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Nitric Oxide Synthase Variants and Disease-Free Survival among Treated and Untreated Breast Cancer Patients in a Southwest Oncology Group Clinical Trial

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Abstract

Purpose: Numerous chemotherapeutic agents are cytotoxic through generation of reactive species, and variability in genes related to oxidative stress may influence disease-free survival (DFS). We examined relationships between DFS and variants in *NOS3*, as well as *NQO1, NQO2*, and *CBR3*, among treated and untreated breast cancer patients in a Southwest Oncology Group clinical trial (S8897).

Experimental Design: In the parent trial, women were assigned according to prognostic features; the high-risk group was randomized to cyclophosphamide, i.v. methotrexate, and 5-fluorouracil or to cyclophosphamide, i.v. doxorubicin, and 5-fluorouracil ± tamoxifen, and the low-risk group did not receive adjuvant therapy. We extracted DNA from normal lymph node tissue and examined functional polymorphisms in *NOS3, NQO1, NQO2*, and *CBR3*, in relation to DFS, using Cox proportional hazard model.

Results: There were significant interactions between DFS, adjuvant therapy, and *NOS3* Glu298Asp and -786 polymorphisms, alone and in combination (*P* for interaction = 0.008). When *NOS3* genotypes were combined, women with genotypes encoding for lower nitric oxide who received chemotherapy had a >2-fold increase in hazard of progression (hazard ratio, 2.32; 95% confidence interval, 1.26-4.25), whereas there was reduced risk for those who did not receive adjuvant therapy (hazard ratio, 0.42; 95% confidence interval, 0.19-0.95). There were no associations between the other genotypes and DFS in either group.

Conclusion: Variants encoding lower activity of *NOS3* may affect outcomes in breast cancer patients, with the direction of risk differing depending on chemotherapy status. These results may mirror the known dual functions of nitric oxide and nitric oxide synthase, depending on oxidative environment. (Clin Cancer Res 2009;15(16):5258–66)

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Translational Relevance

In this ancillary study to a clinical trial, germ-line variants encoding lower activity of *NOS3* are related to better prognosis in untreated patients, but conversely, with worse outcomes in patients who are treated with chemotherapy, perhaps by altering sensitivity normally induced by NOS3-generated production of nitric oxide. The heterogeneity of effects by whether or not patients received chemotherapy cautions against studies of predictors of breast cancer survival among groups of patients with varied treatments, or for whom treatment regimens are unknown, because the effects can vary dramatically based on treatment status. This growing body evidence of the important role of endogenous oxidants in chemotherapy treatment outcomes warrants increased investigation of potential interactions with supplement use by patients undergoing chemotherapy treatment, and may add weight to the recommendations that patients receiving adjuvant chemotherapy should be cautioned about the use of antioxidant supplements.

Breast cancer is the most commonly diagnosed cancer and the second leading cause of cancer-related death in American women (1, 2). Adjuvant systemic therapy, in particular chemotherapy and endocrine therapy for hormone receptor–positive patients, reduces the risk of recurrence and mortality for women with early-stage disease (3). However, only a fraction of patients benefit from adjuvant systemic treatment, either because their prognosis is so favorable that they are not likely to relapse without treatment or because the therapy is ineffective. Many somatic factors, such as nodal status, tumor size and grade, and expression of hormone receptors and HER2, are used to determine prognosis and prediction for specific therapies (4). The role of inherited genetic factors has not been thoroughly investigated in relation to breast cancer treatment outcomes.

Cyclophosphamide and anthracyclines exert their cytotoxic effects through apoptosis via intrinsic mitochondrial pathways, mediated through the production of reactive oxygen species (ROS) and concomitant oxidative stress (5). As an activated analogue of cyclophosphamide, 4-hydroperoxy-cyclophosphamide mediates caspase-independent T-cell apoptosis involving oxidative stress–induced nuclear relocation of mitochondrial apoptosis (5). Murata and colleagues showed generation of hydrogen peroxide during degradation of 4-hydroperoxy-cyclophosphamide and showed that blocking this by addition of catalase abrogated DNA damage in a leukemic cell line (6). It has also been shown that cyclophosphamide-mediated apoptosis in human granules cells in culture involves oxidative stress and glutathione depletion (7). Among various combinations of intracellular mechanisms for cytotoxic effects of anthracyclines, a number of oxidoreductases catalyze the one-electron reduction of the quinone moiety of anthracyclines (ring C) to generate a semiquinone free radical that covalently interacts with and damages DNA. In the presence of oxygen, this free radical can be oxidized back to the quinone, concomitantly generating ROS such as superoxide. Doxorubicin semiquinone can also be subjected to reductive deglycosidation to form 7-deoxyaglycone, which in turn forms ROS by intercalating into biological membranes (reviewed in ref. 8).

Thus, common variants in genes that generate or protect from ROS may play a role in breast cancer treatment outcomes. Among them, endothelial nitric oxide synthase (NOS3), NAD(P)H dehydrogenase, quinone 1 (NQO1), NAD(P)H dehydrogenase, quinone 2 (NQO2), and carbonyl reductase 3 (CBR3) are plausible candidates because of their potential role during the detoxification of quinones and oxidative stress (reviewed in ref. 9). NOS3 generates low amounts of short-lived nitric oxide (NO) by converting L-arginine to citrulline in endothelial tissue (10). The *NOS3* 786 T > C promoter polymorphism results in reduced promoter activity, and a 894 G > T polymorphism in *NOS3* exon 7 results in an amino acid substitution that alters susceptibility to cleavage, leading to decreased endothelial NO production (11–13). Similar to NOS3, NQO1 and NQO2, as oxidoreductases, catalyze the one-electron reduction of the quinone moiety of anthracyclines (doxorubicin), which is associated with metabolic detoxification or activation of quinones and quinone-based antitumor drugs (8, 14). A 609 C > T polymorphism in *NQO1* leads to a proline-to-serine substitution (15) that results in rapid degradation of the variant enzyme (16, 17). CBR activity metabolizes a broad spectrum of xenobiotic carbonyl compounds, including various pharmacologic agents (18) such as haloperidol and doxorubicin (19). CBRs catalyze the two-electron reduction of the side-chain C-13 carbonyl group to form anthracycline-alcohol metabolites, transforming doxorubicin to doxorubicinol, which has diminished antineoplastic activity (8, 18). Variable cytosolic CBR activities have been documented in breast and lung tumors, as well as in human liver (20, 21). Blanco and colleagues found that a common genetic polymorphism in *CBR3* (*CBR3* V244M) results in protein isoforms with distinctive catalytic properties toward the quinone menadione, doxorubicin, and the NADP(H) cofactor (22).[14]

In this study, in the context of a completed clinical trial [Southwest Oncology Group (SWOG) 8897], we investigated whether women with functional genetic polymorphisms that result in less protection from chemotherapy-induced oxidative damage would presumably have better tumor cell kill and, subsequently, less disease recurrence than those with common alleles. We hypothesized that genotypes would affect outcomes of patients treated with chemotherapy through indirect effects on treatment-induced cytotoxicity, but would have no effects among untreated patients.

Materials and Methods

Clinical information. These analyses were conducted within the context of a completed clinical trial for breast cancer (S8897), which was led by SWOG within the North American Breast Cancer Intergroup (INT0102). Methods and results are reported according to the REMARK criteria (23). Complete details of S8897 have been reported elsewhere (24). As shown in Fig. 1, women with node-negative breast cancer who were eligible for the trial were assigned to groups based on presumed risk of recurrence (low, indeterminate, high) according to standard prognostic features (tumor size and hormone receptor status). Those in the indeterminate group were then assigned to either the low-risk

[14] http://www.ncbi.nih.gov/pubmed/18457324?ordinalpos=1&itool=EntrezSystem2PEntrezPubmedPubmed_ResultsPanelPubmed_DefaultReportPanelPubmed_RVDocSum

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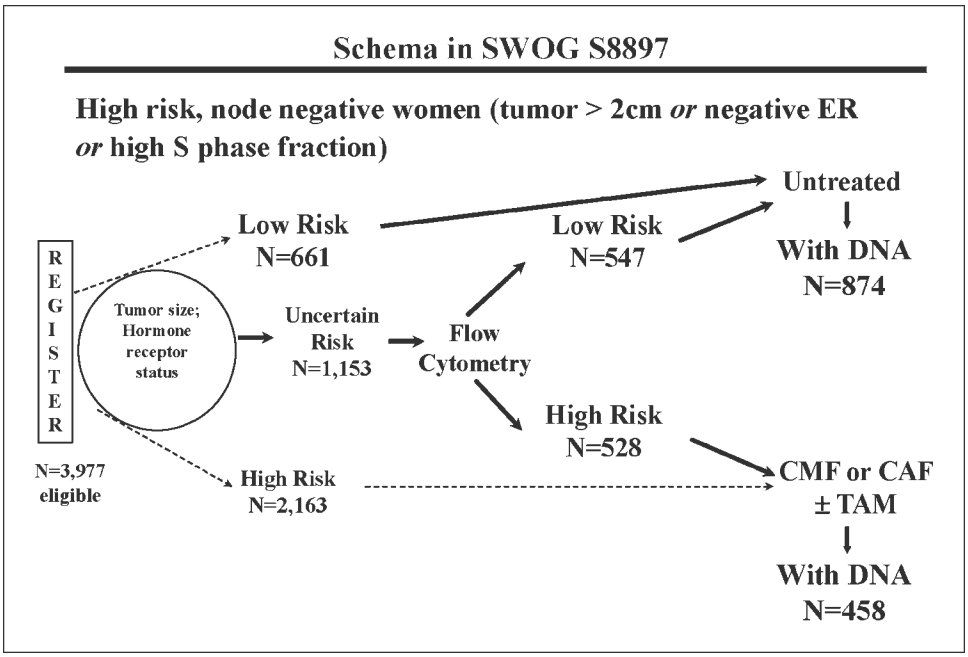

Fig. 1. Schema in SWOG S8897.

or the high-risk group based on flow cytometry results. Those in the low-risk group (n = 1208) were assigned to follow-up with no adjuvant therapy, and those in the high-risk group (n = 1153) were randomly assigned to adjuvant chemotherapy consisting of cyclophosphamide, i.v. methotrexate, and 5-fluorouracil (CMF) or cyclophosphamide, i.v. doxorubicin, and 5-fluorouracil (CAF). CMF chemotherapy was administered per guidelines established by Bonadonna et al. (25), with cyclophosphamide 100 mg/m^2 orally on days 1 to 14, methotrexate 40 mg/m^2 i.v. on days 1 and 8, fluorouracil 600 mg/m^2 i.v. on days 1 and 8, repeated at 28-d intervals for six cycles; and CAF chemotherapy per guidelines established by Bull et al. (26), with cyclophosphamide 100 mg/m^2 orally on days 1 to 14, doxorubicin 30 mg/m^2 i.v. on days 1 and 8, fluorouracil 500 mg/m^2 i.v. on days 1 and 8, repeated at 28-d intervals for six cycles. Doses were delayed for up to 2 wk for granulocytopenia or thrombocytopenia, and then given at reduced levels if granulocytopenia or thrombocytopenia persisted. Doses on day 8 were decreased or withheld accordingly.

Hematopoietic growth factors were not used. In tamoxifen arms, tamoxifen 20 mg/d was started on day 29 of cycle 6 and was continued for 5 y. Distributions of age, race, and type of primary surgery were similar among all treatment groups. The end points for the trial were disease-free survival (DFS) as defined prospectively in the S8897 protocol. DFS was calculated from the date of randomization to the date of first recurrence or death due to any cause, and included local, regional, or distant recurrence or a new breast primary, but not a new nonbreast primary cancer. As previously reported (24), the median follow-up time was 10.8 y, and 10-y estimates indicated that CAF was not significantly better than CMF for DFS.

Specimens and genotyping. As shown in Fig. 1 and as previously reported (27), only patients with archived, uninvolved lymph node tissue available in the SWOG tissue bank from which DNA extraction could be done were included in the current genotyping study. This group included women initially assigned to the low-risk group (n = 1208), and those who were in the indeterminate group, subsequently assigned to low-risk (n = 547) or high-risk (n = 528) category. Thus, participants in the high-risk group included in our analyses all had hormone receptor–positive tumors, generally between 1 to 2 cm in size, whereas those in the low-risk group had either hormone receptor–positive tumors or tumors too small for evaluation. Patients consented to the use of their tissue for ancillary research studies, and this pharmacogenetic study was approved by the Institutional Review Board at Roswell Park Cancer Institute. Two 5-μm slides of normal lymph node tissue were procured from the SWOG tissue bank in San Antonio, were deparaffinized, and removed from slides; DNA was extracted as previously described (28). DNAs were plated in a blinded fashion for genotyping, with duplicates from 5% of samples included for quality control. Genotyping for *NOS3*, *NQO1*, *NQO2*, and *CBR3* was done using Sequonom high-throughput matrix assisted laser desorption/ionization time-of-flight mass spectrometry. A total of 458 high-risk and 874 low-risk participants had sufficient amplifiable DNA, and genotyping rates were ≥97.5% for all polymorphisms assessed, with differences in genotyping rates between those in the treated and those in the untreated arms not exceeding 3.2%.

Statistical analyses. The effects of genetic polymorphisms on DFS were evaluated by contrasting those with genotypes associated with higher ROS, assumed to have better tumor cell kill and thus better survival, against women with lower oxidative stress–related genotypes. Kaplan-Meier estimates of DFS for the subgroups of patients with common and variant alleles were generated, with differences tested using log-rank tests. Cox regression models were used to estimate hazard ratios (HR) and 95% two-sided confidence intervals, with adjustments for age (continuous), race (Caucasian versus non-Caucasian), menopausal status, time from initial surgery to treatment in the treated group, and randomization assignment (CAF versus CMF; tamoxifen versus no tamoxifen) in the treated group. Separate analyses were fit for the treated (high-risk) and untreated (low-risk) groups. We also used a composite model that tested an explicit interaction between treatment and genotypes on DFS. We further evaluated if relationships varied by whether women received CAF or CMF or were assigned to tamoxifen or not. To evaluate whether multiple polymorphisms in the same pathway may have synergistic or additive effects, "high-risk" alleles of each gene were tallied. The number of high-risk alleles for each individual ranged from 0 to 12 and was subsequently categorized as 0 to 3, 4, 5, and ≥6.

Results

The characteristics of the SWOG S8897 participants for whom genotyping and vital status data were available are shown in Table 1. Women in the treated group tended to be

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Table 1. Demographic and clinical characteristics of patients participating in SWOG 8897 from whom DNA was available

Characteristic	% Treated group (*n* = 458)	% Untreated group (*n* = 874)
Age		
<40	15	8
40-49	37	31
50-59	23	25
60-69	19	24
≥70	6	12
Median (range)	49 (27-85)	54 (24-89)
Race		
White, non-Hispanic	88	92
Black, non-Hispanic	6	4
Hispanic	2	2
Other	4	2
Menopausal status		
Premenopausal	51	38
Postmenopausal	49	62
Postmenopausal estrogen		
Yes	14	18
No	86	82
Primary treatment		
BSP, delayed RT	18	23
BSP, RT before registration	12	13
Mastectomy	70	64
Receptor status		
Receptor positive	100	-
ER and PgR negative	0	-
Node involvement		
No	100	100
Yes	0	0
Tumor size, cm		
≤1	25	59
1.1-1.9	75	40

Abbreviations: ER, estrogen receptor; PgR, progesterone receptor; BSP, breast sparing surgery; RT, radiation therapy.

younger, premenopausal, and to have larger tumor size than women in the untreated group. When examining associations between genotypes and DFS, we noted a statistically significant interaction (*P* for interaction = 0.003) between treatment status and the *NOS3* Glu298Asp (G to T) polymorphism, with diametrically opposite associations between the treated and untreated groups. As shown in Fig. 2, women who were homozygous for T alleles, associated with lower activity, and who received no adjuvant systemic treatment had better survival than those with GG/GT genotypes [adjusted HR, 0.67; 95% confidence interval (95% CI), 0.42-1.07; Table 2]. In contrast, women treated with CMF or CAF who were homozygous for the variant TT genotype had poorer survival compared with those with GG/GT genotypes (HR, 1.76; 95% CI, 1.14-2.92; Table 2). A second promoter polymorphism in *NOS3* (-786 T to C) was also associated with better survival among women who did not receive treatment (HR, 0.59; 95% CI, 0.38-0.91), but had no effect in the treated arm (HR, 1.15; 95% CI, 0.69-1.91; *P* for interaction = 0.049; Table 2). When *NOS3* genotypes were combined, associations were further strengthened. As shown in Table 3, women who did not receive adjuvant therapy and had both low-activity genotypes (*NOS3* -786 CC and 894 TT) were at significantly decreased risk of recurrence (HR, 0.42; 95% CI, 0.19-0.95) compared with women with other genotypes (-786 TT + TC and 894 GG + GT). Women assigned to chemotherapy who had *NOS3* -786 CC and 894 TT genotypes had a >2-fold increase in risk of recurrence compared with those with common alleles (HR, 2.32; 95% CI, 1.26-4.25; *P* for interaction = 0.008).

No significant associations were observed between the genotypes for *NQO1* Pro187Ser, *NQO2* Lys47Phe, and *CBR3* Val244Met and Val93Val and breast cancer survival in either group (Table 4). When calculating the total number of "at-risk" alleles (those associated with reduced activity of *NOS3*, *NQO1*, *NQO2*, and *CBR3*), and categorizing into four groups (0-3, 4, 5, and 6+), women in the untreated arm with more than six risk alleles had a 2-fold risk reduction in mortality compared with women having zero to three risk alleles (*P* for trend = 0.001), although the magnitude of reduction was not greater than that associated with the *NOS3* CC and TT genotypes combined (data not shown). Among women who were randomized to chemotherapy, there were no significant effects of combined variant alleles (*P* for trend = 0.244).

When evaluating potential modification of results by type of chemotherapy received and further randomization to tamoxifen, we noted that there were no differences by whether or not women received CAF or CMF, although there was a suggestion of stronger effects for CBR3 among women who received the anthracycline-containing regimen (data not shown). Women receiving CAF and homozygous for the *CBR3* A allele had a nonsignificantly increased risk of recurrence and mortality [odds ratio (OR), 1.54; 95% CI, 0.86-2.77] compared with *G* allele carriers, but risks were not elevated for those receiving CMF adjuvant chemotherapy (OR, 0.86; 95% CI, 0.42-1.74). For *NOS3 894T* polymorphism, relationships were strongest among women who were further randomized to tamoxifen treatment (OR, 2.44, 95% CI 1.17-5.05) than those who were not (OR, 1.48, 0.79-2.75). Type of chemotherapy and tamoxifen treatment did not modify any of the null relationships observed between DFS and *NQO1* and *NQO2* polymorphisms.

Discussion

In this ancillary study to SWOG clinical trial 8897, we noted associations between DFS and inherited germ-line polymorphisms in both the promoter and coding regions of *NOS3* that result in decreased activity. Combined risk variants were associated with a >2-fold reduction in hazard of disease-related death among women who did not receive adjuvant systemic therapy, but among women who received alkylating agent–based adjuvant chemotherapy, low-activity *NOS* genotypes were associated with a 2-fold increase in risk of recurrence. We did not find any statistically significant associations between other candidate genes (*NQO1*, *NQO2*, and *CBR3*) and breast cancer outcomes among both untreated and treated groups, although there was a suggestion of an association between DFS and *CBR3* polymorphisms among women who received CAF, in contrast to those receiving CMF.

The differential effects observed with *NOS3* are likely explained by the multiple roles of NO in tumorigenesis and progression, which seem to be substantially modified by tumor environment, cell type, NO concentrations produced, and type of adjuvant treatments received (29, 30). Among women in the untreated group, relationships between *NOS3* variants resulting

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in lower endothelial NO levels and better DFS may be due to the role of NO in disease progression, which seem to be modified by concentrations produced. Low levels have been associated with increased vascular endothelial growth factor synthesis and neovascularization, tumor angiogenesis, blood flow, immune surveillance, enhanced tumor growth, invasion, and metastasis (29, 31–33). Low levels of NO can also enhance the nitrosylation and activation of Ras oncogenes, which are required for tumor growth and maintenance (34, 35). Furthermore, increased NO levels are associated with better tumor vasculature; thus, in the untreated group, higher levels could result in increased angiogenesis. These mechanism could result in slower tumor growth and reduced risk of metastasis among those with genotypes encoding lower levels of NO, resulting in better prognosis.

The contrasting data in the chemotherapy-treated patients, showing that lower levels of NOS3 were associated with poorer survival, are consistent with the theory that NOS3 enzymatic activity may mediate sensitivity to alkylating agent–based chemotherapy by virtue of production of NO. Several studies have shown that NO production interacts with other chemotherapy-induced radicals to form peroxynitrite, which damages lipids, DNA, and proteins via direct oxidative reactions or indirect, radical-mediated mechanisms (36, 37). In addition, enhanced tumor vasculature with higher levels of NO would result in better drug delivery; in this case, independent of prognosis in untreated women, those who receive chemotherapy would be expected to have a worse outcome if they have variants encoding lower levels of NOS3, compared with those who produce ROS, which is precisely what we observed.

Our data are consistent with previously published human studies and *in vitro* data. Co-author Choi previously reported that *NOS3* haplotypes containing low-activity -786C or 894T alleles were associated with greater risk of recurrence among 873 Asian breast cancer patients who received adjuvant chemotherapy (38). Furthermore, NOS3 protein expression either in the tumor itself or in the surrounding vasculature has been associated with favorable prognosis in patients who received chemotherapy (39, 40). Taken together with the present results, these data suggest that patients with major alleles, and therefore normal NOS3 enzymatic expression and activity, seem to benefit from chemotherapy more than those with genotypes encoding lower enzyme expression.

Findings from several cell line studies also provide support for an interaction between NOS3 function and chemotherapy on therapeutic outcomes, potentially mediated by hypoxia-induced

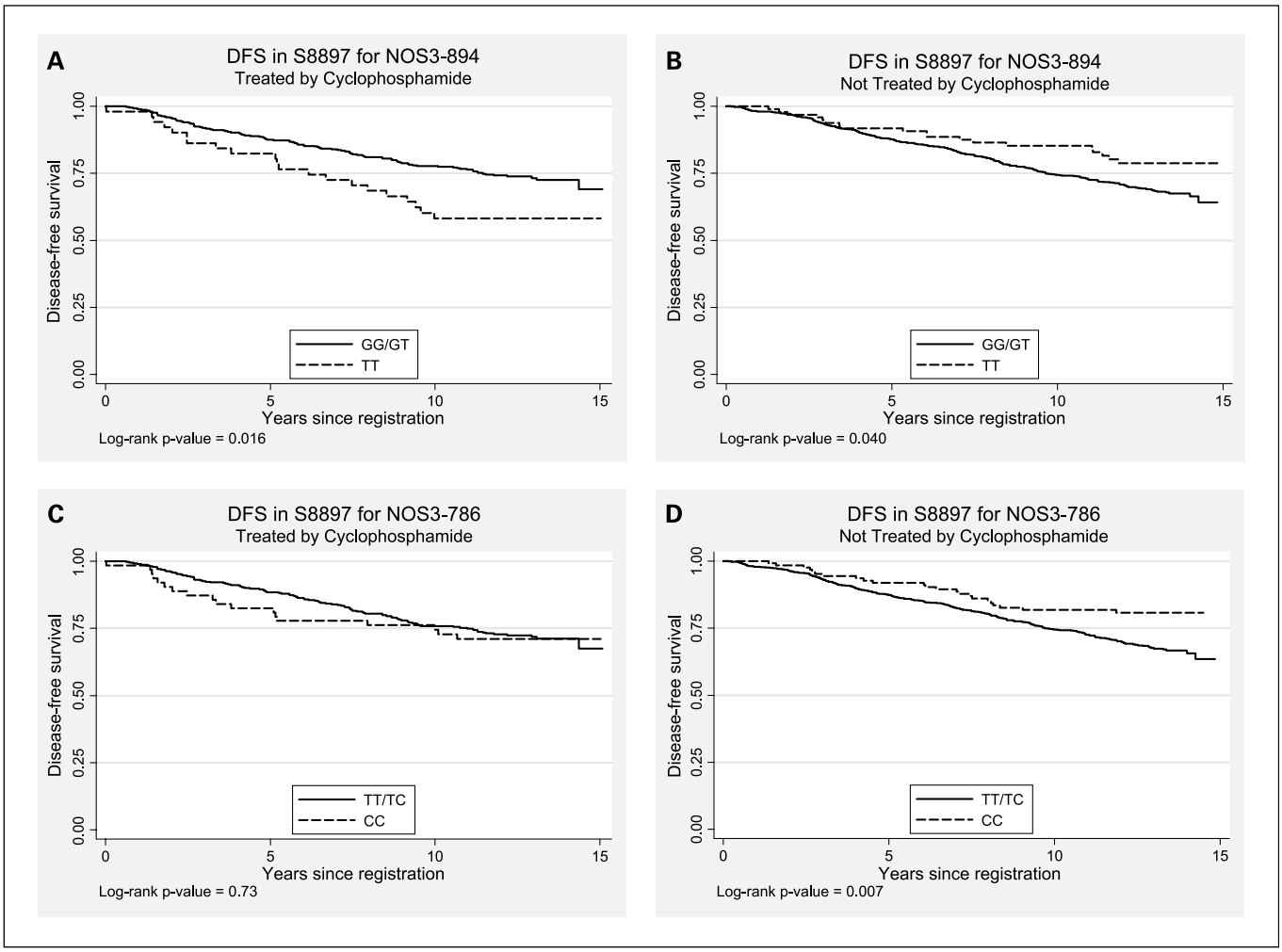

Fig. 2. Disease-free survival by *NOS* genotypes.

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Table 2. Disease-free survival hazard ratios for *NOS3* genotypes among women randomized to CMF or CAF ± tamoxifen and among women not receiving adjuvant therapy

Genotypes	Treated (n = 458)			Untreated (n = 874)		
	Censored	Failures	aHR (95% CI)	Censored	Failures	aHR (95% CI)
NOS3 -786 T > C			n = 445			n = 862
TT	123 (38.2)	56 (45.5)	1.0	230 (37.7)	112 (44.4)	1.0
TC	154 (47.8)	49 (39.8)	0.74 (0.50-1.12)	278 (45.6)	117 (46.4)	0.89 (0.68-1.15)
CC	45 (14.0)	18 (14.6)	0.98 (0.57-1.70)	102 (16.7)	23 (9.1)	0.55 (0.35-0.87)
P_{trend}			0.575			0.015
$P_{interaction}$						0.089
TT + TC	277 (86.0)	105 (85.4)	1.0	508 (83.3)	229 (90.9)	1.0
CC	45 (14.0)	18 (14.6)	1.15 (0.69-1.91)	102 (16.7)	23 (9.1)	0.59 (0.38-0.91)
$P_{interaction}$						0.049
NOS3 894 G > T			n = 438			n = 862
GG	154 (48.7)	65 (53.3)	1.0	290 (47.5)	132 (52.4)	1.0
GT	131 (41.5)	36 (29.5)	0.71 (0.47-1.08)	241 (39.5)	101 (40.1)	0.97 (0.75-1.26)
TT	31 (9.8)	21 (17.2)	1.54 (0.94-2.53)	79 (13.0)	19 (7.5)	0.66 (0.41-1.07)
P_{trend}			0.526			0.171
$P_{interaction}$						0.005
GG + GT	285 (90.2)	101 (82.8)	1.0	531 (87.1)	233 (92.5)	1.0
TT	31 (9.8)	21 (17.2)	1.76 (1.10-2.83)	79 (13.0)	19 (7.5)	0.67 (0.42-1.07)
$P_{interaction}$						0.003

NOTE: HR controlling for age, race, menopausal status, time between surgery and registration, and types of treatment among the treated arm.
Abbreviation: aHR, adjusted hazard ratio.

chemoresistance through the low-concentration NO-cyclic guanosine 3′,5′-monophosphate signaling pathway, which leads to the phosphorylation of various target molecules that regulate cell function and gene expression (41). Matthews et al. (42) showed that hypoxia-mediated acquisition of resistance to doxorubicin and 5-fluorouracil in human breast carcinoma could be attenuated by low concentrations of the NO mimetics glyceryl trinitrate or diethylenetriamine-NO adduct, suggesting that suppression of endogenous NO production is important in the development of hypoxia-induced drug resistance. Recently, NO mimetics were also found to attenuate drug resistance associated with spheroid culture of human MDA-MB-231 breast carcinoma cells, which are associated with a high level of resistance to antitumor drugs (43). Interestingly, incubation with glyceryl trinitrate or diethylenetriamine-NO adduct decreased survival of cancer cells by up to 47%, but the survival of MDA-MB-231 cells was not affected by incubation with glyceryl trinitrate or diethylenetriamine-NO adduct in the absence of doxorubicin (43). The inverse association between low concentrations of NO and increasing rates of chemoresistance is unlikely specific to a particular chemoregimen because NO mimetics have been shown to be effective in reducing hypoxia-induced acquired resistance to doxorubicin, 5-fluorouracil, and paclitaxel (41–43), supporting our findings that the low-activity *NOS3* 894 T allele was associated with worse DFS for both CAF- and CMF-treated patients.

In addition to NOS3, there are two other isoforms of NOS; neuronal NOS (NOS1 or nNOS) and inducible NOS (NOS2 or iNOS). NOS1 and NOS3 are constitutive and expressed in neuronal and vascular endothelial cells, respectively. NOS2, however, is transcriptionally regulated and induced by factors related to hypoxia and oxidative stress. In addition to the research showing dual roles for NOS3, there has also been much interest and research in NOS2 in cancer etiology and treatment outcomes due to its role in immunologic responses. Similar to the proneoplastic and antineoplastic properties of NOS3 and NO, iNOS has been shown to exhibit the same characteristics. There have been numerous *in vitro* and *in vivo* studies that have

Table 3. Associations between *NOS3* combined genotypes and DFS, treated and untreated arms in a recessive model

Genotype		Treated			Untreated		
***NOS3* -786**	***NOS3* 894**	Censored	Failures	aHR (95% CI)	Censored	Failures	aHR (95% CI)
TT + TC	GG + GT	253 (80.6)	93 (78.2)	1.0	467 (76.8)	215 (85.7)	1.0
TT + TC	TT	18 (5.7)	8 (6.7)	1.19 (0.57-2.47)	39 (6.4)	13 (5.2)	0.84 (0.48-1.48)
CC	GG + GT	30 (9.6)	6 (5.0)	0.63 (0.27-1.44)	62 (10.2)	17 (6.8)	0.67 (0.41-1.10)
CC	TT	13 (4.1)	12 (10.1)	2.32 (1.26-4.25)	40 (6.6)	6 (2.4)	0.42 (0.19-0.95)
P for interaction							0.008

NOTE: HR controlling for age, race, menopausal status, time between surgery and registration, and types of treatment among the treated arm.

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Table 4. Disease-free survival hazard ratios for *NQO1, NQO2*, and *CBR3* genotypes among women randomized to CMF or CAF ± tamoxifen and among women not receiving adjuvant therapy

Genotypes	Treated (n = 458)			Untreated (n = 874)		
	Censored	Failures	aHR (95% CI)	Censored	Failures	aHR (95% CI)
NQO1 609 C > T			n = 447			n = 863
CC	210 (65.2)	90 (72.0)	1.0	388 (63.7)	172 (67.7)	1.0
CT	97 (30.1)	28 (22.4)	0.71 (0.47-1.10)	191 (31.4)	71 (28.0)	0.85 (0.65-1.13)
TT	15 (4.7)	7 (5.6)	1.10 (0.51-2.39)	30 (4.9)	11 (4.3)	0.74 (0.40-1.36)
P_{trend}			0.368			0.162
$P_{interaction}$						0.522
CC + CT	307 (95.3)	118 (94.4)	1.0	579 (95.1)	243 (95.7)	1.0
TT	15 (4.7)	7 (5.6)	1.21 (0.56-2.60)	30 (4.9)	11 (4.3)	0.77 (0.42-1.42)
$P_{interaction}$						0.345
NQO2 14055 T > C		n = 450			n = 867	
TT	209 (64.5)	86 (68.3)	1.0	366 (60.0)	166 (66.1)	1.0
TC	105 (32.4)	37 (29.4)	0.87 (0.59-1.28)	212 (34.8)	73 (29.1)	0.77 (0.58-1.01)
CC	10 (3.1)	3 (2.4)	0.76 (0.24-2.39)	32 (5.3)	12 (4.8)	0.81 (0.45-1.45)
P_{trend}			0.407			0.082
$P_{interaction}$						0.905
TT + TC	314 (96.9)	123 (97.6)	1.0	578 (94.8)	239 (95.2)	1.0
CC	10 (3.1)	3 (2.4)	0.79 (0.25-2.49)	32 (5.3)	12 (4.8)	0.88 (0.49-1.58)
$P_{interaction}$						0.894
CBR3 ex1-11 C > T		n = 447			n = 860	
CC	116 (36.0)	48 (38.4)	1.0	194 (31.8)	87 (34.8)	1.0
CT	143 (44.4)	54 (43.2)	0.90 (0.61-1.33)	311 (51.0)	131 (52.4)	0.93 (0.71-1.22)
TT	63 (19.6)	23 (18.4)	0.93 (0.56-1.53)	105 (17.2)	32 (12.8)	0.74 (0.49-1.11)
P_{trend}			0.692			0.167
$P_{interaction}$						0.706
CC + CT	259 (80.4)	102 (81.6)	1.0	505 (82.8)	218 (87.2)	1.0
TT	63 (19.6)	23 (18.4)	0.98 (0.63-1.55)	105 (17.2)	32 (12.8)	0.73 (0.52-1.08)
$P_{interaction}$						0.566
CBR3 ex3-155G > A		n = 445			n = 863	
GG	125 (39.1)	46 (36.8)	1.0	268 (43.9)	100 (39.7)	1.0
GA	144 (45.0)	55 (44.0)	1.01 (0.68-1.50)	270 (44.2)	125 (49.6)	1.14 (0.88-1.49)
AA	51 (15.9)	24 (19.2)	1.19 (0.76-1.86)	73 (11.9)	27 (10.7)	1.01 (0.66-1.55)
P_{trend}			0.543			0.613
$P_{interaction}$						0.596
GG + GA	269 (84.1)	101 (80.8)	1.0	538 (88.1)	225 (89.3)	1.0
AA	51 (15.9)	24 (19.2)	1.19 (0.76-1.86)	73 (11.9)	27 (10.7)	0.95 (0.63-1.41)
$P_{interaction}$						0.404

shown the tumorigenic properties of iNOS, particularly in rodent models (reviewed in ref. 44). Other studies have shown that iNOS inhibits tumor formation, growth, and metastasis (44). It is likely that the tumor microenvironment also influences the effects of high levels of NO generated by iNOS, and in fact, there is preclinical research to exploit iNOS in chemotherapy regimens.

The effect of NOS3 genotypes on DFS seemed to be more pronounced among women receiving tamoxifen, possibly because tamoxifen treatment interrupts the activation of NOS3 by estrogens, which would be beneficial for preventing hypoxia- related chemoresistance. Estradiol, and potentially other steroid hormones such as dehydroepiandrosterone sulfate, can stimulate NOS3 expression in breast cancer cell lines through both estrogen receptor–dependent and estrogen receptor–independent phosphorylation by the phosphoinositide 3-kinase-Akt pathway (45–50). Therefore, disruption of the estrogen-estrogen receptor complex by tamoxifen might lead to reduced levels of NO generated from NOS3, which would further reduce NO levels in low-activity *NOS3* 894 T allele carriers, resulting in worse disease prognosis. Because aromatase inhibitors directly deplete estrogen, the potential modification of aromatase inhibitor efficacy by *NOS3* polymorphisms merits investigation.

Consistent with the role of CBR3 in the pharmacodynamics of doxorubicin, increased hazards associated with the *CBR3* -155 A allele, although not statistically significant, were only observed among those receiving CAF chemotherapy, with no associations observed among the CMF or the untreated group. These findings would be consistent with the expectation that the high activity *CBR3* A allele would lead to faster conversion of doxorubicin to doxorubicinol, which is less antineoplastic but plays a key role in anthracycline-related chronic cardiotoxicity (51, 52). A recent study examining *CBR1* and *CBR3* genotypes with the pharmacokinetics and pharmacodynamics of doxorubicin in a group of 101 breast cancer patients found the *CBR3* A variant to be associated with higher doxorubicinol area under the curve and CBR3 expression in breast tumor tissues (53). CBR3 variants may play a more important role in treatment-related toxicities than in tumor cell kill. There is relatively low expression of CBR3 in liver, and the activity of this enzyme *in vitro* is very modest. However, due to the relatively small number of women who were homozygous for the *CBR3* variant alleles, these findings need to be confirmed in a larger

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population. Furthermore, because epirubicin has been shown to provide some survival advantage over methotrexate (54) and the anthracycline used in this trial, doxorubicin, did not, it is possible that results may not be generalizable to all anthracyclines used in chemotherapy regimens.

In this study, we used genetic polymorphisms as surrogates for systemic enzymatic levels. Because we did not have tumor tissue available, we were not able to measure mRNA or protein in the breast tumors or to correlate levels with genotypes. However, there are ample data supporting the functional effects of both polymorphisms evaluated (refs. 11–13), and expression levels of NOS3 in blood mononuclear cells were lower among those with T-786C alleles, in a dose-dependent manner, in both patients with Alzheimer's disease and healthy controls (55). NOS3 gene expression levels and protein concentrations and enzyme activity were also genotype dependent in human cultured endothelial cells (56).

It is possible that the differential results for *NOS3* genotypes among treated and untreated groups could result from bias if the frequencies of the genotypes varied with disease characteristics, which were determinants of whether or not patients were randomized to adjuvant chemotherapy. This could be a concern given that all treated patients had larger (1-2 cm) hormone receptor–positive tumors, whereas the low-risk untreated group consisted of patients with smaller tumors (≤1 cm). However, frequencies of the variant minor alleles for the *NOS3* 894 G > T and -786 T > C polymorphisms were virtually identical (-786 C allele: 37.0% in treated, 37.4% in untreated; 894 T allele: 30.9% in treated, 31.2% in untreated), eliminating this potential source of bias. Because one of the aims of this study was to assess modification of outcomes by adjuvant treatment regimens received, DFS, rather than mortality, was used as the outcome of interest because patients may choose to cross over to another therapeutic agent if recurrence occurred, which would complicate the assessment of outcome associations from the first agent (57). A key strength of this pharmacogenetic study was the population source, which provided comparisons between individuals with similar risk for recurrence based on their tumor characteristics and the standardization of treatments received, thereby eliminating biases and confounding due to treatment heterogeneity (58). As well, the presence of an untreated group in the study population was unique, which allowed for the examination of genotype as a modifier of treatment outcomes.

The results of this correlative study, which are consistent with our a priori hypothesis, suggest that inherited, germ-line variants encoding lower activity of the oxidative stress gene *NOS3* may affect outcomes in patients with early-stage breast cancer. These variants may be related to better prognosis in untreated patients by virtue of reducing malignant tumor biology mediated by the activity of this gene, but conversely with worse outcomes in patients who are treated with chemotherapy, perhaps by altering sensitivity normally induced by NOS3 generated production of NO. The heterogeneity of effects by whether or not patients received chemotherapy cautions against studies of predictors of breast cancer survival among groups of patients with varied treatments, or for whom treatment regimens are unknown, because the effects can vary dramatically based on treatment status.

It is likely that oral cyclophosphamide was an important contributor to antitumor effect in these patients because the differences observed apply equally in the arms with and without doxorubicin, and 5-fluorouracil effects are not thought to be mediated through generation of ROS. Whereas we do not recommend any change in standard clinical practice based on these results, this growing body of evidence of the important role of endogenous oxidants in chemotherapy treatment outcomes warrants increased investigation of potential interactions with supplement use by patients undergoing chemotherapy treatment. These findings, and other investigations showing that genotypes related to higher levels of oxidative stress are associated with better survival (59), may add weight to the recommendations that patients receiving adjuvant chemotherapy should be cautioned about the use of antioxidant supplements (60).

Our results suggest that although inhibition of ROS might be an attractive strategy to prevent cancer, it may have adverse effects on the efficacy of adjuvant chemotherapy. However, to date, there are few rigorous data in the literature to support these recommendations. Indeed, based on these findings and others regarding endogenous antioxidants, SWOG is now conducting a prospective clinical trial addressing nonprescribed antioxidant therapy in women participating in a randomized chemotherapy trial (S0221).

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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Nitric Oxide Synthase Variants and Disease-Free Survival among Treated and Untreated Breast Cancer Patients in a Southwest Oncology Group Clinical Trial

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