Manganese superoxide dismutase Ala-9Val polymorphism and risk of breast cancer in a population-based case–control study of African Americans and whites

Robert C Millikan1, Jon Player1, Allan René de Cotret1, Patricia Moorman2, Gary Pittman3, Vani Vannappagari1, Chiu-Kit J Tse1 and Temitope Keku4

Introduction: A polymorphism in the manganese superoxide dismutase (MnSOD) gene, Ala-9Val, has been examined in association with breast cancer risk in several epidemiologic studies. Results suggest that the Ala allele increases the risk of breast cancer and modifies the effects of environmental exposures that produce oxidative damage to DNA.

Methods: We examined the role of the MnSOD Ala-9Val polymorphism in a population-based case–control study of invasive and in situ breast cancer in North Carolina. Genotypes were evaluated for 2025 cases (760 African Americans and 1265 whites) and for 1812 controls (677 African Americans and 1135 whites).

Results: The odds ratio for MnSOD Ala/Ala versus any MnSOD Val genotypes was not elevated in African Americans (odds ratio = 0.9, 95% confidence interval = 0.7–1.2) or in whites (odds ratio = 1.0, 95% confidence interval = 0.8–1.2). Greater than additive joint effects were observed for the Ala/Ala genotype and smoking, radiation to the chest, and occupational exposure to ionizing radiation. Antagonism was observed between the Ala/Ala genotype and the use of nonsteroidal anti-inflammatory drugs.

Conclusions: The MnSOD genotype may contribute to an increased risk of breast cancer in the presence of specific environmental exposures. These results provide further evidence for the importance of reactive oxygen species and of oxidative DNA damage in the etiology of breast cancer.

Keywords: African Americans, breast cancer, manganese superoxide dismutase polymorphism
convert superoxide (O$_2^−$) to peroxide (H$_2$O$_2$) and molecular oxygen (O$_2$). There are three superoxide dismutase enzymes: cytosolic superoxide dismutase, mitochondrial superoxide dismutase (manganese superoxide dismutase [MnSOD]), and extracellular superoxide dismutase [6].

The MnSOD gene on chromosome 6q25 encodes human MnSOD (also known as SOD2). MnSOD is synthesized in the cytoplasm as a precursor protein and is transported to the mitochondria, where it is processed and assembled into an active homotetramer. Studies of MnSOD protein expression showed higher levels in non-neoplastic breast epithelial cells compared with levels in invasive breast cancer [6]. MnSOD overexpression in breast cancer cell lines leads to upregulation of GAD153 (which is involved in the repair of double strand breaks in DNA) and to a variety of redox-sensitive transcription factors [7]. MnSOD also induces expression of the matrix metalloproteinase MMP-2, which helps to mediate cell migration and adhesion to the extracellular matrix [8]. Several investigators hypothesize that MnSOD may act as a tumor suppressor gene in breast epithelial cells [6,7,9].

A common polymorphism exists in the human MnSOD gene. This Ala-9Val polymorphism is a single nucleotide substitution of C → T at nucleotide 47, changing the encoded amino acid from Ala (GCT) to Val (GTT) [10,11]. The amino acid change occurs within the N-terminal mitochondrial targeting sequence, a 24-amino acid signal sequence that targets the MnSOD precursor protein for transport into the mitochondria. Mitochondrial localization of MnSOD is required to protect cells from ionizing radiation and other forms of oxidative damage [12]. The variant residue is nine amino acids upstream of the cleavage site, hence the polymorphism designation Ala-9Val. Computer models predicted that the Val allele would encode a beta-sheet conformation of the MnSOD precursor protein that exhibited impaired transport into the mitochondria, while the alpha-helical structure of the Ala-containing precursor would show correct transport [10]. Recent experiments by Sutton and colleagues [13] confirmed that, in rat liver, the human Val-containing MnSOD protein has difficulty crossing the mitochondrial inner membrane, leading to decreased formation of active MnSOD within the mitochondrial matrix. The MnSOD Ala-containing protein showed normal transport and generated 30–40% more active MnSOD protein than did the Val form of the enzyme.

Several epidemiologic studies have examined the association of the MnSOD Ala-9Val polymorphism and cancer. The Ala allele was associated with an increased risk of breast cancer in two populations [14,15] but not in another population [16]. The Ala allele was associated with an increased risk of prostate cancer [17] and of early-onset colorectal cancer [18], while the Val allele was associated with an increased risk of lung cancer [19] and of bladder cancer [20]. Studies of the role of MnSOD in noncancer outcomes also showed mixed results [21]. We conducted genotyping for MnSOD Ala-9Val genotypes in the Carolina Breast Cancer Study (CBCS), a population-based case–control study of breast cancer in African Americans and whites. We estimated odds ratios (ORs) for the MnSOD genotype and joint effects with several environmental exposures.

Materials and methods

Study design and participants.

The CBCS is a population-based case–control study of breast cancer conducted in North Carolina [22]. Participants provided informed consent using forms approved by the Institutional Review Board of the University of North Carolina School of Medicine, in compliance with the Helsinki Declaration. Breast cancer cases were identified in cooperation with the North Carolina Central Cancer Registry, and controls were identified using Division of Motor Vehicles lists (for women younger than age 65 years) and using Health Care Financing Administration lists (for women aged 65 years or older). Details of recruitment of participants and response rates have been published previously [23,24].

A total of 1803 cases of invasive breast cancer (787 African Americans and 1016 whites) and 1564 controls (718 African Americans and 846 whites) were enrolled between 1993 and 2001, and a total of 508 cases of in situ breast cancer (107 African Americans and 401 whites) and 458 controls (70 African Americans and 388 whites) were enrolled between 1996 and 2001. Controls were frequency matched to in situ cases based on age (±5 years) and on race. In the invasive study, the response rates were 76.0% for cases and 55.0% for controls. In the in situ study, the response rates were 82.7% for cases and 65.2% for controls.

Home interviews were conducted to obtain blood samples and information on breast cancer risk factors. Response rates for blood draws that contained usable DNA combining all phases of the study were 89% for cases and 90% for controls. DNA samples were provided for a total of 2045 cases (768 African Americans and 1277 whites) and for 1818 controls (681 African Americans and 1137 whites).

Laboratory methods

DNA was extracted from whole blood using an automated ABI-DNA extractor (Applied Biosystems Nuclei Acid Purification System; Applied Biosystems, Foster City, CA, USA) in the University of North Carolina SPORE Tissue Procurement Facility. Genotyping for the MnSOD Ala-9Val
polymorphism (accession number S77127) was conducted using an ABI 7700 Sequence Detection System or the ‘Taqman™’ assay (Applied Biosystems). PCR primers and probes were designed using Primer Express™ software (Applied Biosystems). The assay design and conditions were based on the allelic discrimination protocol from Applied Biosystems.

The Ala (C) allele-specific probe was labeled on the 5′ end with the VIC reporter dye and contained the nucleotide sequence 5′-CACAAGCGGAGCC-3′, with 69.2% G–C content and a melting temperature of 65.1°C. The Val (T) allele-specific probe was labeled on the 5′ end with the FAM reporter dye and contained the nucleotide sequence 5′-CCAAAAACCGGAGCC-3′, with 64.3% G–C content and a melting temperature of 65.8°C. Both probes contained the minor groove binding nonfluorescing quencher dye on the 3′ end. Forward and reverse primers were used to amplify the region surrounding the polymorphism. The nucleotide sequence for the forward primer was 5′-GGCTGTGCTTCTGCTTCA-3′, and the melting temperature was 59.2°C with 52.4% G–C content. The nucleotide sequence for the reverse primer was 5′-TTCTGCGTGGAGCCGATG-3′, and the melting temperature was 59.3°C with 57.9% G–C content.

PCR reactions were performed in a 15.0 µl reaction volume using the hot-start format. The reaction components were as follows: 1 x Taqman Universal PCR Master Mix, 900 nM each primer, 250 nM Ala (VIC), 150 nM Val (FAM) probe, and 15.0 ng genomic DNA. PCR reactions were run on a PerkinElmer GenAmp® 9700 thermocycler (Perkin Elmer, Boston, MA, USA) using the 9600 mode under the following conditions: 50°C for 2 min (AmpErase® UNG activation), 95°C for 10 min (AmpliTaq® Gold activation), followed by 35 cycles of 95°C for 15 s (denature) and 57°C for 1 min (anneal/extend). Synthetic oligonucleotides of 73 base pairs corresponding to the MnSOD-9Val and MnSOD-9Ala alleles were included as positive controls in each plate. Samples that could not be scored were repeated.

MnSOD genotype data were obtained on a total of 3837 participants (2025 cases and 1812 controls). A total of 26 samples were not readable due to poor PCR amplification. Genotyping was repeated on a random 10% sample and results were identical to the original run.

Due to failure to observe Hardy–Weinberg equilibrium in a portion of the samples (see Results), the accuracy of the Taqman™ genotyping protocol was verified in three ways. First, genotyping was repeated on 20% of samples (n = 772) using the PCR-restriction fragment length polymorphism assay developed by Ambrosone and colleagues [14]. Five samples showed different results in the two assays (99.9% agreement). The five samples were repeated using the Taqman™ assay, and the results were identical to the original Taqman™ results. Only the Taqman™ results were therefore used in subsequent statistical analysis.

For the second verification method, 15 DNA samples from the Coriell Cell Repository (Camden, NJ, USA) were genotyped using the Taqman™ assay. The results corresponded to the direct DNA sequencing results provided by the NCI SNP500 project (http://SNP500cancer.nci.nih.gov).

Finally, we verified our Taqman genotyping procedure using direct DNA sequencing. Five DNA samples for each MnSOD genotype (Ala/Ala, Ala/Val and Val/Val) were amplified, were tagged with M13 forward and reverse universal primers, and were analyzed on the ABI 3730 DNA Analyzer using the ABI Prism™ BigDye™ Version 1.1 Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq® DNA Polymerase, FS (Applied Biosystems) (primer sequences and reaction conditions provided upon request). All direct DNA sequencing results corresponded to the previous genotyping results.

Statistical analysis

Differences in MnSOD allele frequencies between cases and controls were determined using a chi-square test. Departures from Hardy–Weinberg equilibrium were determined by comparing the observed genotype frequencies with expected genotype frequencies calculated using observed allele frequencies. Since deviations from Hardy–Weinberg equilibrium were observed (see Results), genotype frequencies in cases and controls were compared using the Cochran–Armitage test for trend with a two-sided P value [25].

Age was based on age at diagnosis in cases or age at selection in controls. Race was classified according to self-report. Less than 2% of participants reported Native American or other race, and these were classified as whites. The stage at diagnosis in cases was classified according to the American Joint Committee on Cancer system based on review of medical records; data was missing for 107 cases. Additional covariates included family history (one or more first-degree relatives with breast cancer), menopausal status, duration of active cigarette smoking (years), any use of oral contraceptives, any use of hormone replacement therapy (postmenopausal women), a composite of age at first full-term pregnancy (AFFTP) and parity (nulliparous, parity = 1 and AFFTP < 26 years, parity = 1 and AFFTP ≥ 26 years, parity ≥ 2 and AFFTP < 26 years, and parity ≥ 2/AFFTP ≥ 26 years), age at menarche (continuous), lactation (ever, never), and alcohol use (ever, never). Body mass index (kg/m²) and waist–hip ratio were calculated using measurements taken at the time of interview and were included as continuous variables.
High-dose radiation exposure to the chest was estimated by asking about medical procedures, including coronary catheterization, coronary angioplasty, and treatment of the upper body with radiation (excluding treatment or diagnosis for breast cancer). Occupational exposure was based upon a history of working for 6 months or longer at occupations with potential exposure to ionizing radiation (nurses, medical doctors, X-ray technicians and laboratory technicians) as described previously [26].

Participants were asked any use of vitamin supplements for the previous 5 years, including multivitamins, vitamin A, vitamin C, vitamin E, and beta-carotene and calcium, as detailed previously [27].

The use of NSAIDs was determined by asking participants in phase 2 of the invasive study and the carcinoma in situ study about use of a variety of medications using color charts, as described previously [28]. Both prescription NSAIDs and nonprescription items, such as aspirin and acetaminophen, were included. Women who used NSAIDs for less than 3 months or who reported sporadic use (≤ 7 days per month) were classified as ‘occasional users’. Women who used NSAIDs at least 8 days a month for 3 months or more were classified into two groups: those who used NSAIDs for less than 3 years’ total duration were designated ‘short-term users’, while those who used NSAIDs for 3 years or longer were ‘long-term users’.

Participants were asked about their usual servings per week of fruits and vegetables; a food frequency questionnaire or other comprehensive dietary history was not obtained. For this analysis, participants were classified according to the median consumption of fruits and vegetables in controls during summer months.

The ORs for breast cancer were calculated using unconditional logistic regression as implemented in the SAS software program (version 8.1; SAS Institute, Cary, NC, USA). Offset terms were incorporated into models using the SAS procedure, PROC GENMOD, to account for the sampling probabilities used to define eligible cases and controls [29]. Potential confounding was evaluated by selecting covariates for final models that resulted in a change of 10% or more for the beta coefficients for the MnSOD genotype or the relevant environmental exposure.

Interaction was evaluated on the multiplicative scale by comparing models with the main effects for the MnSOD genotype and environmental factors with models containing the main effects and interaction terms using a likelihood ratio test. \( P < 0.10 \) for likelihood ratio tests was considered statistically significant. Joint effects of the MnSOD genotype and environmental factors were estimated on an additive scale using a common referent group [30]. Interaction contrast ratios (ICRs) and 95% confidence intervals (CIs) were calculated in the SAS software program as described by Lundberg and colleagues [31]. The ICR is a measure of departure from additive joint effects: ICR > 0 implies greater than additive effects (positive interaction on an additive scale or synergy), ICR = 0 implies additive effects (no interaction), and ICR < 0 implies less than additive effects (antagonism) [30]. For the present study, we consider ICR ≥ 0.5 as evidence for a positive interaction on an additive scale. To estimate ORs for vitamin and mineral supplements, a common referent group of never users was employed as described previously [27].

Joint effects using an additive scale are presented for each exposure and MnSOD genotype. Stratified results using a multiplicative scale (two different referent groups) are also presented for NSAID use and the MnSOD genotype to assist in data interpretation.

Results

The characteristics of study participants and the response rates [24], as well as ORs for breast cancer and vitamin use, for smoking, for alcohol consumption, for exposure to ionizing radiation, for hormone use, and for NSAIDs have been previously published for the CBCS [26–28,32–35]. Briefly, modest inverse associations were observed for use of multivitamins, vitamin C, and vitamin E [27]. Weak positive associations were observed for smoking for longer than 20 years [32], for alcohol consumption [33], for high-dose radiation to the chest, and for occupational exposure to ionizing radiation [26]. No association was observed for hormone replacement therapy [34], and a weak positive association was found among younger women for the use of oral contraceptives [35]. An inverse association was observed for the use of NSAIDs [28]. No associations were found for summer or winter fruit and vegetable intake, and results have not been previously published for these variables.

Genotype and allele frequencies for MnSOD are presented in Table 1 for both African American and white study participants. The MnSOD-9Val allele was slightly more common among African American controls compared with white controls. The Val allele and the Val/Val genotype frequencies were higher among African American cases compared with controls, but the differences were not statistically significant. MnSOD allele and genotype frequencies were similar in cases compared with controls among whites. The MnSOD genotype frequencies did not differ according to stage at diagnosis of breast cancer in African American or white cases (data not shown).

The frequency of the Ala allele in white controls was within the previously reported range for Europeans and American
whites (0.44–0.51) [14–18]. Tests for Hardy–Weinberg equilibrium showed a borderline, statistically significant departure from equilibrium distributions in African American controls (\( P = 0.08 \)) and a significant departure in white cases (\( P = 0.005 \)). In white cases, we observed an excess of Val/Ala heterozygotes (681 observed versus 631 expected). In African American controls, there was also an excess of heterozygotes (357 observed versus 335 expected). There was a nonsignificant excess of Val/Ala heterozygotes in African American cases (\( P = 0.67 \)) and white controls (\( P = 0.26 \)). In order to verify our Taqman™ genotyping results, we repeated MnSOD genotyping on 772 study participants using a PCR-restriction fragment length polymorphism assay (as described in Materials and methods); the results showed excellent agreement. In most previous studies, MnSOD genotypes appear to be in Hardy–Weinberg equilibrium, but these studies have not included populations with large numbers of African Americans.

The ORs for breast cancer and MnSOD genotypes in African Americans and whites are presented in Table 1. The ORs were close to the null value, with a slight inverse association in African Americans and a slight positive association in whites for Ala-containing genotypes compared with Val/Val. The OR for MnSOD Ala/Ala versus any MnSOD Val combining African American and white study participants was 1.0 (95% CI = 0.8–1.1), and the OR was similar for cases of carcinoma in situ versus controls (OR = 0.8, 95% CI = 0.6–1.1) and for invasive cancer cases versus controls (OR = 1.0, 95% CI = 0.9–1.2).

| MnSOD genotype and allele | Cases | Controls | Odds ratio (95% confidence interval)\(^a\) |
|---------------------------|-------|----------|----------------------------------|
| **African Americans**     | \( n = 760 \) | \( n = 677 \) |                                 |
| Genotype frequencies\(^b\) |       |          |                                  |
| Val/Val                   | 259 (34%) | 196 (29%) | Reference                         |
| Val/Ala                   | 372 (49%) | 357 (53%) | 0.8 (0.6–1.0)                     |
| Ala/Ala                   | 129 (17%) | 124 (18%) | 0.8 (0.6–1.1)                     |
| Cochran–Armitage trend test, \( P = 0.08 \) | | | |
| Val/Val or Val/Ala        | 631 (83%) | 553 (82%) | Reference                         |
| Ala/Ala                   | 129 (17%) | 124 (18%) | 0.9 (0.7–1.2)                     |
| Allele frequencies\(^c\)  |       |          |                                  |
| Val                       | 0.59 (0.56–0.61) | 0.55 (0.53–0.58) |                                  |
| Ala                       | 0.41 (0.39–0.44) | 0.45 (0.42–0.47) |                                  |
| Chi-square test, \( P = 0.08 \) | | | |
| **Whites**                | \( n = 1265 \) | \( n = 1135 \) |                                 |
| Genotype frequencies\(^b\) |       |          |                                  |
| Val/Val                   | 273 (21%) | 266 (23%) | Reference                         |
| Val/Ala                   | 681 (54%) | 586 (52%) | 1.2 (0.9–1.4)                     |
| Ala/Ala                   | 311 (25%) | 283 (25%) | 1.1 (0.8–1.4)                     |
| Val/Val or Val/Ala        | 954 (75%) | 852 (75%) | Reference                         |
| Ala/Ala                   | 311 (25%) | 283 (25%) | 1.0 (0.8–1.2)                     |
| Cochran–Armitage trend test, \( P = 0.59 \) | | | |
| Allele frequencies\(^c\)  |       |          |                                  |
| Val                       | 0.49 (0.47–0.51) | 0.49 (0.47–0.51) |                                  |
| Ala                       | 0.51 (0.50–0.55) | 0.51 (0.49–0.53) |                                  |
| Chi-square test, \( P = 0.60 \) | | | |

\(^a\) Adjusted for offsets and age. \(^b\) Data presented as \( n \) (%). \(^c\) Data presented as frequency (95% confidence interval).
The ORs for MnSOD stratified on menopausal status are presented in Table 2. Values close to the null were observed in each group. The ORs did not differ among in situ or invasive breast cancer cases versus controls, and they did not change after adjustment for known breast cancer risk factors or any of the covariates presented in Materials and methods (data not shown).

The ORs estimating the joint effects of the MnSOD genotype and several environmental exposures are presented in Table 3. The ORs were elevated for combinations of MnSOD Ala/Ala genotype and for smoking duration longer than 20 years, for high-dose radiation to the chest, and for occupational exposure to ionizing radiation. The ICRs suggested positive interaction on an additive scale for each of these exposures and the MnSOD Ala/Ala genotype.

A slight inverse association was observed for the combination of the MnSOD Ala/Ala genotype and hormone replacement therapy, but the ICR was close to zero. The ORs for the joint effects of the MnSOD genotype and NSAID use are presented in Table 4 for participants in phase 2 of the invasive study and in the carcinoma in situ study. ORs for combinations of the MnSOD genotype and NSAID use are presented on a multiplicative scale and on an additive scale. On a multiplicative scale, a strong inverse association of NSAID use and breast cancer was observed among participants with any MnSOD Val genotype, but not the MnSOD Ala/Ala genotype. The P value for the likelihood ratio test was 0.09.

Joint effects were calculated on an additive scale using a common referent group of any MnSOD Val genotype and never using NSAIDs. ICRs>0 were observed for each category of NSAID use and the MnSOD Ala/Ala genotype. The P value for the likelihood ratio test was 0.09.

To compare our results with the two previous studies of MnSOD genotypes and breast cancer, we calculated the ORs for the MnSOD genotype and environmental factors using the methods of Ambrosone and colleagues [14] and Mitrunen and colleagues [15].

| MnSOD genotype | Cases | Controls | Odds ratio (95% confidence interval) |
|----------------|-------|----------|------------------------------------|
| Premenopausal  |       |          |                                    |
| Val/Val        | 224   | 187      | Reference                           |
| Val/Ala        | 479   | 417      | 1.0 (0.8–1.2)                      |
| Ala/Ala        | 201   | 179      | 1.0 (0.7–1.3)                      |
| Val/Val or Val/Ala | 703   | 604      | Reference                           |
| Ala/Ala        | 201   | 179      | 1.0 (0.8–1.2)                      |
| Postmenopausal |       |          |                                    |
| Val/Val        | 308   | 275      | Reference                           |
| Val/Ala        | 574   | 526      | 1.0 (0.8–1.2)                      |
| Ala/Ala        | 239   | 228      | 0.9 (0.7–1.2)                      |
| Val/Val or Val/Ala | 882   | 801      | Reference                           |
| Ala/Ala        | 239   | 228      | 0.9 (0.8–1.2)                      |

* Adjusted for offsets, age and race.
Ambrosone and colleagues [14] calculated ORs for the *MnSOD* genotype after stratifying on environmental exposures, using a separate referent group of the *MnSOD* Val/Val genotype within each stratum. ORs were calculated for the *MnSOD* genotype across strata of fruit and vegetable intake and antioxidant use in premenopausal women and postmenopausal women. In the present study, ORs for the *MnSOD* genotype varied only slightly.

## Table 3
Joint effects of the manganese superoxide dismutase (*MnSOD*) genotype and environmental risk factors on breast cancer

| Factor                                | Val/Val or Val/Ala MnSOD genotype | Ala/Ala MnSOD genotype |
|---------------------------------------|-----------------------------------|------------------------|
|                                       | Cases/controls                    | Odds ratio (95% CI)    | Cases/controls | Odds ratio (95% CI) | ICR (95% CI) |
| Vitamin usea                          |                                   |                        |               |                    |             |
| No vitamin use                        | 626/543                           | Referent               | 148/143       | 0.9 (0.7–1.2)      |             |
| Any vitamin use                       | 959/862                           | 0.9 (0.8–1.1)          | 292/264       | 0.9 (0.7–1.1)      | 0.1 (−0.2, 0.4) |
| Multivitamin use                      | 791/695                           | 1.0 (0.8–1.1)          | 235/200       | 0.9 (0.7–1.2)      | 0.1 (−0.2, 0.4) |
| Vitamin A                             | 58/48                             | 1.1 (0.7–1.7)          | 17/16         | 0.9 (0.4–1.9)      | −0.1 (−1.0, 0.7) |
| Vitamin C                             | 405/363                           | 1.0 (0.8–1.2)          | 129/103       | 1.1 (0.8–1.5)      | 0.3 (−0.1, 0.7) |
| Vitamin E                             | 401/325                           | 1.1 (0.9–1.3)          | 123/101       | 1.1 (0.8–1.5)      | 0.1 (−0.3, 0.6) |
| Beta-carotene                         | 59/58                             | 1.0 (0.7–1.6)          | 17/15         | 1.1 (0.5–2.4)      | 0.2 (−0.8, 1.1) |
| Calcium supplements                   | 413/412                           | 0.8 (0.7–1.0)          | 137/123       | 0.9 (0.7–1.3)      | 0.2 (−0.2, 0.6) |
| Smokingb                              |                                   |                        |               |                    |             |
| No active or passive                  | 311/268                           | Referent               | 87/87         | 0.8 (0.6–1.2)      |             |
| Passive only                          | 540/478                           | 1.0 (0.8–1.3)          | 143/144       | 1.0 (0.7–1.3)      |             |
| Duration of active smoking            |                                   |                        |               |                    |             |
| ≤ 10 years                            | 186/180                           | 0.9 (0.7–1.3)          | 59/56         | 0.9 (0.6–1.3)      |             |
| > 10 years to 20 years                | 175/166                           | 1.0 (0.8–1.4)          | 52/49         | 0.8 (0.6–1.4)      |             |
| > 20 years                            | 366/309                           | 1.2 (0.9–1.5)          | 96/71         | 1.5 (1.0–2.2)      | 0.5 (−0.1, 1.0) |
| Alcohol consumptionc                  |                                   |                        |               |                    |             |
| No                                    | 507/454                           | Referent               | 122/139       | 0.7 (0.6–1.0)      |             |
| Yes                                   | 1076/949                          | 1.0 (0.8–1.2)          | 318/268       | 1.0 (0.8–1.3)      | 0.3 (0.0, 0.6) |
| High-dose radiation to chesta         |                                   |                        |               |                    |             |
| No                                    | 1468/1299                         | Referent               | 395/387       | 0.9 (0.8–1.1)      |             |
| Yes                                   | 116/106                           | 1.1 (0.8–1.4)          | 45/20         | 2.3 (1.3–4.1)      | 1.3 (0.0, 2.7) |
| Occupational exposure to ionizing radiationa |                                   |                        |               |                    |             |
| No                                    | 1497/1334                         | Referent               | 413/391       | 0.9 (0.8–1.1)      |             |
| Yes                                   | 88/71                             | 1.1 (0.8–1.6)          | 27/16         | 1.6 (0.8–3.1)      | 0.6 (−0.5, 1.7) |
| Oral contraceptives (regular use)a    |                                   |                        |               |                    |             |
| No                                    | 561/511                           | Referent               | 136/146       | 0.9 (0.6–1.1)      |             |
| Yes                                   | 1016/885                          | 1.1 (0.9–1.4)          | 304/258       | 1.1 (0.9–1.4)      | 0.1 (−0.2, 0.5) |
| Hormone replacement therapy (postmenopausal women, regular use)a |                                   |                        |               |                    |             |
| No                                    | 436/375                           | Referent               | 117/106       | 1.0 (0.7–1.3)      |             |
| Yes                                   | 446/426                           | 0.8 (0.7–1.0)          | 122/122       | 0.7 (0.5–1.0)      | −0.1 (−0.5, 0.3) |

CI, confidence interval; ICR, interaction contrast ratio. a Adjusted for offsets, age, race, family history, age at menarche, age at first full-term pregnancy (AFTP)/parity composite, smoking duration, and alcohol use. b Adjusted for offsets, age, race, family history, age at menarche, AFTP/parity composite, and alcohol use. c Adjusted for offsets, age, race, family history, age at menarche, AFTP/parity composite, and smoking duration.
Table 4

| Use of NSAIDs | Val/Val or Val/Ala MnSOD genotype | Ala/Ala MnSOD genotype |
|---------------|----------------------------------|------------------------|
| Multiplicative scale | Cases/controls | Odds ratio (95% CI) | Cases/controls | Odds ratio (95% CI) | ICR (95% CI) |
| Never | 105/48 | Referent | 19/16 | Referent | |
| Occasional | 511/420 | 0.6 (0.4–0.8) | 142/127 | 0.8 (0.4–1.9) | |
| Short term | 148/141 | 0.4 (0.3–0.7) | 43/36 | 0.9 (0.4–2.4) | |
| Long term | 218/200 | 0.4 (0.3–0.7) | 58/58 | 0.9 (0.4–2.1) | |
| Additive scale | Never | 105/48 | Referent | 19/16 | 0.5 (0.2–1.3) | |
| Occasional | 511/420 | 0.5 (0.4–0.8) | 142/127 | 0.5 (0.3–0.7) | 0.4 (0.1, 0.9) |
| Short term | 148/141 | 0.4 (0.3–0.7) | 43/36 | 0.5 (0.3–1.0) | 0.5 (0.1, 1.1) |
| Long term | 218/200 | 0.4 (0.3–0.7) | 58/58 | 0.4 (0.2–0.7) | 0.4 (0.1, 0.9) |

CI, confidence interval; ICR, interaction contrast ratio. *Adjusted for offsets, age, race, family history, age at menarche, age at first full-term pregnancy/party composite, smoking duration, alcohol use, lactation, menopausal status, oral contraceptive use, education, body mass index, and waist–hip ratio.

discussion

Discussion

Using data from a population-based case–control study of invasive and in situ breast cancer in North Carolina, we did not observe an association between the MnSOD genotype and breast cancer (ignoring environmental factors) in African American or white women, nor among premenopausal or postmenopausal women. Ambrosone and colleagues [14] reported a weak positive association in women from New York state that was stronger in premenopausal than in postmenopausal women. Mitrunen and colleagues [15] reported a weak positive association in women from Finland that was similar in premenopausal and postmenopausal women, while Egan and colleagues [16] observed no association in premenopausal or postmenopausal women from Massachusetts and New Hampshire.

The number of participants in the CBCS was larger than in previous studies. The ORs for genotypes tend to converge towards the null as the sample size increases in association studies [36–38], and the phenomenon has been seen previously for several loci in breast cancer [39]. We did not observe strong joint effects for the MnSOD genotype and vitamin use or summer vegetable and fruit consumption. Ambrosone and colleagues [14] reported elevated ORs for the MnSOD Ala/Ala genotype in women with low consumption of fruits and vegetables and dietary sources of carotenoids, ascorbic acid, and alphatocopherol. Egan and colleagues [16] did not observe strong differences in the ORs for MnSOD stratified according to fruit and vegetable intake. Mitrunen and colleagues [15] reported that the OR for the MnSOD genotype was stronger in persons who took vitamin A, vitamin C, and vitamin E supplements. Fruit and vegetable intake and dietary intake of vitamin A, vitamin C, and vitamin E in the studies of Ambrosone and colleagues [14] and of Egan and colleagues [16] were estimated using detailed food frequency questionnaires. Mitrunen and colleagues [15] did not collect information on dietary intake of fruits and vegetables, and in the present study only a few basic questions were asked about fruit and...
vegetable intake. The latter two studies thus had insufficient power to fully investigate the joint effects of the MnSOD genotype and intake of dietary antioxidants.

Modest joint effects for the MnSOD genotype and smoking were observed in our study. Mitrunen and colleagues [15] reported an elevated OR for the MnSOD genotype among smokers, while Egan and colleagues [16] reported a stronger association for the MnSOD genotype in never smokers than in ever smokers. Neither study addressed the duration of smoking.

We did not observe evidence for joint effects of the MnSOD Ala/Ala genotype and use of alcohol, oral contraceptives, or of hormone replacement therapy. Mitrunen and colleagues [15] reported elevated ORs for the MnSOD genotype in ever drinkers, in users of oral contraceptives, and for postmenopausal estrogen use. Egan and colleagues [16] reported a slightly elevated OR for the MnSOD genotype in women who used oral contraceptives, but only a weak association among users of hormone replacement therapy. We did not observe strong joint effects for the MnSOD genotype and exogenous hormone use in the present study. One explanation may be that we did not observe strong main effects (ignoring MnSOD genotypes) for oral contraceptives [35] or for hormone replacement therapy [34] in the CBCS. We also did not observe greater than additive joint effects for the MnSOD genotype and indices of increased endogenous hormone exposure (early age at menarche, nulliparity, late age at first pregnancy, late age at menopause; data not shown). Relatively few persons reported high levels of alcohol consumption in our study population [33], decreasing the power to observe joint effects with the MnSOD genotype.

We observed moderately elevated joint effects of exposure to ionizing radiation and the MnSOD Ala/Ala genotype. Previous studies of the MnSOD genotype and breast cancer did not investigate interactions with radiation exposure. Our results are consistent with laboratory evidence that MnSOD expression plays a role in adaptive responses to ionizing radiation [40]. Green and colleagues [41] recently showed, however, that the Ala-9Val polymorphism in MnSOD was not correlated with sensitivity to radiotherapy in breast cancer patients. The present results suggest that the MnSOD genotype could influence the risk of breast cancer associated with low-dose occupational and medical sources of ionizing radiation, but additional epidemiologic studies are needed.

We observed evidence suggesting that the MnSOD Ala/Ala genotype antagonizes a protective effect of NSAID use and breast cancer. Several previous epidemiologic studies of breast cancer, as well as the CBCS, demonstrated protective effects of NSAID use (for a review, see [28]). In the CBCS, an inverse association was observed for occasional and regular users, and for users of prescription NSAIDs as well as nonprescription NSAIDs. The effects were strongest among women with the longest NSAID use [28]. Proposed mechanisms for the protective effect of NSAID use include decreased production of inflammatory cytokines, decreased cell proliferation, and reduced production of ROS. Interactions between NSAIDs and the MnSOD genotype suggest that both factors operate on a common biochemical pathway or series of pathways. Reduced ROS and decreased oxidative damage may thus underlie the protective effect of NSAID use and breast cancer. The gene–environment interaction also provides evidence that the protective effect of NSAIDs and breast cancer observed in the present study and in previous epidemiologic studies may be causal, and may not be due to confounding by lifestyle or other factors.

Epidemiologic studies of the MnSOD Ala-9Val polymorphism are difficult to interpret in light of recent data suggesting that the Val allele leads to impaired protein transport and to reduced MnSOD activity, while the Ala allele has normal activity [13]. Indeed, while studies of breast cancer have consistently implicated the Ala allele in increasing risk, studies of other health outcomes show differing associations [13,17,18,21]. The transport studies of Sutton and colleagues [13] were conducted in rat liver, which may not be generalizable to human breast tissue or other tissues. Since MnSOD removes the superoxide anion, a potential source of DNA damage, one would predict that that the MnSOD Val allele would lead to an increased risk of cancer. On the other hand, MnSOD also generates hydrogen peroxide that can be toxic if not removed [13].

As suggested by Wang and colleagues [19], the effects of the MnSOD polymorphism may depend upon the tissue and tumor site, and may perhaps even depend on the host species. The effects of the MnSOD genotype also appear to depend upon environmental exposures that increase or decrease the levels of ROS, and such exposures may differ across populations. Finally, there may be unidentified variants in linkage disequilibrium with the MnSOD-9Val allele that contribute to transport and/or enzymatic activity. The complete story of the MnSOD genotype is probably quite complex, a situation that has proven true for many or most single-nucleotide polymorphisms [42,43].

There are several weaknesses to our study. MnSOD genotypes were not in Hardy–Weinberg equilibrium. One possible explanation for this is laboratory error [44]. We compared our genotyping results with a ‘gold standard’ of direct DNA sequencing, and the results were identical. Genotyping error can thus be practically excluded.
A second weakness of our study is that we had limited power to examine joint effects of the MnSOD genotype and dietary intake of antioxidants (due to lack of data) and alcohol use (due to low levels of alcohol consumption in our study population). Estimates of the joint effects of the MnSOD genotype and exposure to ionizing radiation were imprecise due to the small number of exposed participants.

Finally, the MnSOD Ala-9Val polymorphism may interact with polymorphisms in other genes involved in modulating levels of oxidative damage that were not measured in our study. We did not observe evidence for joint effects of the MnSOD genotype and polymorphisms in the glutathione S-transferases GSTM1, GSTT1, or GSTP1 (data not shown). Genotyping for additional loci that could interact with MnSOD was not conducted for study participants.

Conclusions
In a population-based case–control study of invasive and in situ breast cancer in African American women and in white women, we observed weak joint effects for the MnSOD Ala/Ala genotype and several environmental exposures. Our results are in agreement with previous epidemiologic studies of breast cancer that showed no main effects for the MnSOD genotype [16] and interactions between the MnSOD Ala/Ala genotype and environmental exposures that alter endogenous levels of ROS species [14–16]. The present study adds to previous knowledge by including exposure to ionizing radiation as a potential environmental exposure that may be mediated in part by MnSOD genotype. We also identified a possible antagonistic interaction between NSAID use and the MnSOD genotype.

Taken together, these four studies help to implicate ROS as important biologic intermediates in the etiology of breast cancer. However, the increased risk associated with the MnSOD-9 Ala allele is difficult to interpret given that the Val allele (but not the Ala allele) is associated with reduced MnSOD enzymatic activity in rat liver model systems [13].

As pointed out by St Clair and Kasarskis [21], more functional studies of the MnSOD-9 polymorphism are needed. Epidemiologic studies of MnSOD will shed additional light on the role of ROS in the etiology of breast cancer and other diseases, and could help to understand the mechanisms of NSAIDs and other medications that reduce the risk of breast cancer.

Authors’ contributions
RM, PM, VV, and TK participated in the interpretation of results and writing of the manuscript.

C-KJT conducted the statistical analyses.

JP, ARdC and GP conducted the laboratory analyses.

Competing interests
None declared.

Acknowledgements
The study was supported by the Specialized Program of Research Excellence (SPORE) in Breast Cancer (NIH/NCI P50-CA58223), by the Center for Environmental Health and Susceptibility (NIEHS P30-ES10126) and by the Superfund Basic Research Program (NIEHS P42-ES050946). The authors thank Allison Eaton, Kristin Heard, and Gillian Gilson (University of North Carolina High Throughput Genotyping Core Laboratory), Daynise Skeen and Dr Lynn Dressler (University of North Carolina SPORE Tissue Procurement Facility) for technical assistance, and thank Dr Charles Poole for helpful discussions.

References
1. Feig D, Reid T, Loeb L: Reactive oxygen species in tumorigenesis. Cancer Res 1994, 54:1890s-1894s.
2. DeZwart L, Meerman J, Commandeur J, Vermeulen N: Biomarkers of free radical damage: applications in experimental animals and in humans. Free Radic Biol Med 1999, 26:202-226.
3. Forsberg L, de Faire U, Morgenstern R: Oxidative stress, human genetic variation, and disease. Arch Biochem Biophys 2001, 389:94-93.
4. Cooke M, Evans M, Dizdaroglu M, Lunec J: Oxidative DNA damage: mechanisms, mutation, and disease. FASEB J 2003, 17:1195-1214.
5. Kinnula V, Crapo J: Superoxide dismutases in the lung and human lung diseases. Am J Respir Crit Care Med 2003, 167:1600-1619.
6. Soini Y, Vakkala M, Kahlos K, Paakkio P, Kinnula V: MnSOD expression is less frequent in tumour cells of invasive breast carcinomas than in in situ carcinomas or non-neoplastic breast epithelial cells. J Pathol 2001, 195:156-162.
7. Li Z, Khaletsky A, Wang J, Wong J, Oberley L, Li J: Genes regulated in human breast cancer cell lines overexpressing manganese-containing superoxide dismutase. Free Radic Biol Med 2001, 30:260-267.
8. Zhang H, Zhao W, Venkataraman S, Robbins M, Buettner G, Marshall J, Graham S, Laughlin R, Nemoto T, Shields P: Oxidative stress, human manganese superoxide dismutase gene. Oncogene 1995, 10:1899-2000.
9. Shimoda-Matsubayashi S, Matsumine H, Kobayashi T, Nakagawa-Hattori Y, Shimizu Y, Mizuno Y: Structural dimorphism in the mitochondrial targeting sequence in the human manganese superoxide dismutase gene. Biochem Biophys Res Commun 1996, 226:561-565.
10. Rosenblum J, Gilula N, Lerner R: On signal sequence polymorphisms and diseases of distribution. Proc Natl Acad Sci USA 1996, 93:4471-4473.
11. Wong G: Protective roles of cytokines against radiation: induction of mitochondrial MnSOD. Biochem Biophys Acta 1995, 1271:205-209.
12. Sutton A, Khoury H, Prip-Buus C, Capanec C, Pessayre D, Degoul F: The Ala16Val genetic dimorphism modulates the import of human manganese superoxide dismutase into rat liver mitochondria. Pharmacogenetics 2003, 13:145-157.
13. Ambrosone C, Freudenhain J, Thompson P, Bowman E, Vena J, Marshall J, Graham S, Laughlin R, Nemoto T, Shields P: Manganese superoxide dismutase (MnSOD) gene polymorphisms, dietary antioxidants, and risk of breast cancer. Cancer Res 1999, 59:602-606.
14. Mitrunen K, Sullivan P, Kataja V, Eskelinen M, Kosma V-M, Benhamou S, Uusitupa M, Hirvonen A: Association between manganese superoxide dismutase (MnSOD) gene polymorphism and breast cancer risk. Carcinogenesis 2001, 22:827-829.
15. Egan K, Thompson P, Titus-Ernstoff L, Moore J, Ambrosone C: MnSOD polymorphism and breast cancer in a population-based case–control study. Cancer Lett 2003, 199:27-33.
17. Woodson K, Tangrea J, Lehman T, Madali R, Taylor K, Snyder K, Taylor P, Vitamo J, Albanes D: Manganese superoxide dismutase (MnSOD) polymorphism, alpha-tocopherol supplementa-
tion and prostate cancer risk in the Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study (Finland). Cancer Causes Control 2003, 14:513-518.
18. Stoehlker J, Ingles S, Park D, Zhang W, Lenz H: The –9Ala/ 
9Val polymorphism in the mitochondrial targeting sequence of 
the manganese superoxide dismutase gene (MnSOD) is 
associated with age among Hispanics with colorectal ca-
cinoma. Oncol Rep 2002, 9:235-238.
19. Wang L, Miller D, Sai Y, Liu G, Su L, Wain J, Lynch T, Christiani D: Manganese superoxide dismutase alanine-to-valine polymor-
phism at codon 16 and lung cancer risk. J Natl Cancer Inst 2001, 93:4471-4473.
20. Hung R, Boffetta P, Brennan P, Malaveille C, Gelati U, Placidi E, 
Carta A, Hautefeuille A, Porru S: Genetic polymorphisms of 
MPO, COMT, MnSOD, NQO1, interactions with environmental 
exposures and bladder cancer risk. Carcinogenesis, 2004, in press.
21. St Clair D, Kasarskis E: Genetic polymorphism of the human 
manganese superoxide dismutase: what difference does it make? Pharmacogenetics 2003, 13:129-130.
22. Newman B, Moorman PG, Millikan R, Oarish BF, Geradts J, 
Aldrich TE, Liu ET: The Carolina Breast Cancer Study: integrat-
ing population-based epidemiology and molecular biology. 
Breast Cancer Res Treat 1995, 35:51-60.
23. Moorman PG, Newman B, Millikan RC, Tse C-KJ, Sandler D: Parti-
ticipation rates in a case–control study: the impact of age, 
race, and race of interviewer. Annals Epidemiol 1999, 9:188-
195.
24. Millikan R, Eaton A, Worley K, Biscocho L, Hodgson E, Huang 
W-Y, Geradts J, Iacocca M, Cowan D, C, Conway K, Drexler L: HER2 
codon 655 polymorphism and risk of breast cancer in 
African Americans and whites. Breast Cancer Res Treat 2003, 
79:355-364.
25. Schaid D, Jacobsen S: Biased tests of association: compar-
isations of allele frequencies when departing from Hardy–Wein-
berg proportions. Am J Epidemiol 1999, 149:706-711.
26. Duell E, Millikan R, Pittman G, Winkel S, Lunn R, Tse C-K, Eaton 
A, Mohrenweiser H, Newman B, Bell D: Polymorphisms in the 
DNA repair gene XRCC1 and breast cancer. Cancer Epidemiol 
Biomarkers Prev 2001, 10:217-222.
27. Moorman P, Ricciuti M, Millikan R, Newman B: Vitamin supple-
ment use and breast cancer in a North Carolina population. 
Public Health Nutr 2001, 4:821-827.
28. Moorman P, Grubber J, Millikan R, Newman B: Association 
between non-steroidal anti-inflammatory drugs (NSAIDs) and 
invasive breast cancer and carcinoma in situ of the breast. 
Cancer Causes Control 2003, 14:915-922.
29. Weinberg C, Sandler D: Randomized recruitment in 
case–control studies. Am J Epidemiol 1991, 134:421-432.
30. Rothman K, Greenland S: Modern Epidemiology, second edition. 
Philadelphia, PA: Lippincott-Raven; 1998.
31. Lundberg M, Frelund P, Hallqvist J, Diderichsen F: A SAS 
program calculating three measures of interaction with confi-
dance intervals. Epidemiology 1996, 7:655-656.
32. Millikan RC, Pittman GS, Newman B, Tse C-KJ, Selmin O, Rock-
hill B, Savitz D, Moorman PG, Bell DA: Cigarette smoking, N-
acetyltansferases 1 and 2 and breast cancer risk. Cancer 
Epidemiol Biomarkers Prev 1998, 7:371-378.
33. Kinney AY, Millikan RC, Lin YH, Moorman PG, Newman B: 
Alcohol consumption and breast cancer among black and 
white women in North Carolina. Cancer Causes Control 2000, 
11:345-357.
34. Moorman P, Kuwabara H, Millikan R, Newman B: Menopausal 
hormones and breast cancer in a biracial population. Am J 
Public Health 2000, 90:966-971.
35. Moorman P, Millikan R, Newman B: Oral contraceptives and 
breast cancer among African-American women and white 
women. J Natl Med Assoc 2001, 93:329-334.
36. Colhoun H, McKerrow P, Smith G: Problems of reporting 
genetic associations with complex outcomes. Lancet 2003, 
361:865-872.
37. Ioannidis J, Ntzani E, Trikalinos T, Contopoulos-Ioannidis D: Repli-
cation validity of genetic association studies. Nat Genet 2001, 
29:306-309.