruit intake, which could increase the likelihood of type I error. Nevertheless, this study is still one of the largest among published studies for postmenopausal breast cancer assessing interactions between genotypes associated with anti- and pro-oxidant status and fruit and vegetable intake. Although multiple comparisons and therefore inflated type I error is relevant and possible in all studies, confidence in findings from this study is increased by testing a hypothesis based on biological plausibility and results from previous studies, along with relatively consistent interactions that were observed across the examined genotypes. Another limitation is that a single diet assessment was used to assess vegetable and fruit intake that could introduce misclassification into our dietary exposures. Since the dietary data were captured prior to cancer diagnosis, however, this misclassification is unlikely to be differential between cases and controls and therefore risk estimates are unlikely to be biased by this limitation. Furthermore, compared with the other examined SNPs, concordance rates for replicate samples for the *HO-1* repeat length polymorphism genotypes were lower at 80% compared with the 100% concordance rates observed with the other SNPs. The *HO-1* polymorphism is a repeat rather than a SNP, so the genotype is based on the assignment of repeat lengths, which varies between 114 and 148 in 2 bp units. About half of the discordance in replicates resulted from either an error in one allele or from a difference in repeat size of no more than 4 bp. The lower concordance rate was unlikely to have biased study findings since the distribution of *HO-1* (*GT*) dinucleotide repeats did not differ between cases and controls ($P = 0.48$). The published distribution for this study population was bimodal, with one peak located at 23 *GT* repeats and one located at 30 repeats, similar to previous reports (26,34). The major strength of this study is that it is nested in a large cohort study, and therefore the epidemiologic data collected represent lifestyle patterns, including diet, prior to the development of breast cancer.

In summary, there is a growing body of evidence demonstrating effect modification of breast cancer risk associated with oxidative stress-related genotypes by consumption of fruits and vegetables, which highlights the potential importance of vegetable and fruit consumption in reducing breast cancer risk in certain subgroups. Further, results characterizing women according to *CAT*, *MPO*, *NOS3* and *HO-1* at-risk allele groups indicates that a multigenetic approach appears to be more informative than examining single SNPs.

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