Manganese Superoxide Dismutase Polymorphism, Plasma Antioxidants, Cigarette Smoking, and Risk of Breast Cancer

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Abstract

Oxidative stress may be involved in the development of breast cancer. Manganese superoxide dismutase (MnSOD) is one of the primary enzymes that directly scavenge potential harmful oxidizing species. A valine (Val) to alanine (Ala) substitution at amino acid 16, occurring in the mitochondrial targeting sequence of the *MnSOD* gene, has been associated with an increase in breast cancer risk. We conducted a nested case-control study within the Nurses' Health Study cohort to examine the role of this polymorphism and its interaction with environmental factors with breast cancer risk. *MnSOD* genotype data are available from 968 incident cases of breast cancer diagnosed after blood collection in 1989 and 1990, but before June 1, 1998 and 1,205 matched controls. Compared with women homozygous for the Val allele, women homozygous for the Ala allele were not at an increased risk of breast cancer (multivariate odds ratio, 0.96; 95% confidence interval, 0.74-1.24). We did not observe any significant interactions between *MnSOD* genotype with alcohol consumption, postmenopausal hormone use, plasma antioxidant levels, or dietary sources of antioxidants. We did observe evidence that the *MnSOD* Ala allele may modify the relation of cigarette smoking with breast cancer risk. A nonsignificant increased risk of breast cancer among current smokers was limited to women homozygous for the Ala alleles compared with Val/Val never smokers (multivariate odds ratio, 1.41; 95% confidence interval 0.77-2.60; *P* for interaction = 0.03). These data suggest that the Ala allele of *MnSOD* may modify breast cancer risk among current smokers, but is not an independent risk factor for breast cancer. (Cancer Epidemiol Biomarkers Prev 2004;13(6):989–96)

Introduction

Evidence indicates that oxidative stress may be involved in the development of breast cancer (1). In the cell, there exists a balance between reactive oxygen species (ROS) and oxidative defenses. ROS are generated through the metabolism of estradiol, polyunsaturated fats, ethanol, and calories, all of which have been considered potential risk factors for the development of breast cancer (2). In addition to dietary antioxidants, there are endogenous mechanisms, primarily antioxidant enzymes, such as manganese superoxide dismutase (MnSOD), catalase, and glutathione peroxidase, which scavenge ROS (3). Together, variation in genetic makeup of antioxidant enzymes and environmental exposure to ROS may play a role in breast cancer development.

MnSOD is one of the primary enzymes that directly converts potential harmful oxidizing species to harmless metabolites (4). The importance of *MnSOD* in cancer and tumorigenesis is supported by substantial data, implicating it as a tumor suppressor gene. It has been reported that in many tumors, chromosome 6, particularly 6q25, the region to which *MnSOD* maps, is often lost (5). Many types of cancer cells have reduced MnSOD activity compared with normal cells (6). Overexpression of the *MnSOD* gene *in vitro* has been shown to reverse the transformed phenotype, to increase apoptosis, to prevent induced neoplastic transformation, and to reduce metastatic potential in a various cell types including breast cancer (5-8).

A valine (Val) to alanine (Ala) substitution at amino acid 16 occurs in the mitochondrial targeting sequence of the *MnSOD* gene (9). This signal sequence is hypothesized to target proteins encoded in the nucleus to different sites of action in the cell. Rosenblum et al. (9) predict that the Val to Ala substitution alters the secondary structure of the protein, which may affect the localization and transport of the enzyme into the mitochondria, where it exerts its antioxidant action. Recent *in vitro* data support this hypothesis. Sutton et al. (10) reported that *MnSOD* Ala alleles were 30% to 40% more efficiently localized to the mitochondrial matrix compared with *MnSOD* Val alleles. In contrast, Hong et al. (11) reported that urine concentrations of 8-hydroxydeoxyguanosine (8-OH-dG), a marker of

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oxidative DNA damage, were significantly increased in women with *MnSOD* Ala alleles, providing physiologic evidence that this polymorphism is an important modifier of oxidative stress.

Three studies to date have examined the role of this polymorphism and risk of breast cancer. Ambrosone et al. (2) reported that premenopausal women (cases = 114) who were homozygous for the Ala allele had a relative risk of 4.3 [95% confidence interval (CI), 1.7-10.8] as compared with women with a Val allele. The authors observed that the Ala allele of the *MnSOD* gene had a more pronounced deleterious effect in premenopausal women who consumed the lowest level of total fruits and vegetables (2).

Mitrunen et al. (12) conducted a similar study examining the same polymorphism and risk of breast cancer in a Finnish population (cases = 479). This group also observed an increased risk of breast cancer associated with the Ala allele, although the associations were more modest than those reported by Ambrosone et al. They reported that having an Ala allele increased the risk of breast cancer by 50% [odds ratio (OR) = 1.5 (95% CI, 1.1-2.0)], although the positive association was observed primarily in postmenopausal women.

The most recent study by Egan et al. (13) did not support an overall association between this polymorphism and breast cancer in a population-based case-control study (cases = 476).

We evaluated the role of *MnSOD* genotype and breast cancer in the Nurses' Health Study and investigated potential interactions with this *MnSOD* polymorphism and environmental factors hypothesized to play a role in oxidative stress including plasma antioxidant levels, cigarette smoking, and alcohol consumption.

Materials and Methods

Study Design and Population. We conducted a nested case-control study within the Nurses' Health Study cohort. The Nurses' Health Study was initiated in 1976, when 121,700 US registered nurses ages 30 to 55 returned an initial questionnaire. Every 2 years, the information on reproductive variables, cigarette smoking, postmenopausal hormone use, and dietary information (since 1980) are updated. Incident breast cancer cases were identified through self-report and are confirmed by medical record review. Between 1989 and 1990, blood samples were collected from 32,826 women. In general, blood samples were returned within 26 hours of blood being drawn; immediately centrifuged; aliquoted into plasma, red blood cells, and buffy coat fractions; and stored in liquid nitrogen freezers.

Eligible cases in this study consist of women with pathologically confirmed incident breast cancer from the subcohort of women who returned a blood sample and were diagnosed by June 1, 1998. Cases were excluded if they had any other prior cancer diagnosis except for non-melanoma skin cancer. Controls were randomly selected from the subcohort of women returning a blood sample and had no diagnosis of cancer except for non-melanoma skin cancer. Controls were matched to cases on year of birth, menopausal status, postmenopausal hormone use at time of blood draw, time of day, month, and fasting status at time of blood draw. To maximize logistical efficiency of the overall study design, selection of breast cancer cases and controls included in this study is identical to those involved in plasma hormone analyses. Although blood draw characteristics are unlikely to confound the MnSOD-breast cancer relationship, matching on these characteristics is necessary for analyses involving plasma hormones. This nested case-control study consists of a total of 968 incident cases and 1,205 controls for which *MnSOD* genotype data are available.

Genotype and Laboratory Data. DNA was extracted from buffy coat fractions using the Qiagen QIAamp Blood kit (Qiagen, Chatsworth, CA). Genotyping of the Val to Ala polymorphism of *MnSOD* was conducted using four different methods, reflective of the highest throughput method available in the laboratory at the time samples became available. Cases and their matched controls were always genotyped at the same time by the same method. For each genotyping method, a random 10% of the samples were included in the 96-well plates as duplicates to serve as quality control samples. The quality control samples served as internal controls to validate the genotyping methods; there was 100% concordance of the quality control samples. Lab personnel were blinded to the status (case, control, or quality control) of samples.

Genotyping of the Val to Ala polymorphism was completed for cases and controls collected up until June 1, 1992 by fluorescent allele specific PCR (Appendix 1; ref. 14). Cases identified between June 1, 1992 and June 1,1994 and their matched controls were genotyped by dideoxy terminator sequencing on an ABI Prism 377 DNA sequencer (Applied Biosystems, Foster City, CA; Appendix 2). The genotyping of cases identified between June 1, 1994 and June 1, 1996 and their matched controls were conducted by Pyrosequencing (Appendix 3; ref. 15). Cases identified between June 1, 1996 and June 1, 1998 and matched controls were genotyped using TaqMan technology (Applied Biosystems; Appendix 4).

Plasma antioxidants (α-carotene, β-carotene, β-cryptoxanthin, lycopene, lutein/zeaxanthin, retinol, α-tocopherol, γ-tocopherol) were assessed by reversed-phase high-performance liquid chromatography (HPLC) methods described by El-Sohemy et al. (16). Coefficients of variation from quality control samples of the plasma antioxidants included in this data set had a median of 7.4% (range 7.1% to 11.0%). Lutein and zeaxanthin are isomers and are not separated by the method used; they were analyzed together as lutein/zeaxanthin. Total carotenoids in this analysis are the sum of individual concentrations of α-carotene, β-carotene, β-cryptoxanthin, lycopene, and lutein/zeaxanthin. Plasma folate levels were determined by radioassay kit (Bio-Rad, Richmond, CA) at the Jean Mayer USDA Human Nutrition Research Center on Aging at Tufts University. The mean coefficient of variation from 75 pairs of replicate samples included in these assays was 6.5 % for plasma folate (17).

Statistical Analysis. A Mantel-Haenszel χ^2 test across matched case-control sets was used to determine differences in genotype frequencies between cases and controls.

ORs and 95% CIs were determined by using both conditional and unconditional logistic regression. Indicator

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variables were created to code for the three possible genotypes (Val/Val, Val/Ala, Ala/Ala). The Val/Val genotype was designated as the reference group, because it is hypothesized to be the genotype with the lowest risk (2, 12). Genotype was also evaluated as a dichotomous variable grouping the Val/Val and Val/Ala genotypes together as the reference group, for comparison with data from Ambrosone et al. (2).

As described previously, cases and controls were matched on potential confounders and blood draw characteristics, as was necessary for other analyses requiring plasma. Although blood draw characteristics are unlikely to be related to *MnSOD* genotype or breast cancer risk, and results did not differ when variables related to blood draw were excluded, all analyses were conducted taking into account this matching. In addition to the matching factors, we controlled in multivariate analyses for the following potential confounders and breast cancer risk factors: body mass index (BMI) at age 18 (kg/m^2, continuous), weight gain since age 18 (<5, ≥5 to <20, ≥20 kg), age at menarche (<12 years, 12, 13, >13), parity/age at first birth (nulliparous, one to four children/age at first birth ≤24, one to four children/age at first birth >24, five or more children/age at first birth ≤24, five or more children age at first birth >24), family history of a first-degree relative (yes/no), and personal history of benign breast disease (yes/no). Analyses including postmenopausal women were also adjusted for age at menopause (≤45 years, 46 to 50, 51 to 60) and duration of postmenopausal hormone use (never, past use <5 years duration, past use ≥5 years duration, current use <5 years duration, current use ≥5 years duration).

Interactions between genotype and breast cancer risk factors were assessed by conducting two statistical tests of interaction. First, we used a likelihood ratio test (LRT) to compare the multivariate models with each level of genotype cross-classified with each level of breast cancer risk factor to the model with indicator variables for both the *MnSOD* genotype and breast cancer risk factor main effects (18). This test, which we will refer to as a nominal LRT (LRT_1), makes no assumptions of order in the exposure variables. In addition, we used a LRT to determine statistical significance of the interaction terms assuming an order of the *MnSOD* genotype and of the stratification variable, if a natural order could be assumed. We will refer to this test as an ordinal LRT (LRT_2). In some instances, a natural order was not assumed, as was the case with the smoking variables. In this case, the ordinal LRT maintains the assumption of order on genotype, but treats smoking exposure levels as nominal.

Results

MnSOD genotype data were available for 968 cases and 1,205 controls. At the time of blood collection, 460 women were premenopausal with a mean age of 48.6 (SD = 3.28) and 1,502 women were postmenopausal with a mean age of 60.8 (SD = 5.10).

Compared with controls, cases tended to have an earlier age at menarche, a later age at first birth, later age at menopause, lower mean parity, a lower BMI, and a greater weight gain since age 18, although none of these differences were statistically significant (Table 1). Cases were significantly more likely to have a personal history of benign breast disease as compared with controls, and were also more likely to have a family history of breast cancer (Table 1).

The genotype frequencies among the controls were consistent with those predicted under Hardy-Weinberg equilibrium (P = 0.58). Among controls, the prevalence of the Ala allele was 50%, which is identical to a previous report among a similar population of primarily Caucasian women in the US (50%; ref. 2). There was no significant difference in genotype distributions between cases and controls ($\chi^2_{\text{Cochran-Mantel-Haenzel}}$ = 1.12; df = 2; P = 0.57).

We observed little evidence that women carrying the Ala *MnSOD* allele were at an increased risk of breast cancer (Table 2). Women heterozygous (multivariate OR, 0.88; 95% CI, 0.70-1.10) or homozygous for the Ala allele (multivariate OR, 0.96; 95% CI, 0.74-1.24) were not at an increased risk of breast cancer compared with women homozygous for the wild-type Val allele. The association between *MnSOD* genotype and breast cancer was not statistically different for premenopausal and postmenopausal women (P for interaction = 0.86[LRT_1]).

We investigated the risk of breast cancer associated with *MnSOD* genotype stratified by smoking status and alcohol consumption, lifestyle factors hypothesized to contribute to oxidative stress. The risk of breast cancer associated with *MnSOD* genotype was modified by smoking status (P for interaction = 0.03[LRT_1], 0.006[LRT_2]; Table 3). The significant interaction appeared to be due to both an increased risk of breast cancer among Ala/Ala current smokers (multivariate OR, 1.41; 95% CI, 0.77-2.60) and a reduced risk of breast cancer among Val/Val current smokers (multivariate OR, 0.41; 95% CI, 0.21-0.81) compared with wild-type never smokers. Women homozygous for the Ala allele who currently smoke 15 or more cigarettes per day had an 80% greater risk (multivariate OR, 1.82; 95% CI, 0.83-3.97) of developing breast cancer compared with Val/Val nonsmokers. Among current heavy smokers, there was a marginally significant trend (P = 0.08) of breast cancer with increasing number of Ala alleles; women with two

Table 1. Selected characteristics of breast cancer cases and controls in the Nurses' Health Study

	Cases (n = 968)	Controls (n = 1,205)
Age at menarche (y)*	12.5	12.6
Parity*	2.91	2.98
Age at first birth (y)*,†	25.1	24.9
Age at menopause (y)*,‡	48.2	48.0
BMI (kg/m^2)*	25.4	25.5
Weight gain since age 18 (kg)*	11.7	11.4
Personal history of benign breast disease (%)§	64.9	49.5
Family history of breast cancer (%)§	21.1	13.7

*Mean values.
†Among parous women.
‡Among postmenopausal women.
§P value = 0.001.

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Table 2. Association between *MnSOD* genotype and risk of breast cancer in the Nurses' Health Study (1989 to 1998)

	Cases *N* (%)	Controls *N* (%)	OR (95% CI)	Multivariate OR (95% CI)
All women				
Val/Val	255 (26.3)	297 (24.7)	1.00 (Ref)*	1.00 (Ref)†
Ala/Val	468 (48.4)	612 (50.8)	0.90 (0.72-1.11)	0.88 (0.70-1.10)
Ala/Ala	245 (25.3)	296 (24.6)	0.96 (0.75-1.23)	0.96 (0.74-1.24)
Premenopausal at diagnosis‡				
Val/Val	24 (24.2)	26 (23.6)	1.00 (Ref)§	1.00 (Ref)‖
Ala/Val	42 (42.4)	54 (49.1)	0.85 (0.42-1.72)	0.83 (0.39-1.77)
Ala/Ala	33 (33.3)	30 (27.3)	1.20 (0.56-2.57)	1.02 (0.45-2.29)
Postmenopausal at diagnosis‡				
Val/Val	217 (27.1)	254 (25.0)	1.00 (Ref)§	1.00 (Ref)¶
Ala/Val	391 (48.8)	518 (51.0)	0.89 (0.71-1.12)	0.89 (0.70-1.12)
Ala/Ala	194 (24.2)	244 (24.0)	0.95 (0.73-1.24)	0.95 (0.72-1.24)

*Conditional logistic regression estimates adjusted for matching variables: age, menopausal status, postmenopausal hormone use, date of blood draw, time of blood draw, and fasting status at blood draw.
†Conditional logistic regression adjusted for the matching factors and BMI at age 18, weight gain since age 18, age at menarche, personal history of benign breast disease, first-degree family history of breast cancer, age at first birth/parity, age at menopause, and duration of postmenopausal hormone use.
‡Premenopausal and postmenopausal women do not add to the total number of women, because women with uncertain menopausal status at diagnosis were excluded.
§Unconditional logistic regression adjusted for matching factors.
‖Unconditional logistic regression adjusted for the matching factors and BMI at age 18, weight gain since age 18, age at menarche, personal history of benign breast disease, first-degree family history of breast cancer, and age at first birth/parity.
¶Unconditional logistic regression estimates adjusted for the variables above and age at menopause, and duration of postmenopausal hormone use.

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Ala alleles had a greater than 4-fold increased risk of breast cancer compared with those with no variant alleles (multivariate OR, 4.53; 95% CI, 0.89-23.16).

To better understand the interaction between smoking and *MnSOD* genotype, we also examined duration of smoking as a potential effect modifier (Table 3). The increased risk of breast cancer among Ala/Ala women appeared to be restricted to current smokers, with current smokers for 40 or more years at the greatest risk. In an effort to assess latency, we examined the interaction between *MnSOD* and smoking status 10 years before diagnosis (P for interaction = 0.02[LRT_1], 0.002[LRT_2]). The analysis suggested that women homozygous for the Ala allele were at an increased risk of breast cancer if

Table 3. ORs and 95% CIs for breast cancer according to *MnSOD* genotype and smoking characteristics in the Nurses' Health Study (1989 to 1998)

	MnSOD genotype									*P* for interaction	
	Val/Val			Val/Ala			Ala/Ala				
	Cases	Controls	OR (95% CI)*	Cases	Controls	OR (95% CI)*	Cases	Controls	OR (95% CI)*	LRT_1†	LRT_2‡
Smoking status at diagnosis§										0.03	0.006
Never	124	135	1.00 (Ref)	205	289	0.79 (0.58-1.09)	110	148	0.84 (0.58-1.20)		
Past	116	124	1.06 (0.74-1.53)	216	254	0.94 (0.68-1.29)	102	119	0.95 (0.65-1.39)		
Current	15	38	0.41 (0.21-0.81)	45	59	0.83 (0.52-1.35)	32	24	1.41 (0.77-2.60)		
<15 cigarettes/d	7	19	0.36 (0.14-0.92)	12	23	0.58 (0.27-1.27)	11	12	1.01 (0.42-2.45)	0.12	0.02
15+ cigarettes/d	8	19	0.47 (0.19-1.16)	33	36	0.99 (0.57-1.72)	21	12	1.82 (0.83-3.97)		
Smoking duration (y)§											
Never	124	135	1.00 (Ref)	205	289	0.79 (0.58-1.09)	110	148	0.83 (0.58-1.20)	0.05	0.03
Past <20	56	64	0.98 (0.62-1.54)	92	136	0.72 (0.49-1.05)	55	60	1.01 (0.64-1.60)		
Past 20+	58	59	1.12 (0.71-1.77)	122	114	1.22 (0.84-1.76)	44	59	0.83 (0.51-1.34)		
Current, <40	10	20	0.50 (0.22-1.16)	24	29	0.80 (0.43-1.50)	13	12	1.17 (0.50-2.76)		
Current, 40+	5	18	0.30 (0.11-0.87)	21	30	0.86 (0.45-1.63)	18	12	1.55 (0.69-3.47)		
Smoking status 10 y prior§										0.02	0.002
Never	124	136	1.00 (Ref)	205	289	0.80 (0.58-1.10)	110	148	0.84 (0.59-1.21)		
Past	96	101	1.11 (0.75-1.63)	169	207	0.88 (0.63-1.23)	66	95	0.76 (0.50-1.15)		
Current, <15	12	20	0.63 (0.29-1.38)	20	28	0.93 (0.48-1.78)	24	15	2.08 (1.01-4.28)		
Current, 15+	23	40	0.63 (0.35-1.14)	72	78	1.05 (0.69-1.61)	44	33	1.48 (0.87-2.53)		

*Adjusted for matching factors, BMI at age 18, weight gain since age 18, age at menarche, personal history of benign breast disease, first-degree family history of breast cancer, age at first birth/parity, age at menopause, and duration of postmenopausal hormone use and alcohol consumption.
†LRT comparing the main effects model to the model with each level of smoking cross-classified with *MnSOD* genotype.
‡LRT of the interaction terms between smoking status and MnSOD genotype, where only genotype is ordered.
§Numbers do not add to the total number of women, because women with missing exposure data were excluded from the analysis.

they were current smokers 10 years prior. Women homozygous for the variant allele who were current light smokers had a 2-fold increased risk (multivariate OR, 2.08; 95% CI, 1.01-4.28), whereas heavier smokers had a nonsignificant increased risk (multivariate OR, 1.48; 95% CI, 0.87-2.53) compared with wild-type never smokers.

There was no striking interaction between *MnSOD* genotype and alcohol consumption, even after adjustment for the interaction between smoking and *MnSOD* genotype (*P* for interaction = 0.42[LRT_1]).

Because the data reported by Ambrosone et al. (2) suggested an interaction between dietary sources of antioxidants and *MnSOD* genotype primarily among premenopausal women, we investigated this potential interaction. There was no evidence that premenopausal or postmenopausal women homozygous for the variant *MnSOD* alleles were at an increased risk of breast cancer if they were low consumers of dietary sources of antioxidants (Table 4).

Plasma antioxidant levels may serve as a better marker of bioavailable antioxidants than dietary intake levels; therefore, we also evaluated the association between *MnSOD* genotype and breast cancer according to plasma antioxidant levels. There was no evidence that plasma levels of α-carotene, β-carotene, β-cryptoxanthin, lycopene, lutein/zeaxanthin, retinol, α-tocopherol, or γ-tocopherol modify the association between *MnSOD* genotype and breast cancer risk (Table 5). The women in the lowest tertile of plasma α-carotene with Ala/Ala genotype were at a suggestive increased risk of breast cancer (multivariate OR, 1.42; 95% CI, 0.90-2.25). Plasma folate levels were available for cases (*N* = 698) diagnosed through June 1, 1996 and their matched controls. There was no significant interaction between *MnSOD* genotype and plasma folate levels among this subset of women (data not shown).

A previous study reported a significant interaction between *MnSOD* genotype and ever use of postmenopausal hormones (12). We evaluated the relationship

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Table 4. ORs and 95% CIs for breast cancer according to *MnSOD* genotype and nutritional factors among premenopausal and postmenopausal women in the Nurses' Health Study (1989 to 1998)

	MnSOD genotype				*P* for interaction*
	Val/Val or Val/Ala		Ala/Ala		
	Cases/Controls	OR (95% CI)	Cases/Controls	OR (95% CI)	
Premenopausal†					
Vitamin C supplements‡					0.48
No	44/51	1.00 (Ref)	25/22	1.09 (0.50-2.38)	
Yes	13/17	0.76 (0.30-1.94)	5/4	1.64 (0.37-7.38)	
Vitamin E supplements‡					0.71
No	42/59	1.00 (Ref)	27/23	1.40 (0.66-3.00)	
Yes	14/9	1.88 (0.67-5.32)	3/3	1.78 (0.30-10.68)	
Multivitamins‡					0.34
No	37/50	1.00 (Ref)	19/19	1.02 (0.43-2.39)	
Yes	25/24	1.48 (0.66-3.32)	12/7	3.01 (0.94-9.68)	
Total fruits and vegetables‡,§					0.15
Low	26/32	1.00 (Ref)	11/17	0.72 (0.26-2.05)	
High	32/39	0.88 (0.41-1.91)	19/11	1.87 (0.67-5.20)	
Vitamin C dietary intake ‡,§					0.76
Low	29/35	1.00 (Ref)	16/15	1.12 (0.43-2.90)	
High	33/39	0.88 (0.41-1.87)	14/11	1.22 (0.44-3.39)	
Postmenopausal‖					
Vitamin C supplements‡					0.48
No	372/486	1.00 (Ref)	128/162	1.05 (0.79-1.39)	
Yes	181/228	1.00 (0.78-1.28)	47/67	0.87 (0.58-1.32)	
Vitamin E supplements‡					0.54
No	443/574	1.00 (Ref)	149/185	1.05 (0.81-1.37)	
Yes	103/136	0.91 (0.68-1.23)	26/41	0.79 (0.47-1.33)	
Multivitamins‡					0.52
No	349/426	1.00 (Ref)	116/150	0.96 (0.72-1.29)	
Yes	235/323	0.82 (0.65-1.04)	71/88	0.92 (0.65-1.33)	
Total fruits and vegetables‡,§					0.13
Low	313/343	1.00 (Ref)	99/127	0.91 (0.66-1.26)	
High	234/372	0.67 (0.53-0.85)	82/100	0.88 (0.62-1.24)	
Vitamin C dietary intake‡,§					0.06
Low	290/358	1.00 (Ref)	90/132	0.84 (0.61-1.16)	
High	289/380	0.86 (0.68-1.08)	95/100	1.13 (0.81-1.58)	

*LRT comparing the main effects model to the model with each level of exposure cross-classified with *MnSOD* genotype.
†Unconditional logistic regression estimates adjusted for matching factors, age at menarche, personal history of benign breast disease, family history of breast cancer, parity/age at first birth, BMI at age 18, weight gain since age 18, and smoking status.
‡Numbers do not add to the total number of women, because women with missing exposure data were excluded from the analysis.
§Cutpoints for low and high consumption per day are based on median values for premenopausal and postmenopausal women, respectively: total fruits and vegetables, 4.7 and 5.6 servings; vitamin C dietary intake, 162 and 205 mg.
‖Unconditional logistic regression estimates adjusted for factors above and age at menopause and duration of postmenopausal hormone use.

Table 5. ORs and 95% CIs for breast cancer according to *MnSOD* genotype and tertiles of plasma antioxidants in the Nurses' Health Study (1989 to 1998)

	MnSOD genotype						*P* for interaction	
	Val/Val		Val/Ala		Ala/Ala			
	Cases/Controls	OR (95% CI)*	Cases/Controls	OR (95% CI)*	Cases/Controls	OR (95% CI)*	LRT_1[†]	LRT_2[‡]
Plasma α-carotene[§]							0.35	0.23
Low tertile	78/88	1.00 (Ref)	162/162	1.09 (0.74-1.60)	90/67	1.42 (0.90-2.25)		
Medium tertile	99/71	1.47 (0.94-2.31)	164/167	1.03 (0.70-1.52)	80/77	1.08 (0.68-1.71)		
High tertile	77/76	0.96 (0.60-1.52)	137/156	0.84 (0.56-1.25)	70/83	0.93 (0.58-1.47)		
Plasma β-carotene[§]							0.93	0.70
Low tertile	78/83	1.00 (Ref)	154/166	0.93 (0.63-1.38)	79/64	1.18 (0.73-1.88)		
Medium tertile	110/79	1.35 (0.87-2.11)	179/160	1.11 (0.75-1.65)	87/78	1.18 (0.74-1.85)		
High tertile	66/72	0.81 (0.50-1.31)	130/159	0.72 (0.48-1.08)	74/85	0.84 (0.53-1.34)		
Plasma β-cryptoxanthin[§]							0.06	0.40
Low tertile	94/79	1.00 (Ref)	163/167	0.77 (0.53-1.13)	85/69	0.97 (0.61-1.52)		
Medium tertile	89/65	1.05 (0.67-1.67)	143/168	0.64 (0.43-0.95)	82/77	0.87 (0.55-1.36)		
High tertile	71/91	0.53 (0.34-0.84)	157/150	0.74 (0.50-1.10)	73/81	0.66 (0.42-1.05)		
Plasma lycopene[§]							0.47	0.42
Low tertile	78/86	1.00 (Ref)	167/160	1.15 (0.78-1.70)	64/63	1.15 (0.71-1.86)		
Medium tertile	92/73	1.33 (0.85-2.09)	147/165	0.95 (0.64-1.42)	94/77	1.30 (0.83-2.04)		
High tertile	84/76	1.22 (0.77-1.92)	149/160	0.97 (0.65-1.44)	82/87	1.08 (0.69-1.69)		
Plasma lutein/zeaxanthin[§]							0.30	0.58
Low tertile	87/77	1.00 (Ref)	172/160	0.90 (0.61-1.33)	85/77	0.92 (0.59-1.44)		
Medium tertile	91/77	0.95 (0.60-1.49)	134/167	0.62 (0.42-0.93)	80/71	0.95 (0.60-1.51)		
High tertile	76/81	0.67 (0.42-1.06)	157/158	0.75 (0.51-1.12)	75/79	0.74 (0.47-1.18)		
Plasma carotenoids[§]							0.86	0.40
Low tertile	93/89	1.00 (Ref)	168/167	0.97 (0.67-1.42)	81/62	1.24 (0.78-1.95)		
Medium tertile	84/74	1.05 (0.68-1.64)	152/158	0.90 (0.61-1.31)	85/83	0.94 (0.61-1.46)		
High tertile	77/72	0.93 (0.59-1.47)	143/160	0.74 (0.50-1.09)	74/82	0.86 (0.55-1.35)		
Plasma retinol[§]							0.12	0.75
Low tertile	78/70	1.00 (Ref)	163/156	0.89 (0.60-1.34)	98/83	1.09 (0.69-1.71)		
Medium tertile	87/81	0.92 (0.58-1.47)	149/152	0.85 (0.56-1.29)	60/82	0.63 (0.39-1.02)		
High tertile	89/84	0.91 (0.57-1.45)	151/177	0.73 (0.48-1.10)	82/62	1.17 (0.72-1.91)		
Plasma α-tocopherol[§]							0.92	0.51
Low tertile	84/77	1.00 (Ref)	156/158	0.89 (0.60-1.32)	86/77	1.02 (0.65-1.61)		
Medium tertile	86/84	0.90 (0.58-1.42)	158/159	0.89 (0.60-1.32)	77/74	0.95 (0.60-1.51)		
High tertile	84/74	0.98 (0.62-1.55)	149/168	0.74 (0.49-1.09)	77/76	0.91 (0.57-1.45)		
Plasma γ-tocopherol[§]							0.53	0.87
Low tertile	86/81	1.00 (Ref)	151/159	0.91 (0.62-1.35)	87/72	1.20 (0.76-1.88)		
Medium tertile	84/69	1.21 (0.77-1.91)	150/169	0.89 (0.60-1.31)	62/75	0.85 (0.53-1.36)		
High tertile	84/85	1.06 (0.68-1.65)	162/157	1.03 (0.69-1.52)	91/80	1.19 (0.76-1.85)		

*Adjusted for matching factors, personal history of benign breast disease, first-degree family history of breast cancer, age at menopause, age at menarche, BMI at age 18, weight gain since age 18, age at first birth/parity, duration of postmenopausal hormone use, alcohol consumption, and smoking status.
†LRT comparing the main effects model to the model with each level of exposure cross-classified with *MnSOD* genotype.
‡LRT of the interaction term between tertiles of antioxidants and *MnSOD* genotype.
§Numbers do not add to the total number of women, because women with missing exposure data were excluded from the analysis.

between *MnSOD* genotype and risk of breast cancer among postmenopausal women, according to postmenopausal hormone use (data not shown). There was no evidence that ever use, current use, or duration of current postmenopausal hormone use modified the *MnSOD* genotype and breast cancer relationship.

Discussion

We did not find evidence of an association between *MnSOD* polymorphism and breast cancer. These results are in contrast to two prior studies, which both reported an increased risk of breast cancer associated with the Ala allele of *MnSOD* (2, 12). Ambrosone et al. (2) found a 3.5-fold increased risk of breast cancer associated with being homozygous for the Ala allele compared with any carriers of the wild-type allele among premenopausal women and a near 2-fold increased risk among postmenopausal women. The second study among Finnish Caucasians reported a more modest effect, which was observed primarily among postmenopausal women (OR, 1.7; 95% CI, 1.2-2.5 for Ala allele carriers compared with noncarriers; ref. 12). Although both Ambrosone et al. and our study report identical allele frequencies among the controls, our genotype distributions are different. In addition, the Ambrosone study (N = 114) and our study (N = 99) have comparably low numbers of premenopausal cases, suggesting that the disparity in these results may be attributable to chance. The discrepancy between our studies warrants further investigations in studies with greater numbers of premenopausal breast cancer cases.

While there was no main effect of *MnSOD* genotype in this study, there was evidence that this polymorphism may modify risk of breast cancer, primarily among

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smokers. The role of smoking in breast cancer has been controversial, with epidemiologic studies reporting inverse, null, and positive associations (19). *In vivo* and *in vitro* studies provide support of the biological plausibility of the smoking and breast cancer relationship. Tobacco smoke contains potential breast cancer carcinogens and highly reactive free radicals (19, 20). These carcinogens are lipophilic and can be stored in the breast adipose tissue and the mammary epithelial cells are capable of metabolizing and activating these compounds. Higher frequencies of smoking specific DNA adducts of *p53* have been reported in breast tissue of smokers as compared with nonsmokers, providing further evidence that tobacco smoke may have a biological effect in breast tissue (19). In addition, MnSOD expression is induced by cigarette smoke (21) and there are cell culture data demonstrating that overexpression of *MnSOD* protects against cytotoxicity induced by cigarette smoke (20).

In this study, there was a statistically significant interaction between *MnSOD* genotype and cigarette smoking status. This interaction is attributed to an increased risk among current smokers homozygous for the variant alleles, as well as a reduced risk observed among current smokers who are homozygous for the wild-type allele. The smaller *P* value obtained from the ordinal LRT is due to the trend of increasing breast cancer risk with increasing numbers of Ala alleles among current smokers.

The increased risk of breast cancer we observed among women homozygous for the variant allele and smokers is consistent with the a priori hypothesis that due to their genotype, these women may be less efficient in their ability to scavenge ROS produced by tobacco smoke, and incur DNA damage that increases their risk of breast cancer. In contrast, the decreased risk observed in homozygous wild-type smokers was an unexpected observation. Smoking is hypothesized to have antiestrogenic properties. It is possible that women with fully functional MnSOD (i.e., wild-type) may experience reduced oxidative stress, and thus experience an anti-estrogenic effect of the cigarette smoke, reducing their risk of breast cancer.

The increased risk associated with the Ala/Ala genotype among smokers in this study is consistent with results reported by Mitrunen et al. (12) indicating an increased risk of breast cancer among postmenopausal smokers carrying Ala alleles (OR, 3.7; 95% CI, 1.4-9.9). Although these results are based on small numbers of current smokers, they do suggest that the variant allele of *MnSOD* is less efficiently able to scavenge ROS produced by cigarette smoking. As discussed previously, epidemiologic data about the relationship between smoking and breast cancer have been inconsistent and controversial. These data suggest that one possible explanation for the inconsistency in the literature may be due to differences in genetic susceptibility between populations.

We found no evidence that the consumption of fruits and vegetables, vitamin supplements, or plasma antioxidant levels modify the *MnSOD* breast cancer relationship. This is in contrast to the study of Ambrosone et al. (2), which reported a 6-fold increased risk of breast cancer among premenopausal women homozygous for the Ala allele and consuming low levels of fruits and vegetable. The study of Mitrunen et al. (12) was unable to assess interactions with fruits and vegetables, yet they were able to evaluate interactions with antioxidant supplement use. They reported no significant interactions between genotype and vitamins A, C, or E supplement use. We have previously reported an inverse association between plasma α-carotene levels and breast cancer risk in this study population;[6] however, there was no statistical interaction between *MnSOD* genotype and plasma antioxidant levels including α-carotene.

The strengths of the study include its relatively large size, prospectively collected exposure data, and plasma collected before cancer diagnosis. In addition, we were able to evaluate interactions with not only dietary intake of antioxidants, but also with plasma levels of eight plasma antioxidants. In conclusion, we did not find a main effect of *MnSOD* genotype and breast cancer risk. The significant interaction between genotype and smoking suggests that the Ala allele of *MnSOD* may modify breast cancer risk among current smokers. The evidence about smoking and breast cancer has remained inconclusive, with the majority of studies reporting no association. Additional studies examining variation in other genes involved in the reduction of ROS (e.g., catalase, glutathione peroxidase) may be helpful in elucidating this potential gene-environment interaction.

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Appendix 1

Genomic DNA was amplified using two forward primers with different fluorescent labels. The primer specific for the Ala allele was tet-5′-CAGCAGGCAGCTGGCT-CCGGC-3′, and the primer specific for the Val allele was fam-5′-GCAGGCAGCTGGCTCCGGT-3′. The reverse primer used was 5′-GCGTTGATGTGAGGTTCCAGG-3′. The PCR cycling had an initial denaturation at 94°C for 4 minutes, followed by 30 cycles 94°C for 30 seconds, 72°C for 1 minute. These cycles were followed by a final round of elongation at 72°C for 6 minutes. The PCR products were then diluted and run on a Long Ranger (FMC BioProducts, Rockland, ME) sequencing gel and genotypes were determined using automated fluorescence detection (Genescan: Applied Biosystems).

Appendix 2

Genomic DNA was amplified using the following primers: 5′-ACCAGCAGGCAGCTGGCTCCG-3′ (forward) and 5′-GCGTTGATGTGAGGTTCCAG-3′

[6] R.M. Tamimi, S.E. Hankinson, H. Campos, D. Spiegelman, S. Zhang, G.A. Colditz, W.C. Willet, D. J. Hunter. Plasma antioxidants and risk of breast cancer, submitted for publication.

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(reverse). The reverse primer was also used as the sequencing primer. Reactions were run on an ABI Prism 377 DNA sequencer (Applied Biosystems) and genotypes were determined with ABI Prism Sequencing Analysis version 3.4 (Applied Biosystems).

Appendix 3

Genomic DNA was amplified using a biotinylated forward primer (5′-ACCAGCAGGCAGCTGGCTCCG-3′) and an unlabeled reverse primer (5′-GCGTTGATGT-GAGGTTCCAG-3′). The PCR reaction had an initial denaturation at 95°C for 5 minutes, followed by 50 cycles of melting (95°C for 1 minute), annealing (64°C for 1 minute), and elongation (72°C for 2 minutes). These cycles were followed by a final round of elongation at 72°C for 7 minutes. The Val/Ala polymorphism was detected using a PSQ 96 SNP reagent kit and Pyrosequencer PSQ 96 (Uppsala, Sweden). Following the manufacturer's directions, the amplified DNA was used as a template for pyrosequencing with the sequencing probe (5′-CCAGATACCCCAAA-3′).

Appendix 4

The TaqMan system uses fluorescently labeled allele specific oligonucleotides to discriminate between alleles using the ABI Prism 7900HT Sequence Detection system and software (Applied Biosystems). PCR amplification was carried out on 5 to 20 ng DNA using 1× TaqMan universal PCR master mix (No Amp-erase UNG), 900 nmol/L forward (5′-GGCTGTGCTTTCTCGTC-TTCA-3′) and reverse (5′-TCTGCCTGGAGCCCAGA-TAC-3′) primers, 200 nmol/L of the FAM-labeled probe (CCCAAAGCCGGAGC), and 200 nmol/L of the VIC-labeled probe (CCAAAACCGGAGCCA) in a 5-μl reaction; the polymorphic base is shown underlined. Amplification conditions on a AB 9700 dual plate thermal cycle (Applied Biosystems) were as follows: 1 cycle of 95°C for 10 minutes, followed by 50 cycles of 92°C for 15 seconds and 58°C for 1 minute. TaqMan primers and probes were designed using the Primer Express Oligo Design software v2.0 (ABI Prism).

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