

Genetic Variability in Iron-Related Oxidative Stress Pathways (*Nrf2*, *NQO1*, *NOS3*, and *HO-1*), Iron Intake, and Risk of Postmenopausal Breast Cancer

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Abstract

Oxidative stress resulting from excess reactive oxygen species and/or deficiencies in antioxidant capabilities may play a role in breast cancer etiology. In a nested case-control study of postmenopausal women (505 cases and 502 controls) from the American Cancer Society Prevention II Nutrition Cohort, we examined relationships between breast cancer risk and genetic polymorphisms of enzymes involved in the generation and removal of iron-mediated reactive oxygen species. Using unconditional logistic regression, genetic variations in *Nrf2* (11108C>T), *NQO1* (609C>T), *NOS3* (894G>T), and *HO-1* [(GT)_n, dinucleotide length polymorphism] were not associated with breast cancer risk in a multivariate model. A significant dose trend (*P* trend = 0.04), however, was observed for total number of putative "at-risk" alleles (*Nrf2* T, *NQO1* T, *NOS3* T, and *HO-1* LL and LM genotypes), with those carrying three or more at-risk alleles having an odds ratio (OR) of 1.56 [95%

confidence interval (95% CI), 0.97-2.51] compared with those having none. When examined in relation to iron, carriage of three or more high-risk alleles in the highest tertile of iron intake (OR, 2.27; 95% CI, 0.97-5.29; *P* trend = 0.02; *P* interaction = 0.30) or among users of supplemental iron (OR, 2.39; 95% CI, 1.09-5.26; *P* trend = 0.02; *P* interaction = 0.11) resulted in a greater than 2-fold increased risk compared with women with no high-risk alleles. Increased risk was also observed among supplement users with the *HO-1* LL or LM genotypes (OR, 1.56; 95% CI, 1.01-2.41; *P* interaction = 0.32) compared with S allele carriers and MM genotypes combined. These results indicate that women with genotypes resulting in potentially higher levels of iron-generated oxidative stress may be at increased risk of breast cancer and that this association may be most relevant among women with high iron intake. (Cancer Epidemiol Biomarkers Prev 2007;16(9):1784-94)

Background

There is evidence that oxidative stress, resulting from either an excess of reactive oxygen species (ROS) or a deficiency in antioxidant capabilities, may play a role in the etiology of breast cancer (1, 2). The balance of endogenous oxidants and antioxidants is likely affected by variation in genes involved in the generation and removal of oxidative species, with ultimate effects dependent in part on the presence of relevant exogenous exposures (3-5). Indeed, we have noted such interactions in studying associations between breast cancer risk and dietary antioxidants in concert with polymorphisms in myeloperoxidase (6) and catalase (7).

One source of ROS generation among postmenopausal women is high dietary iron and high iron stores (8). Iron can cause oxidative tissue damage by catalyzing Haber-

Weiss and Fenton reactions that convert hydrogen peroxide (H₂O₂) to free radicals (9-12). In women, neoplastic breast tissue contains higher levels of iron and ferritin compared with normal tissue (13, 14), and rats fed excess iron develop earlier and more numerous mammary tumors (15), whereas those fed iron-deficient diets are protected (16). Currently, some epidemiologic studies support a role for excessive dietary iron intake and risk of total cancers (17-19) as well as risk of lung and colon cancers (17, 20-23), but there are few data on relationships with breast cancer (24-26).

Several enzymes are important in the formation and reduction of iron-generated ROS (Fig. 1). NAD(P)H:quinone oxidoreductase 1 (NQO1) may be particularly relevant for breast carcinogenesis because of its role in reduction of endogenous catechol estrogens generated in the metabolism of estrogen. By catalyzing the obligatory two-electron reduction of catechol estrogens and other quinones, the reactive semiquinone intermediate that drives the Fenton reaction is bypassed, and superoxide-mediated release of iron from ferritin stores is prevented (27). In animals, *NQO1* suppression increases estradiol-dependent tumor formation (28, 29). A C609T polymorphism (rs1800566) in *NQO1* (30) leads to a proline to serine substitution in the NQO1 protein that results in

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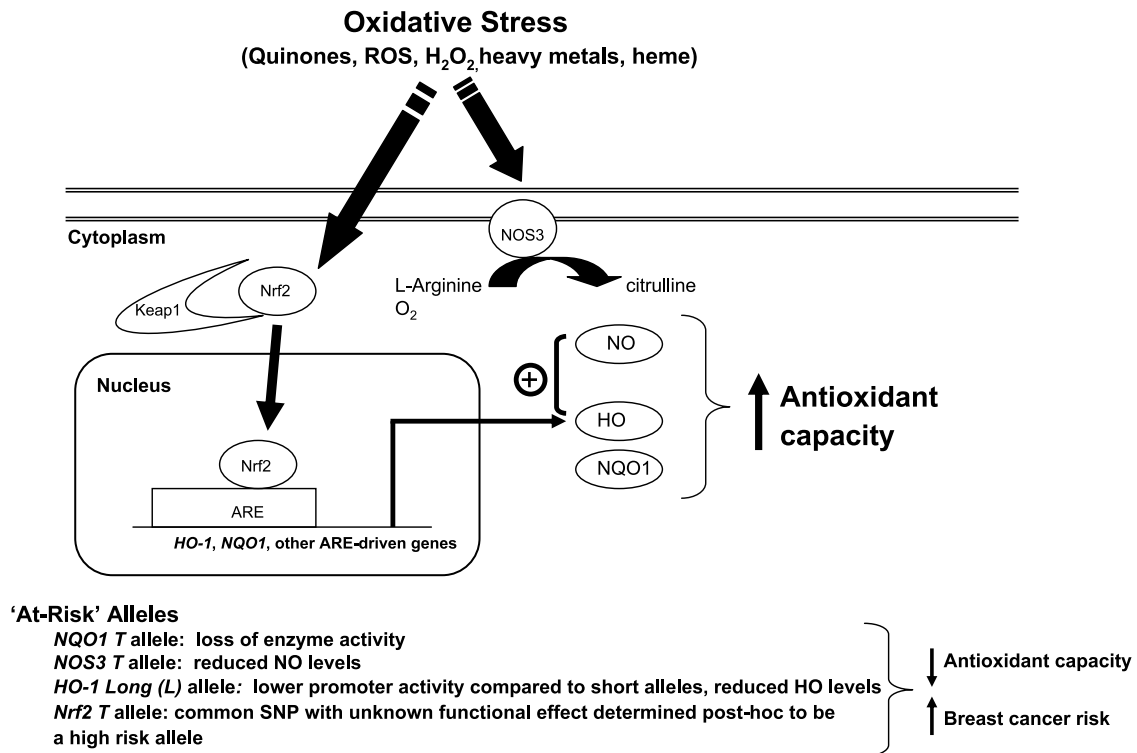


Figure 1. Genes related to generation and reduction of iron-mediated ROS and hypothesized alleles for increased breast cancer risk (see text for detailed discussion). Increased oxidative stress will promote nuclear accumulation of Nrf2, which activates transcription of *NQO1*, *HO-1*, and other antioxidant response element-driven genes as well as *NOS3*. Enzyme products of these genes have antioxidant properties and can reduce iron-generated ROS by several mechanisms. As well, a costimulatory relationship exists between the *NOS3* and *HO-1* pathways, with NO up-regulating HO activity, possibly by increasing expression of *Nrf2*, and HO reciprocally up-regulating *NOS3* activity.

loss of virtually all enzyme activity due to rapid degradation of the variant enzyme (31, 32).

NQO1 expression is regulated, to some extent, by nuclear factor erythroid-related factor 2 (Nrf2), a transcription factor that binds to the antioxidant response element (33). Antioxidant response elements are regulatory sequences found on the promoters of several phase 2 detoxification genes (33), including *NQO1*. Oxidative stress promotes nuclear accumulation of Nrf2 and activates transcription of *NQO1* and other antioxidant response element-driven genes (34). Nrf2 also induces *ferritin-H* and *ferritin-L* genes, leading to increased sequestering of iron (35-37). Rodent models show that *Nrf2*^{-/-} mice are extremely susceptible to oxidative stress challenges and xenobiotics (38, 39). A common C to T polymorphism (*IVS1+11108 C>T*, rs1806649) in the *Nrf2* gene has been identified and may be relevant for breast cancer etiology.

Endothelial nitric oxide (NO) synthase (*NOS3*) generates low amounts of short-lived NO by converting L-arginine to citrulline in endothelial tissue (40). At low levels, NO is considered to be cytoprotective and can act as an antioxidant by scavenging for ROS (41) and can bind to iron to reduce redox cycling (42, 43). *NOS3* expression has been detected in breast tumors (44-46) and is positively associated with estrogen and progesterone receptor status (45, 46) and negatively correlated

with histologic grade and lymph node status (46). Loss of *NOS3* expression has been associated with breast cancer progression in estrogen-independent cancers (47). A *G894T* polymorphism in exon 7 of *NOS3* results in a *Glu298Asp* substitution (rs1799983; ref. 48) that alters susceptibility to cleavage (49) and leads to reduced NO levels (50).

Heme oxygenase (HO) may be important in iron-related carcinogenesis because it catalyzes the rate-limiting step in heme degradation and provides cellular protection against both heme- and nonheme-mediated oxidant injury (51-53). HO-1 is the inducible form of HO and is rapidly up-regulated by NO, heavy metals, ROS, hemin, and other stress conditions (54-58). Expression levels are high in tumor cells (59, 60), and deficiency has been linked to endothelial damage (61). A microsatellite length polymorphism (rs3074372; ref. 62) modulates HO-1 response to exogenous stimuli, whereby the number of (GT)_n repeats is inversely related to activity (63). When stimulated by H₂O₂ *in vitro*, short *HO-1* alleles (<25 GT repeats) show increased promoter activity compared with longer alleles (63, 64). *HO-1* expression, like *NQO1*, is up-regulated by Nrf2-antioxidant response element.

Because associations between polymorphisms in genes coding for enzymes involved in iron-mediated oxidative stress have not been previously evaluated with respect to

breast cancer risk, we investigated this hypothesis in the context of the American Cancer Society's Cancer Prevention Study II (CPS-II) Nutrition Cohort and also assessed dietary iron intake as a potential effect modifier of these relationships.

Materials and Methods

Study Population. These analyses were conducted using data and samples from the CPS-II nutrition cohort, a subgroup of the larger CPS-II cohort, which prospectively examines cancer incidence in ~184,000 men and women enrolled by the American Cancer Society between 1992 and 1993 (65). At the time of enrollment, most participants were ages 50 to 74 and completed a 10-page self-administered mailed questionnaire that collected data on demographic, medical, lifestyle, and dietary factors. Follow-up questionnaires were sent to all participants in 1997 and every 2 years thereafter to update exposure information and to ascertain newly diagnosed cancers. Between June 1998 and June 2001, 39,300 blood samples were collected from a subset of 142,000 surviving participants, and these analyses are based on the 21,963 women who contributed a specimen. Cancer cases were identified through self-report and verified through medical records, linkage with state cancer registries, or death certificates (65). Pilot results from this cohort found the sensitivity of self-reported breast cancer to be 91% (66). Detailed description of participant recruitment, follow-up, and characteristics has been previously reported (65). For all breast cancer cases, questionnaire data were collected before cancer diagnosis. Collection of DNA from buffy coat occurred after breast cancer diagnosis in some cases.

Between 1992 and 2001, 509 postmenopausal women diagnosed with breast cancer were identified. For each case, a control was randomly selected from postmenopausal women who had provided a blood sample and were cancer-free at the time of diagnosis of the matching case using risk-set sampling (67). Controls were matched to cases by date of birth (± 6 months), date of blood collection (± 6 months), and race/ethnicity (Caucasian, African-American, Hispanic, Asian, other/unknown). Women with any prior cancer other than nonmelanoma skin cancer were excluded from analysis, as were seven cases and four controls who were later found to be premenopausal or not to have breast cancer (for cases). A total of 502 cases and 505 controls remained in the analyses.

Dietary Assessment. Dietary data collected in 1992 were obtained using a modification of the brief 60-item Health Habits and History Questionnaire developed by Block et al. (68). This semiquantitative 68-item food frequency questionnaire queried on frequency of consumption as well as portion size. Nutrient intakes were estimated using the Diet Analysis System version 3.8a (69).

Total iron intake included contributions from diet as well as multivitamin supplements. Estimates for dietary iron intake were adjusted for total energy using the residual method (70). Iron intake from multivitamin supplements was ascertained from the questionnaire, with supplement users being defined as individuals

taking multivitamin supplements at least once each week. Supplemental iron intake was calculated based on brand formulations (Stresstabs type, Therapeutic, Theragran type, or One-A-Day or Centrum type) and number of pills consumed and frequency of intake (none, 1-3 per week, 4-6 per week, 1 per day, 2 per day, 3 per day, 4 per day, 5+ per day) and was not adjusted for energy intake. Total iron intake from diet and supplements was calculated as energy-adjusted dietary iron intake plus iron intake from supplements. The same method was used to calculate total intakes of β -carotene, vitamins C and E, calcium, and zinc. For vitamins C and E, calcium and β -carotene intakes from single supplement preparations were also included in calculations. As previously described, the food frequency questionnaire was validated using four or multiple 24-h recalls over a 1-year period in a subset of the CPS cohort (71).

Genotyping. DNA previously extracted from buffy coat was genotyped for single nucleotide polymorphisms in *NQO1*, *NOS3*, and *Nrf2* using Taqman (Applied Biosystems). Genotyping failed for all three single nucleotide polymorphisms in one case and three controls, although results were obtained for *HO-1* repeats in these individuals. Genotyping failure rates were 3.2% ($n = 32$), 1.6% ($n = 16$), and 0.006% ($n = 6$) for *NOS3*, *NQO1*, and *Nrf2*, respectively. The *HO-1* repeat was genotyped as described in Yamada et al. (63), and the failure rate was 3.7% ($n = 40$). Laboratory personnel were blinded to case-control status, and 10% blind duplicates were randomly interspersed among the samples for quality control. Overall concordance was 100% for the three single nucleotide polymorphisms and 80% for the *HO-1* repeat.

Statistical Analysis. The allelic distributions among controls were tested for Hardy-Weinberg equilibrium, and χ^2 tests were used to compare variant allele frequencies and genotype distributions between cases and controls. We used unconditional logistic regression, which gave similar risk estimates as conditional regression in overall models. Multivariate models were adjusted for known risk factors for breast cancer and/or determinants of body iron stores, including age, family history of breast cancer in a mother or sister (yes, no), hormone replacement therapy (ever, never), body mass index (continuous, log transformed), age at menarche (continuous, log transformed), age at menopause (continuous), parity (yes, no), and race (Caucasian, other). Ninety-six individuals (9.5%) had missing values for one or more covariates, with half ($n = 48$, 9.6%) being cases and half ($n = 48$, 9.5%) being controls. Reported findings excluded participants with missing values, but results were not appreciably different from age- and race-adjusted models with no exclusions for missing data as well as from multivariate analyses that also controlled for hormone replacement therapy (12 exclusions), age at menarche (10 exclusions), and parity (16 exclusions). The three latter covariates were the only factors that changed the risk estimates by $\geq 10\%$ (data not shown). Dietary intake of potential enhancers (vitamin C) and inhibitors (calcium) of iron absorption was also considered as potential confounders, along with levels of dietary antioxidant intake (β -carotene, vitamins C and E, and zinc). Intakes of β -carotene, vitamins C and E, zinc, and calcium were log transformed to approximate a normal

distribution before their inclusion as (continuous) covariates in adjusted models. In addition to using β -carotene and vitamin C as markers of fruit and vegetable intake, we also considered adjustment for total number of fruit and vegetable servings because some (26), but not all (8), studies have reported fruit intake to be associated with high iron stores.

Genotypes for *NQO1*, *NOS3*, and *Nrf2* were evaluated with those heterozygous or homozygous for variant alleles contrasted against those homozygous for common alleles as the referent group. For *HO-1*, the number of (GT)_n dinucleotide repeats was classified into three categories as previously described (63): those with <25 repeats were considered as "short" (S) alleles, 25 to <30 repeats were considered as "medium" (M) alleles, and ≥ 30 repeats were considered as long (L) alleles. Genotype combinations were evaluated with women homozygous for long alleles as the referent group. Women carrying the *HO-1* LL or LM genotypes were also contrasted against those who carried short alleles or two medium alleles. We also contrasted increasing numbers of "at-risk" alleles against those with none. Except for the *Nrf2* T allele, which was determined post hoc to be high risk, at-risk alleles were a priori taken to be the *NQO1* T allele, the *NOS* T allele, and the *HO-1* LL and LM genotypes.

Relationships between genotypes and breast cancer risk were evaluated by tertiles of total iron intake based on distribution in the controls (≤ 9.6 , >9.60 -22.5, and >22.5 mg/d). Twenty-two cases (4.4%) and 21 controls (4.2%) did not have dietary data and therefore were excluded from these analyses. Genotype-disease relationships were subsequently stratified by supplement status, and among nonsupplement users, we further divided participants into lower and higher iron consumers based on the first tertile cutoff (9.6 mg/d iron). Nine participants with information on supplement use (eight nonusers and one user) but without accompanying dietary information were included in analyses comparing supplement users with nonusers, but the eight nonusers with missing dietary information were excluded from the analysis examining low and high iron intake among nonsupplement users. Supplement users were considered as one group because the lowest daily intake of iron within this group was 10.5 mg, and thus, low total iron intake (≤ 9.6 mg/d) could not be evaluated.

All analyses were two-sided tests at the significance level of $P = 0.05$. Odds ratios (OR) and 95% confidence

intervals (95% CI) are reported. We tested for linear trend by determining the number of variant alleles for each genotype and fitting this continuous variable in the model (codominant model for single nucleotide polymorphisms). To test multiplicative interactions, a cross-product term for genotype and iron intake [tertiles for Table 2 analyses and supplement use (yes/no) for Table 3 analyses] was included in multivariate models; genotype was fitted as a continuous variable in the model indicating number of variant alleles. Using the Wald χ^2 test, statistical significance indicated a difference in slopes among genotypes between strata of total iron intake. All analyses were done using Statistical Analysis System statistical package version 9.0 (SAS Institute, Inc.).

Results

Observed frequencies for variant alleles were similar to those previously reported in Caucasian populations: 25% for *Nrf2* (72), 20% for *NQO1* (73, 74), and 30% for *NOS* (75-77). Among controls, genotype frequencies for *NQO1* ($P = 0.53$) and *NOS3* ($P = 0.51$), but not *Nrf2* ($P = 0.05$), were in Hardy-Weinberg equilibrium. However, the variant *Nrf2* allele did not differ between cases and controls ($P = 0.11$), and Hardy-Weinberg assumptions were not similarly violated in cases ($P = 0.65$), as would be expected if the diversion from Hardy-Weinberg equilibrium was caused by genotyping error. When allele frequencies were compared between cases and controls, no significant differences were observed for any of the single nucleotide polymorphisms ($P > 0.11$). As shown in Fig. 2, the number of *HO-1* (GT) dinucleotide repeats ranged from 20 to 42, and distributions did not differ between cases and controls ($P = 0.48$). The distribution was bimodal, with one peak located at 23 GT repeats and one located at 30 repeats, similar to previous reports (63).

***Nrf2*, *NQO1*, *NOS3*, and *HO-1* Genotypes and Breast Cancer Risk.** Associations between genotypes and breast cancer risk are shown in Table 1. *Nrf2*, *NQO1*, and *NOS3* were not significantly related to breast cancer risk. Associations between *NOS3* genotype and breast cancer risk have previously been published for this population (78) but are reproduced here. For *HO-1*, women who were heterozygous for long and short alleles had a

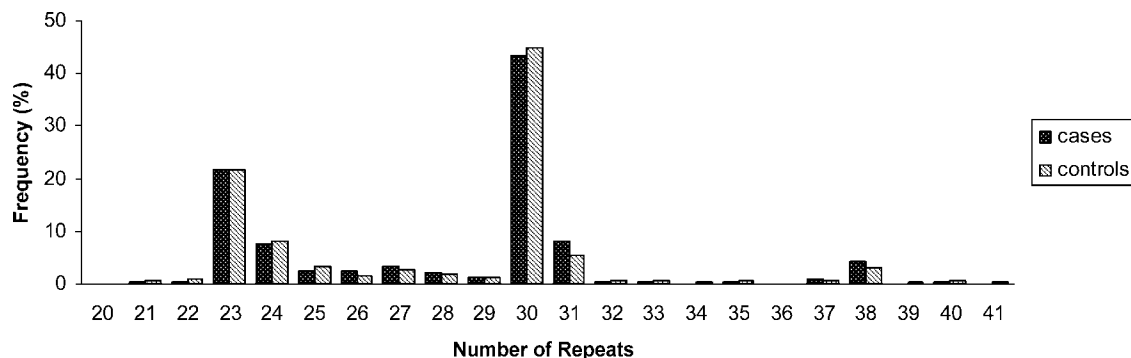


Figure 2. Frequency distribution of the number of (GT)_n repeats in cases ($n = 958$ alleles) and controls (987 alleles).

Table 1. *Nrf2*, *NQO1*, *NOS3*, and *HO-1* genotype and breast cancer risk

Polymorphism	Age-adjusted model*			Fully adjusted model [†]		
	Ca	Co	OR (95% CI) [‡]	Ca	Co	OR (95% CIs)
<i>Nrf2</i>						
CC	255	289	1 (referent)	235	259	1 (referent)
CT	207	172	1.36 (1.05-1.78)	182	157	1.31 (0.99-1.74)
TT	38	40	1.08 (0.67-1.73)	37	38	1.08 (0.66-1.77)
			<i>P</i> trend = 0.12			<i>P</i> trend = 0.20
<i>NQO1</i>						
CC	325	323	1 (referent)	297	293	1 (referent)
CT	157	151	1.03 (0.79-1.36)	140	137	1.00 (0.75-1.33)
TT	14	21	0.66 (0.33-1.32)	13	18	0.71 (0.34-1.49)
			<i>P</i> trend = 0.63			<i>P</i> trend = 0.60
<i>NOS3</i>						
GG	242	236	1.0 (referent)	218	214	1.0 (referent)
GT	200	209	0.94 (0.72-1.22)	179	190	0.95 (0.72-1.26)
TT	47	40	1.15 (0.73-1.81)	47	34	1.33 (0.82-2.16)
			<i>P</i> trend = 0.89			<i>P</i> trend = 0.54
<i>HO-1</i>						
LL	176	167	1 (referent)	160	148	1 (referent)
LM	57	50	1.08 (0.70-1.67)	54	48	0.98 (0.62-1.55)
LS	149	184	0.77 (0.57-1.04)	132	165	0.71 (0.51-0.98)
MM	10	11	0.86 (0.36-2.09)	9	10	0.75 (0.29-1.92)
MS	34	33	0.98 (0.58-1.65)	30	52	0.86 (0.49-1.49)
SS	52	47	1.05 (0.67-1.64)	46	43	0.94 (0.59-1.52)
LS + MM + MS + SS	245	275	1 (referent)	217	250	1 (referent)
LL + LM	233	217	1.20 (0.93-1.55)	214	196	1.29 (0.99-1.70)
High-risk alleles or genotypes [§]						
0	47	60	1 (referent)	41	56	1 (referent)
1	128	145	1.13 (0.72-1.77)	115	128	1.22 (0.76-1.98)
2	172	160	1.37 (0.89-2.13)	156	146	1.45 (0.91-2.31)
3+	155	140	1.41 (0.91-2.21)	143	127	1.56 (0.97-2.51)
			<i>P</i> trend = 0.06			<i>P</i> trend = 0.04

*Adjusted for age.

[†]Fully adjusted model was adjusted for age, family history of breast cancer (yes, no), hormone replacement therapy (yes, no), body mass index (continuous, log transformed), age at menopause, age at menopause, smoking status (ever/never), race (Caucasian, other), and parity (yes/no); 96 participants (48 cases and 48 controls) were excluded from this analysis because of missing covariate information.[‡]OR and 95% CIs calculated by unconditional logistic regression.[§]Sum total of high-risk alleles or genotypes, which were taken to be the *Nrf2* T allele, the *NQO1* T allele, the *NOS3* T allele, and the *HO-1* LL and LM genotypes.

significant reduction in breast cancer risk after adjustment for potential confounders (OR, 0.71; 95% CI, 0.51-0.98). When those with LL or LM genotypes were contrasted against those who carried short alleles or two medium alleles, there was a borderline increased risk of breast cancer (OR, 1.29; 95% CI, 0.99-1.70). When the total number of at-risk alleles for *Nrf2*, *NQO1*, and *NOS3* and genotypes for *HO-1* was counted and contrasted against a referent group of women with no high-risk alleles, there was a significant gene-dose effect showing increased risk (*P* trend = 0.04), with those having three or more high-risk alleles at 56% greater risk of breast cancer compared with the referent group (OR, 1.56; 95% CI, 0.97-2.51). Further adjustment for daily servings of fruits and vegetables did not substantially alter risk estimates (data not shown), with women having three or more high-risk alleles having an adjusted OR of 1.53 (95% CI, 0.93-2.51) compared with those with no high-risk alleles (*P* trend = 0.05). Similarly, risk estimates were not substantially altered by adjustment for intake of β -carotene and vitamins C and E (data not shown).

***Nrf2*, *NQO1*, *NOS3*, and *HO-1* Genotypes and Breast Cancer Risk Stratified by Total Iron Intake from Food and Multivitamin Supplements.** In our study popula-

tion, breast cancer risk was not associated with use of iron-containing multivitamin supplements (*P* = 0.65) or with total daily iron intake, considered either as a continuous variable (log transformed) or as a score variable modeling tertile-specific median intakes (*P* and *P* trends > 0.67). Table 2 shows estimated risk ratios for genotypes stratified by tertiles of total iron intake. Women in the lowest tertile of intake (≤ 9.6 mg/d) consumed all their iron from food sources, whereas women in the highest tertile of intake (> 22.5 mg/d) included only women who obtained some of their total iron intake from multivitamin supplements. *Nrf2*, *NQO1*, and *NOS3* genotypes were not significantly related to breast cancer risk at all levels of iron intake. For *HO-1*, risk was nonsignificantly elevated among those with LL or LM genotypes in the second (OR, 1.59; 95% CI, 0.96-2.63) and third (OR, 1.36; 95% CI, 0.85-2.18) tertile of iron intake when compared with those with short or two medium alleles.

When total number of at-risk alleles were evaluated in relation to iron consumption, risk was elevated among women in the second and third tertile of iron intake, with a clear gene-dose effect only in the highest tertile (*P* trend = 0.02). Women harboring three or more high-risk alleles had a two-fold increase in risk compared with

women with no high-risk alleles (OR, 2.27; 95% CI, 0.97-5.29). Increasing numbers of at risk alleles among women in the lowest tertile of consumption did not increase breast cancer risk, with all risk estimates near unity (P trend = 0.73). Further adjustments for β -carotene, and vitamins C and E, as well as zinc and calcium intakes did not change risk estimates nor did adjustments for daily servings of fruits and vegetables (data not shown).

***Nrf2*, *NQO1*, *NOS3*, and *HO-1* Genotypes and Breast Cancer Risk Stratified by Supplement Use.** Genotype-disease relationships were further examined by supplement use, as shown in Table 3, to determine if there are differential effects of iron due to source. Nonsupplement users were further divided into two groups (≤ 9.6 and >9.6 mg/d), representing low and high dietary iron from food sources. Prevalence of regular multivitamin supplement use among women providing data was 42% and 39% in cases and controls ($\chi^2 = 1.18$, $P = 0.28$),

respectively, similar to the rate reported in postmenopausal women from the main CPS-II cohort (79).

Among women who took iron-containing multivitamin supplements, those homozygous for the *NOS* T allele had a borderline significant 2-fold increase in risk (OR, 2.18; 95% CI, 0.99-4.81) compared with G allele homozygotes, whereas no increase in risk was observed for nonsupplement users. Increased breast cancer risk was also observed among supplement users harboring the *HO-1* LL or LM genotypes (OR, 1.56; 95% CI, 1.01-2.41). Among nonsupplement users, there were no associations with risk among nonsupplement users.

When total number of at-risk alleles were evaluated in relation to supplement use, breast cancer risk was elevated among women taking supplements, with women having three or more at-risk alleles having more than a 2-fold increase in risk (OR, 2.39; 95% CI, 1.09-5.26; P trend = 0.02) compared with those with no at-risk alleles. Further adjustment for β -carotene, vitamins C and E, calcium, and zinc intake, as well as fruit and

Table 2. *Nrf2*, *NQO1*, and *HO-1* genotype and breast cancer risk by tertile of total (dietary and supplemental) iron intake

	Iron intake*								
	≤ 9.6 mg/d			$>9.6-22.5$ mg/d			$>22.5-98.2$ mg/d		
	Ca	Co	OR (95% CI) [†]	Ca	Co	OR (95% CI)	Ca	Co	OR (95% CI)
<i>Nrf2</i> [‡]									
CC	74	77	1 (referent)	70	85	1 (referent)	80	85	1 (referent)
CT	54	51	1.18 (0.71-1.96)	57	47	1.47 (0.87-2.47)	64	53	1.37 (0.84-2.22)
TT	13	15	0.95 (0.41-2.19)	13	15	1.09 (0.47-2.52)	10	7	1.69 (0.58-4.92)
			P trend = 0.81			P trend = 0.37			P trend = 0.15
<i>NQO1</i>									
CC	90	86	1 (referent)	96	94	1 (referent)	101	100	1 (referent)
CT	46	47	0.96 (0.57-1.61)	41	48	0.83 (0.49-1.40)	45	39	1.16 (0.69-1.96)
TT	4	8	0.52 (0.15-1.84)	2	5	0.46 (0.08-2.64)	6	4	1.59 (0.41-6.13)
			P trend = 0.46			P trend = 0.30			P trend = 0.42
<i>NOS3</i>									
GG	69	61	1 (referent)	65	70	1 (referent)	74	73	1 (referent)
GT	52	63	0.75 (0.45-1.26)	65	63	1.11 (0.67-1.84)	56	55	1.03 (0.62-1.71)
TT	15	13	1.11 (0.48-2.56)	11	10	1.10 (0.42-2.88)	20	11	1.92 (0.83-4.43)
			P trend = 0.71			P trend = 0.70			P trend = 0.23
<i>HO-1</i>									
LL	45	45	1 (referent)	50	47	1 (referent)	50	48	1 (referent)
LM	13	14	0.79 (0.31-1.98)	19	16	1.18 (0.53-2.65)	20	17	1.00 (0.46-2.16)
LS	48	46	0.97 (0.53-1.78)	36	60	0.53 (0.29-0.97)	41	53	0.63 (0.35-1.11)
MM	2	5	0.33 (0.06-1.84)	4	2	1.33 (0.21-8.38)	3	3	0.78 (0.14-4.29)
MS	11	9	1.26 (0.47-3.42)	11	7	1.34 (0.46-3.87)	8	14	0.50 (0.19-1.33)
SS	15	18	0.69 (0.30-1.60)	12	15	0.77 (0.31-1.86)	19	9	1.69 (0.69-4.16)
LS + MM + MS + SS	76	78	1 (referent)	63	84	1 (referent)	71	79	1 (referent)
LL + LM	58	59	1.06 (0.64-1.74)	69	63	1.59 (0.96-2.63)	76	65	1.36 (0.85-2.18)
High-risk alleles or genotypes [§]									
0	18	16	1 (referent)	8	17	1 (referent)	14	18	1 (referent)
1	31	34	0.90 (0.38-2.12)	37	39	2.26 (0.83-6.17)	42	50	1.13 (0.49-2.58)
2	50	50	0.95 (0.43-2.10)	55	49	2.83 (1.06-7.53)	44	43	1.51 (0.65-3.47)
3+	43	43	1.08 (0.47-2.46)	40	44	2.24 (0.83-6.04)	54	35	2.27 (0.97-5.29)
			P trend = 0.73			P trend = 0.26			P trend = 0.02

*Energy-adjusted daily total iron intake (diet and supplement) was calculated as energy-adjusted dietary intake plus iron intake from multivitamin supplement use. Intake categories represent tertile of intake in the control group.

[†]OR and 95% CI calculated by unconditional logistic regression adjusted for age, family history of breast cancer (yes, no), hormone replacement therapy (yes, no), body mass index (continuous, log transformed), age at menarche, age at menopause, smoking status (ever/never), race (Caucasian, other), parity (yes/no). Among controls, 67 were excluded from the analyses because of missing diet information ($n = 21$) and missing covariate information ($n = 46$). Among cases, 66 were excluded from the analysis because of missing diet information ($n = 22$) and missing covariate information ($n = 44$).

[‡]To test multiplicative interactions, a cross-product term for genotype (as a continuous variable indicating number of variant alleles) and iron intake (tertile group) was included in multivariate models. P for multiplicative interaction is $P = 0.57$ for *Nrf2*, $P = 0.36$ for *NQO1*, $P = 0.55$ for *NOS3*, $P = 0.52$ for *HO-1* (LL + LM versus LS + MM + MS + SS), and $P = 0.30$ for total number of at-risk alleles or genotypes.

[§]Sum total of high-risk alleles or genotypes, which were taken to be the *Nrf2* T allele, the *NQO1* T allele, the *NOS* T allele, and the *HO-1* LL and LM genotypes.

vegetable consumption, did not change risk estimates (data not shown). Among nonsupplement users, there was indication of a 2-fold increase in risk for those consuming >9.60 mg/d iron, but a gene-dose effect was not observed. There were no associations among women consuming ≤9.60 mg/d iron.

Discussion

In this nested case-control study from the large population-based CPS-II nutrition cohort, we found that genetic variations in *Nrf2*, *NQO1*, *NOS3*, and *HO-1* were not significantly associated with postmenopausal breast cancer risk. A significant dose trend, however, was observed for total number of at-risk alleles, with those carrying three or more at-risk alleles having a 56% increase in breast cancer risk compared with those having no high-risk variants. Among cases and controls, respectively, 30.9% and 27.7% of participants had at least three or more at-risk alleles. When genetic variants were examined according to level of dietary and supplemental iron intake, clear gene-dose effects were evident in the

highest tertile of total iron consumption as well as in supplement users, with carriage of three or more high-risk alleles resulting in more than a 2-fold increase in breast cancer risk compared with women with no high-risk allele. Among supplement users, the *HO-1* *LL* and *LM* genotypes were also significantly associated with a 56% increased breast cancer risk compared with carriers of *S* alleles or two *M* alleles. These findings support the hypothesis that women with genotypes resulting in potentially higher levels of iron-generated oxidative stress and consuming a pro-oxidative diet high in iron may be at increased risk of breast cancer.

We used the availability of functional data to designate "high-risk" alleles for *NQO1*, *NOS3*, and *HO-1*. Enzyme products for these genes all have antioxidant properties, and therefore, alleles considered high risk were those shown functionally to result in lower enzyme activities (31, 32, 50, 63, 64, 80), presumably leading to limited ability to reduce levels of iron-generated and noniron-generated oxidative stress. Without functional data, the effect of the *Nrf2* variant allele on breast cancer risk was unknown and was determined post hoc to be a high-risk allele based on risk estimates above unity in women with

Table 3. *Nrf2*, *NQO1*, *NOS3*, and *HO-1* genotype and breast cancer risk by use of multivitamins containing iron

	Nonsupplement users									Supplement users,* total daily iron intake (10.5-98.2 mg)		
	All			Total daily iron intake						Ca	Co	OR (95% CI)
	Ca	Co	OR (95% CI) [†]	4.8 to ≤9.6 mg			>9.6-22.5 mg					
Ca	Co	OR (95% CI) [†]	Ca	Co	OR (95% CI)	Ca	Co	OR (95% CI)	Ca	Co	OR (95% CI)	
<i>Nrf2</i> [‡]												
CC	134	155	1 (referent)	74	77	1 (referent)	56	75	1 (referent)	95	95	1 (referent)
CT	100	90	1.31 (0.90-1.90)	54	51	1.18 (0.71-1.96)	46	38	1.66 (0.92-3.00)	75	62	1.28 (0.81-2.00)
TT	23	26	1.02 (0.55-1.90)	13	15	0.95 (0.41-2.19)	10	11	1.43 (0.54-3.78)	13	11	1.17 (0.48-2.83)
			<i>P</i> trend = 0.43			<i>P</i> trend = 0.81			<i>P</i> trend = 0.15			<i>P</i> trend = 0.37
<i>NQO1</i>												
CC	171	167	1 (referent)	90	86	1 (referent)	77	77	1 (referent)	120	117	1 (referent)
CT	78	90	0.82 (0.56-1.19)	46	47	0.96 (0.57-1.61)	32	43	0.73 (0.41-1.32)	55	44	1.24 (0.77-2.01)
TT	6	11	0.57 (0.20-1.60)	4	8	0.52 (0.15-1.84)	2	3	0.93 (0.14-6.14)	6	6	0.97 (0.29-3.24)
			<i>P</i> trend = 0.16			<i>P</i> trend = 0.46			<i>P</i> trend = 0.36			<i>P</i> trend = 0.52
<i>NOS3</i>												
GG	124	122	1 (referent)	69	61	1 (referent)	52	59	1 (referent)	88	84	1 (referent)
GT	105	118	0.91 (0.63-1.32)	52	63	0.75 (0.45-1.26)	52	53	1.19 (0.68-2.08)	67	67	0.99 (0.63-1.58)
TT	22	23	0.94 (0.49-1.79)	15	13	1.11 (0.48-2.56)	7	10	0.77 (0.26-2.32)	24	11	2.18 (0.99-4.81)
			<i>P</i> trend = 0.67			<i>P</i> trend = 0.71			<i>P</i> trend = 0.94			<i>P</i> trend = 0.16
<i>HO-1</i>												
LS + MM + MS + SS	132	149	1 (referent)	76	78	1 (referent)	54	69	1 (referent)	80	94	1 (referent)
LL + LM	112	115	1.15 (0.80-1.65)	58	59	1.06 (0.64-1.74)	52	54	1.35 (0.77-2.35)	94	74	1.56 (1.01-2.41)
High-risk alleles or genotypes [§]												
0	26	31	1 (referent)	18	16	1 (referent)	7	14	1 (referent)	15	21	1 (referent)
1	63	71	1.03 (0.55-1.94)	31	34	0.90 (0.38-2.12)	29	35	1.86 (0.62-5.63)	50	54	1.37 (0.63-3.00)
2	97	89	1.28 (0.70-2.35)	50	50	0.95 (0.43-2.10)	47	39	3.06 (1.04-8.96)	53	53	1.55 (0.71-3.40)
3+	72	81	1.08 (0.58-2.00)	43	43	1.08 (0.47-2.46)	29	37	1.90 (0.63-5.70)	65	42	2.39 (1.09-5.26)
			<i>P</i> trend = 0.69			<i>P</i> trend = 0.73			<i>P</i> trend = 0.32			<i>P</i> trend = 0.02

*Supplementary iron intake was based on iron intake from regular multivitamin use and divided into nonusers and users.

[†]OR and 95% CI. All models were adjusted for age, family history of breast cancer (yes, no), hormone replacement therapy (yes, no), body mass index (continuous, log transformed), age at menarche, age at menopause, smoking status (ever/never), race (Caucasian, other), and parity (yes/no). Among controls, 63 were excluded from the analyses because of missing supplement information (*n* = 16) and missing covariate information (*n* = 47). An additional 8 participants (4 cases and 4 controls) were excluded from analyses assessing high and low iron intake among nonsupplement users because of missing dietary information. Among cases, 61 were excluded from the analysis because of missing supplement information (*n* = 17) and missing covariate information (*n* = 44).

[‡]*P* for multiplicative interaction comparing supplement users with all nonusers is *P* = 0.74 for *Nrf2*, *P* = 0.18 for *NQO1*, *P* = 0.19 for *NOS3*, *P* = 0.32 for *HO-1* (LL + LM versus LS + MM + MS + SS), and *P* = 0.11 for total number of high-risk alleles or genotypes.

[§]Sum total of high-risk alleles or genotypes, which were taken to be the *Nrf2* T allele, the *NQO1* T allele, the *NOS3* T allele, and the *HO-1* LL and LM genotypes.

high iron intake. Although the predicted relationship with breast cancer risk is least certain for this allele, its designation as a minor high-risk allele is consistent with the empirical evidence that rarer variants associated with disease will on average confer larger risks (81).

Several findings from our study suggest that the level of iron intake may be more closely associated with breast cancer risk than the source of iron (dietary or supplements). With both *HO-1 LL* and *LM* genotypes, as well as total number of at-risk alleles, breast cancer risk was nonsignificantly raised among nonsupplement users with high iron intake from foods but near unity for those with low intakes. Thus, stronger associations observed among supplement users are likely due to higher levels of iron intake in this group. In assessing interactions with source of dietary iron, we did not separate out intakes of heme and nonheme iron because these levels are difficult to determine accurately with no available database that lists heme and nonheme iron content of foods. However, our observation of elevated breast cancer risk among supplement users suggests that nonheme iron can contribute to increased breast cancer risk, and nonheme iron (from supplement use) has been positively associated with serum ferritin levels (82).

Our findings indicate that genetic variation in genes important for generating and removing iron-generated ROS is important in breast carcinogenesis primarily among those with higher iron intake and, furthermore, that associations with breast cancer risk are strongest when cumulative effects of at-risk alleles are considered. These findings support the notion that multiple genetic "failures" in pathways determining oxidative status are required to raise overall breast cancer risk, with each gene contributing a modest effect. Given that carcinogenesis is a multigenic process, it is unlikely that any one single genetic polymorphism would have a dramatic effect on cancer risk, and many studies to date examining single genes have had limited ability in reproducibly explaining breast cancer risk. By summing high-risk alleles within a hypothesized pathway, we postulate that the effects of individual polymorphisms are amplified (83). This approach assumes the equal contribution of each variant allele to risk, and although this is an oversimplification, the approach provides a proof of principle that cumulative defects increase disease risk. The method has been successfully used to assess bladder cancer risk and DNA repair and cell cycle control genes (84) as well as breast cancer risk associated with functional variants in *BRCA1*, *BRCA2*, and *ATM* genes (85). For association studies, a more comprehensive pathway-based multigenic approach combining multiple polymorphisms has been proposed as potentially better able to deliver more precise delineation of risk groups than assessment of single polymorphisms (84), leading possibly to greater reproducibility of findings.

Failure of previous studies to account for multiple gene effects and for iron intake and/or other contributors to oxidative stress may have led to dilution of gene effects as risk factors for breast cancer and to conclusions that particular genes are not associated with disease risk (73, 74, 86-91). Furthermore, population differences in the underlying prevalence of pro-oxidative exposures may be a key determinant in whether genetic variants have an effect on breast cancer risk and may explain some of the inconsistencies observed across studies.

Similarly, inconsistencies in the literature evaluating relationships between dietary iron and breast cancer risk may, in part, arise from failure to account for genetic variation and other endogenous factors affecting oxidative stress as well as failure to consider both dietary and supplemental iron intake. Few epidemiologic studies have examined the relationship between iron intake and breast cancer risk, and none has considered genetic variation as an effect modifier or considered the use of supplemental iron (24, 25, 92).

Among women taking supplemental iron, relationships between breast cancer risk and individual genetic variants were significant only for *HO-1*. An association between long *HO-1* alleles and breast cancer risk is a novel observation and consistent with previous findings showing the long *HO-1* allele to be a risk factor for chronic pulmonary emphysema (63), lung adenocarcinoma (93), and oral squamous cell carcinoma (94). Induction of *HO-1* is postulated to be cytoprotective due to decreased levels of pro-oxidant heme, increased levels of antioxidant bilirubin (52), and up-regulation of ferritin leading to rapid iron sequestration (95, 96). HO may also decrease cellular iron levels by up-regulation of an iron pump, which increases cellular iron efflux (96), and reduce oxidative stress by up-regulating other antioxidant systems, including superoxide dismutase, catalase, and NOS3 activity (97-99). HO, however, seems to have dual roles, and various studies show that induction of HO is not always beneficial and may lead to pro-oxidative states (100) due to release of ferrous iron (98) and H₂O₂ as by-products (101). This may, in part, explain why individuals in the highest tertile of iron consumption carrying two short *HO-1* alleles were found to be at nonsignificantly elevated risk of breast cancer compared with *LL* individuals. In support of this, homozygous carriers of short repeats have been found to be at greater risk of malignant melanoma compared with *L* and *M* allele carriers (102).

One limitation of the study was that we did not have blood iron measures and therefore could not adjust for serum transferrin saturation levels as an indicator of body stores, which may have attenuated our study results. It is possible that reduced ability to detoxify iron-generated ROS is most pronounced against a background of high dietary iron intake and high transferrin saturation. In an analysis of the National Health and Nutrition Examination Survey I Epidemiologic Follow-up Study, high iron intake was only associated with increased risk of cancer incidence and mortality among those with increased transferrin saturation (103). Those taking supplements regularly, however, might be expected to have high iron stores (26, 82).

Next, we cannot exclude the possibility that other nutrients correlated with iron intake or other nutrients found in multivitamin supplements might be responsible for raising breast cancer risk among those with lower ROS reduction capabilities. However, this possibility seems less likely because our findings were similar for supplement users and for nonusers with high dietary iron intake.

There is a possibility that women who volunteered blood specimens as a subset of the main CPS-II nutrition cohort were different from those who did not. Potential participation bias, however, was unlikely to have affected our findings because the women who provided

blood specimens were similar to the overall population in the distribution of most demographic and lifestyle characteristics (104). As well, the prevalence of regular multivitamin use among women in our nested case-control study was 42% and 39% in cases and controls, respectively, similar to the reported rate of 42.4% in the main study cohort (105). The mean dietary iron intake in our study population of 9.80 mg/d was almost identical to levels reported for the 75,272 white women in the CPS-II nutrition cohort of 9.82 mg/d (65).

A key strength of this study was the nested case-control design in a large prospective cohort, which insured that dietary data captured represented intake patterns before breast cancer development. Although we did not update dietary information using questionnaires collected in 1999 (because many of the cases had already been diagnosed), other studies have shown that middle-aged people maintain a stable nutrient intake for long periods (106). In addition, because baseline characteristics of this study population are largely comparable with those in the large parent population-based cohort (104), our results are unlikely biased by case-control selection. The main limitation of this study was the relatively small sample size, particularly when assessing interaction by iron intake. Thus, findings from this study need to be confirmed in larger studies.

From a public health perspective, our findings that a substantial subpopulation of postmenopausal women may be susceptible to iron-generated oxidative stress indicate that it may be prudent for postmenopausal women to adhere to current recommended dietary allowance guidelines of 8 mg/d for this group. In our study, all women who took an iron-containing multivitamin supplement had iron intakes ≥ 10.5 mg/d, putting them above recommended dietary allowance levels; further, all women in the highest tertile of iron intake took iron-containing supplements. Further study is needed to evaluate and refine risk estimates among nonsupplement users with dietary intakes above recommended levels in at-risk groups.

In summary, our findings show that women with lower overall ROS reduction capabilities, as indicated by total number of at-risk alleles in *Nrf2*, *NQO1*, *NOS3*, and *HO-1*, may be at increased risk for postmenopausal breast cancer when levels of dietary iron are high, particularly at levels usually observed with supplemental iron intake. Further study is required to assess the additional role of body iron stores, if any. This is the first study to show using a multigenic model that polymorphisms associated with iron-generated oxidative stress may be important in breast cancer etiology.

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