ORIGINAL ARTICLE

Polymorphisms of the endothelial nitric oxide synthase gene in breast cancer: a genetic association study and meta-analysis

Elias Zintzaras^{1,2}, Maria Grammatikou³, Georgios D Kitsios^{1,2}, Chrysa Doxani^{1,3}, Nikos Zdoukopoulos¹ and Christos Papandreou³

The endothelial nitric oxide synthase gene (*NOS3*) has been proposed as a candidate gene for breast cancer (BC), however, the specific role of variants and haplotypes has not been clarified. We examined the association of two polymorphisms (4b/a and G894T) and their haplotypes in a case–control sample of 306 patients with BC and in 131 healthy females. In addition, a meta-analysis of studies investigating association between *NOS3* polymorphisms and BC was conducted. The single locus analysis for the two polymorphisms revealed an association only for the 4b/a polymorphism, but adjustment for age diminished this association (odds ratio (OR) (95% confidence interval)=0.98 (0.34–2.81)). The analysis of haplotypes showed an association for two haplotypes involving the 894T allele (bT and aT) ($P<0.05$). The meta-analysis for both polymorphisms produced nonsignificant associations without significant heterogeneity. A positive association was detected for the promoter T786C polymorphism (pooled OR=1.51 (1.07–2.12)), but this comparison was based on few studies. The available evidence from our study and the meta-analysis cannot support a major contributory role of these common *NOS3* polymorphisms in BC, although future larger studies may help in drawing safer conclusions about the genetics of BC. *Journal of Human Genetics* (2010) 55, 743–748; doi:10.1038/jhg.2010.100; published online 19 August 2010

Keywords: breast cancer; endothelial nitric oxide synthase; eNOS; gene; genetic association study; meta-analysis; *NOS3*; polymorphism

INTRODUCTION

Breast cancer (BC) is the most commonly occurring cancer among women, with an estimated incidence rate of one million cases per year worldwide. Although epidemiological studies have established many etiological factors, such as ionizing radiation, exogenous hormones and alcohol consumption, substantial evidence shows that genetic susceptibility has a significant role in an individual's risk of developing BC.¹ Germline mutations in high-penetrance BC susceptibility genes, such as *BRCA1* and *BRCA2*, account for the majority of familial cancers but only for 5–10% of all BC cases. For most sporadic BC cases, genetic susceptibility is still poorly understood and the search for low-penetrance genes by hypothesis-driven candidate-gene studies or agnostic approaches is ongoing.²

On the basis of biochemical evidence implicating nitric oxide in breast carcinogenesis,^{3–5} the endothelial nitric oxide synthase gene (*NOS3*) has emerged as a candidate gene for BC. Among the tested variants of the *NOS3* gene, a non-synonymous single-nucleotide polymorphism (G894T substitution in exon 7—rs1799983) and a variable number of tandem repeats in intron 4 (4b/a) have been commonly tested in association with BC, on the basis of their

potential functional effects⁶ and their relatively high minor allele frequency in various ethnic groups.^{7–15}

Candidate-gene studies have nevertheless a poor replication record, which has necessitated the use of stringent validation criteria. Inconsistent results have been also reported by previous studies on the *NOS3* and BC association.^{7–15} Furthermore, agnostic evidence generated by eight genome-wide association studies (GWASs) has not shown any role for *NOS3* region variants.¹⁶ Consequently, the status of associations for variants from candidate-gene studies remains equivocal. Meta-analysis of individual studies provides a natural framework for testing for replication, by considering the totality of available evidence.^{17,18} Particularly for genetic associations, field synopses that catalog and cumulatively synthesize all available candidate-gene studies have shown that most of the proposed associations are refuted in the long term.^{19–22} In the absence of a comprehensive genetic database for BC, evidence synthesis efforts for intensively researched genes can be informative.

In this project, we aimed to test the proposed association between the two most commonly investigated *NOS3* polymorphisms (G894T and 4b/a) in a genetic association study (GAS) for BC in a homogeneous

¹Department of Biomathematics, University of Thessaly School of Medicine, Larissa, Greece; ²Center for Clinical Evidence Synthesis, The Institute for Clinical Research and Health Policy Studies, Tufts Medical Center, Tufts University School of Medicine, Boston, MA, USA and ³Oncology Clinic, University Hospital of Larissa, University of Thessaly School of Medicine, Larissa, Greece

Correspondence: Dr E Zintzaras, Department of Biomathematics, University of Thessaly School of Medicine, 2 Panepistimiou Street, Biopolis, Larissa 41100, Greece. E-mail: zintza@med.uth.gr

Received 14 January 2010; revised 17 July 2010; accepted 26 July 2010; published online 19 August 2010

population of Caucasian origin (Greeks), by considering gene haplotype analyses as well. Furthermore, to provide conclusive evidence on these associations, we performed a cumulative meta-analysis of all available GAS for BC and NOS3 polymorphisms, which also involved explorations for the presence of heterogeneity and potential bias.^{18,17}

MATERIALS AND METHODS

Genetic association study

Study population. A total of 306 cases with BC and 131 controls were recruited from the University Hospital of Larissa. All patients were females treated with chemotherapy (combinations of 5-fluorouracil, anthracycline, taxanes and cyclophosphamide) for BC and provided a written informed consent. The mean age ($\pm$ s.d.) of cases and controls was 60.9 ± 11.9 and 40.7 ± 16.9 , respectively. Controls were free of chronic illness (renal, cardiovascular, mental, hepatic, endocrine disorders or cancer), without any history of malignancy and unrelated by blood to cases. A blood sample for biochemical measurements and DNA extraction was taken from each individual.

Laboratory assays. Genomic DNA was extracted from whole blood using the QIAamp DNA blood kit (QIAGEN, Valencia, CA, USA) following the manufacturer's instruction. Genotyping of each polymorphism was performed by amplification from 50 to 100 ng of genomic DNA. The primer sequences used and the laboratory conditions for genotyping (polymerase chain reaction, restriction enzymes, agarose electrophoresis) for each NOS3 polymorphism have been previously described.^{23,24} Genotyping was performed by laboratory personnel blinded to clinical status.

Data analysis. The genotypic distribution in the control group was tested for compliance with the Hardy–Weinberg Equilibrium (HWE) and the existence of linkage disequilibrium (LD) between the two polymorphisms in both cases and controls was tested by using exact tests according to Weir.²⁵ Haplotype frequencies were estimated and compared using PHASE v2.1.²⁶ The genotype distribution, allele contrast and dominant and recessive models of the cases were compared with those of the control group using a logistic model. The association between genotype distribution and group effect (cases/controls) was tested using the χ^2 -test. The significance of the genetic contrasts was also evaluated using Fisher's exact test.¹⁷ The comparison associations were expressed in terms of odds ratio (OR), unadjusted and adjusted for age, with the corresponding 95% confidence interval. A *P*-value < 0.05 was considered statistically significant.

Meta-analysis

Identification and eligibility of relevant studies. We searched PubMed (until December 2009) for articles using combinations of the following terms: (gene or polymorphism or variant or allele or mutation) and (endothelial nitric oxide synthase or NOS3 or 'endothelial nitric oxide synthase' or 'nitric oxide synthase') and ('BC' or 'breast neoplasm'). The retrieved studies were then read in their entirety to assess their appropriateness for inclusion in the meta-analysis. All references cited in the studies were also reviewed to identify additional published work not indexed by the PubMed database.

GASs that determined the genotype distribution of NOS3 G894T and 4b/a gene polymorphisms in cases with BC and in controls free from BC were eligible for inclusion in the meta-analysis. We also included studies investigating the T786C polymorphism in order to provide a comprehensive synthesis of NOS3-related GAS, despite the fact that this polymorphism was not considered in our GAS protocol and was thus not analyzed. Only studies in human subjects that have used validated genotyping methods were considered. Case reports, editorials and review articles were excluded. Non-English articles were also excluded. In studies with overlapping cases or controls, the most recent and/or the largest in size study with extractable data was included in the meta-analysis. Family-based association and linkage studies were not considered because of different design considerations.^{27,28}

Data extraction. From each study, the following information was abstracted: first author, journal, year of publication, ethnicity of the study population, demographics, clinical characteristics, matching, validity of the genotyping

method and the number of cases and controls for each polymorphism's genotypes. The frequencies of the alleles and genotypic distributions were extracted or calculated, for both cases and controls. In addition, it was recorded whether the genotyping in each study was performed blinded to clinical status. In studies that investigated more than one polymorphism, information on LD and haplotype estimation (or combined genotypes) was recorded.

Data synthesis. The meta-analysis examined the association between each polymorphism and the risk of BC for (i) the allele contrast (mutant type versus wild-type allele), and (ii) the recessive and (iii) dominant models of the mutant allele. The associations were indicated as a pooled OR with the corresponding 95% confidence interval.^{17,18}

Heterogeneity between studies was tested using the *Q*-statistic, which is a weighted sum of squares of the deviations of individual study OR estimates from the overall estimate.²⁹ When ORs are homogeneous, *Q* follows a χ^2 distribution with *r*–1 (*r* is the number of studies) degrees of freedom (d.f.). If *P* < 0.10 then the heterogeneity was considered statistically significant. Heterogeneity was quantified with the *I*² metric (*I*² = (*Q*–d.f.)/*Q*), which is independent of the number of studies in the meta-analysis.³⁰ *I*² takes values between 0 and 100%, with higher values denoting greater degree of heterogeneity (*I*² = 0–25%: no heterogeneity; *I*² = 25–50%: moderate heterogeneity; *I*² = 50–75%: large heterogeneity; *I*² = 75–100%: extreme heterogeneity). The pooled OR was estimated using random effects (DerSimonian and Laird) models.³¹ Random effects modeling assumes a genuine diversity in the results of various studies, and it incorporates a between-study variance into the calculations.^{17,18}

A cumulative meta-analysis was carried out for the allele contrast of polymorphisms investigated in at least three information steps. In cumulative meta-analysis, studies were chronologically ordered by publication year, then, the pooled ORs were obtained at the end of each year; that is, at each information step. Cumulative meta-analysis provides a framework for updating a genetic effect from all studies as evidence accumulates. Thus, cumulative meta-analysis indicates the trend in estimated risk effect over time.^{17,18} A differential magnitude of effect in large versus small studies for the allele contrast of the most commonly studied polymorphism was checked using the test proposed by Harbord *et al.*³²

The meta-analysis consisted of the main (overall) analysis, which includes all available data and subgroup analyses by 'racial' descent. A sensitivity analysis that examines the effect of excluding specific studies was also carried out.¹⁷ The distribution of the genotypes in the healthy control group was tested to determine whether it is in HWE using an exact test. The meta-analysis was subjected to sensitivity analysis for studies with the controls not in HWE. Analyses were performed using CUMAGAS (<http://biomath.med.uth.gr>) and Compaq Visual Fortran90 with the International Mathematics and Statistics Library (Compaq Computer Corporation, Houston, TX, USA).^{19,20,33}

RESULTS

Genetic association study

The clinical characteristics of cases are shown in Table 1. The distributions of NOS3 G894T and 4b/a genotypes were not associated with clinical status and are presented in Table 2. The genotype distribution in controls was in HWE for both polymorphisms, indicating a lack of stratification and/or genotyping error (*P* = 0.62 for 4b/a and *P* = 0.56 for TG894T).

Table 3 shows the association results for the allele contrast, the recessive and dominant models for the mutant alleles. For G894T polymorphism, none of the genetic contrasts produced significant results. Regarding the 4b/a polymorphism, the recessive model produced a marginal association (unadjusted OR = 1.56 (1.02–2.38)), which was not significant in the age-adjusted model (adjusted OR = 0.98 (0.34–2.81)). In the subgroup analysis by menopausal status (Table 3), both polymorphisms derived nonsignificant association for all examined contrasts.

In the patient population, the two polymorphisms were in LD (*P* = 0.02), whereas no LD was detected in controls (*P* = 0.22).

Table 1 Characteristics of patients with breast cancer

Stage	%
I	11
II	32
III	24
IV	33
<i>ER status</i>	
Positive	84
Negative	16
<i>Histology</i>	
Ductal	84
Lobular	9
Mixed	3
Other	4
<i>Grade</i>	
I	9
II	56
III	35
<i>PR status</i>	
Positive	60
Negative	40
<i>LNs involvement</i>	
Yes ($N > 0$)	62
No ($N \leq 0$)	38

Abbreviations: ER, estrogen receptor; LN, lymph node; PR, progesterone receptor.

Table 2 Distribution (%) of the NOS3 G894T and 4b/a genotypes among case and control subjects

SNP	Genotype	Cases N=306	Controls N=131	P-value
G894T	TT	39	38	0.79
	GT	46	50	
	GG	15	12	
4b/a	aa	3	4	0.26
	ab	27	37	
	bb	70	59	

Abbreviations: BC, breast cancer; NOS3, nitric oxide synthase gene. The P-values for testing the association between genotype distribution of each polymorphism and susceptibility to BC are shown.

Table 3 OR and the corresponding 95% CIs for testing the association between breast cancer and NOS3 G894T and 4b/a polymorphisms for the allele contrast (mutant type vs wild type), the dominant and recessive model

Polymorphism	Genetic contrast	OR (95% CI)	P-value	OR adjusted (95% CI)
<i>(a) Overall</i>				
G894T	Allele contrast	0.94 (0.69–1.26)	0.66	
	Recessive model	1.03 (0.68–1.57)	0.89	1.00 (0.36–2.77)
	Dominant model	0.72 (0.39–1.34)	0.30	0.73 (0.18–2.91)
4b/a	Allele contrast	1.39 (0.97–1.99)	0.07	
	Recessive model	1.56 (1.02–2.38)	0.04	0.98 (0.34–2.81)
	Dominant model	1.06 (0.36–3.13)	0.91	0.57 (0.03–11.62)
<i>(b) In postmenopausal cases</i>				
G894T	Allele contrast	0.88 (0.63–1.24)	0.48	
	Recessive model	0.98 (0.61–1.58)	0.98	0.64 (0.09–4.47)
	Dominant model	0.64 (0.39–1.28)	0.23	0.63 (0.07–5.97)
4b/a	Allele contrast	1.24 (0.82–1.86)	0.31	
	Recessive model	1.37 (0.85–2.23)	0.22	1.54 (0.21–11.41)
	Dominant model	0.90 (0.28–2.92)	0.98	0.26 (0.01–1.02)
<i>(c) In premenopausal cases</i>				
G894T	Allele contrast	0.91 (0.57–1.47)	0.71	
	Recessive model	0.93 (0.48–1.83)	0.87	1.02 (0.37–2.78)
	Dominant model	0.79 (0.30–2.08)	0.62	0.73 (0.18–2.92)
4b/a	Allele contrast	1.16 (0.66–2.03)	0.61	
	Recessive model	1.22 (0.63–2.37)	0.62	0.98 (0.35–2.79)
	Dominant model	1.05 (0.20–5.61)	0.93	0.59 (0.03–10.83)

Abbreviations: CI, confidence interval; NOS3, nitric oxide synthase gene; OR, odds ratio. The ORs adjusted for age are also shown.

Table 4 Estimated haplotype frequencies for the two NOS3 polymorphisms (4b/a and G894T)

4b/a-G894T haplotype	Haplotype frequency			P-value overall
	Cases	Controls	P-value	
bT	0.476	0.448	0.04	0.26
bG	0.352	0.330	0.10	
aT	0.140	0.185	<0.01	
aG	0.033	0.036	0.80	

Abbreviation: NOS3, nitric oxide synthase gene. The P-values for comparing each haplotype between cases and controls, and the P-value for the overall comparison of haplotypes between cases and controls are shown.

The distribution of the estimated haplotype distribution of the two NOS3 polymorphisms for BC cases and controls is presented in Table 4. The global test of association was not significant for the comparison of haplotypes between cases and controls ($P=0.26$). However, the estimated frequencies of haplotypes bT and aT were different in cases and controls ($P=0.04$ and $P<0.01$, respectively). This difference might be due to the LD between the two polymorphisms or due to an interaction between the two polymorphisms, given that the association for individual polymorphisms was generally not significant in the main analyses.

Meta-analysis

Eligible studies and their characteristics. The literature review identified 49 titles in PubMed that met the search criteria. The full articles of

the retrieved studies were read to assess their appropriateness for meta-analysis according to the inclusion criteria. Nine articles were considered to be potentially eligible after the article review.^{7–15} In one article,⁷ the subjects overlapped with the subjects of the more recent and larger study by Li *et al.*,⁸ which was eventually included in the meta-analysis. In another article,¹⁵ the data were not extractable. Seven articles^{8–14} that investigated the association between any of the G894T, 4b/a and T786C polymorphisms and BC met the inclusion criteria. Genotypic information on NOS3 variants was not available (in publication tables or online appendices) from the eight GWAS conducted to date. Thus, in total, eight articles (the seven identified in PubMed and the present GAS, referred to as Zintzaras) were included in the meta-analysis. The eligible studies were published between 2003 and 2009.

The articles were reviewed and data were extracted by two investigators (EZ and GK); disagreements were resolved after a discussion between the authors. Eight studies dealt with G894T (Zintzaras),^{8–14} three with 4b/a (Zintzaras)^{10,11} and three with T786C.^{9,10,12} The studies that investigated 4b/a or T786C had also investigated G894T. Two studies^{9,10} investigated the three polymorphisms together: G894T, 4b/a and T786C. Validated genotyping methods were used in all studies for the determination of genotypes. Three studies stated that the controls were age matched.^{10,12,13} Studies were conducted in populations of various racial descents: five involved Whites (Zintzaras),^{10,11,12,14} one East Asian⁹ and two involved mixed populations.^{8,13}

Overall, the studies provided 3828/3124, 996/824 and 1856/1470 cases/controls for G894T, 4b/a and T786C. The distribution of genotypes in the control group deviated from HWE in one study for G894T.⁹ As lack of HWE indicates possible genotyping errors and/or population stratification, a sensitivity analysis was therefore carried out excluding this study. Three of the studies (including the study by Zintzaras) provided analyses of haplotypes.^{9,10} One study⁹ reported existence of LD between G894T and T786C, whereas another study¹⁰ reported lack of LD between G894T, 4b/a and T786C. The present GAS (Zintzaras) reported lack of LD between G894T and 4b/a in the control group but existence of LD in the case group.

Main results, subgroup and sensitivity analyses. Table 5 shows the results for the association between the different polymorphisms and the risk of BC. For the G894T polymorphism, the allele contrast T versus G showed nonsignificant heterogeneity among studies ($P=0.76$, $I^2=0\%$) and the pooled OR was not significant (OR=1.06 (0.98–1.15)) (Figure 1). The recessive model for the effect of allele T produced a marginal association (OR=1.22 (1.00–1.49)), whereas the dominant model derived nonsignificant results. In subgroup analysis, the association was not significant for Whites. The sensitivity analysis (exclusion of the study with the controls not in HWE) did not alter the pattern of results in all polymorphisms and contrasts.

For the 4b/a polymorphism, overall, the heterogeneity between studies was not significant ($P=0.21$, $I^2=36\%$), and allele b was not

associated with risk of BC compared with the a allele (OR=1.07 (0.85–1.34)). The recessive and dominant models also produced nonsignificant associations.

Finally, a significant association between the T786C polymorphism and the risk of developing BC for the recessive model was found (OR=1.51 (1.07–2.12)). However, the result was based on very few studies and the interpretation of results should be cautious.

Potential bias. None of the studies included in the meta-analysis stated that genotyping was performed blinded to clinical status. In cumulative

Figure 1 Random effects (RE) odds ratio (OR) estimates with the corresponding 95% confidence interval (CI) for the allele contrast of G894T allele T and the risk of breast cancer. The OR estimate of each study is marked with a solid black square. The size of the square represents the weight that the corresponding study exerts in the meta-analysis. The CIs of pooled estimates are displayed as a horizontal line through the diamond; this line might be contained within the diamond if the CI is narrow. The horizontal axis is plotted on a log scale.

Table 5 Random effects ORs with their respective 95% CIs and heterogeneity results (I^2 (%) and P_Q -value) for the genetic contrasts of (a) G894T, (b) 4b/a and (c) T786C NOS3 polymorphisms for breast cancer

Variant	Genetic contrast	Population	Studies	OR (95% CI)	I^2 (%) P_Q -value
G894T	T vs G	All	8	1.06 (0.98–1.15)	0 (0.79)
		All in HWE	7	1.06 (0.97–1.16)	0 (0.69)
		Whites	5	1.10 (0.98–1.23)	0 (0.55)
	Recessive model	All	8	1.22 (1.00–1.49)	14 (0.32)
		All in HWE	7	1.19 (0.99–1.43)	0 (0.55)
		Whites	5	1.21 (0.94–1.57)	19 (0.30)
	Dominant model	All	8	1.03 (0.92–1.14)	0 (0.82)
		All in HWE	7	1.04 (0.92–1.17)	0 (0.75)
		Whites	5	1.10 (0.94–1.29)	0 (0.68)
4b/a	b vs a	All (Whites)	3	1.07 (0.85–1.34)	36 (0.21)
	Recessive model	All (Whites)	3	1.13 (0.85–1.49)	46 (0.16)
	Dominant model	All (Whites)	3	0.83 (0.47–1.48)	0 (0.61)
T786C	C vs T	All	3	1.20 (0.97–1.49)	54 (0.11)
	Recessive model	All	3	1.51 (1.07–2.12)	0 (0.74)
	Dominant model	All	3	1.21 (0.92–1.60)	55 (0.11)

Abbreviations: CI, confidence interval; HWE, Hardy–Weinberg equilibrium; NOS3, nitric oxide synthase gene; OR, odds ratio.

Figure 2 Cumulative meta-analysis for the allele contrast of G894T allele T and the risk of breast cancer. The random effects odds ratio (OR) with the corresponding 95% confidence interval (CI) at the end of each year-information step is shown. The horizontal axis is plotted on a log scale.

meta-analysis, the G894T polymorphism showed a downward trend of association as evidence accumulates and the association remained nonsignificant for the whole period (Figure 2). The test by Harbord *et al.*³² for G894T indicated that there is no differential magnitude of effect in large versus small studies ($P=0.20$).

DISCUSSION

This study evaluated relations between common genetic variants and haplotypes in the NOS3 gene with BC. The single locus analysis among the two most commonly studied polymorphisms (G894T and 4b/a) revealed an association only for the 4b/a polymorphism, but adjustment for age diminished this association. The analysis of haplotypes showed an association for two haplotypes involving the 894T allele.

The subsequent meta-analysis examined the NOS3 G894T, 4b/a and T786C polymorphisms and their relationship to susceptibility for BC. The strength of the present meta-analysis was based on the accumulation of data, providing more information to detect significant differences. Main and subgroup analyses in Whites for the allele contrast, as well as for the recessive and dominant models for G894T and 4b/a polymorphisms, have produced nonsignificant results. However, the T786C polymorphism showed an association, but the result was based on very few studies. There was consistency of genetic effects across different ethnicities as the results in Whites did not differentiate from the overall effect. Although the meta-analysis of G894T and 4b/a polymorphisms produced nonsignificant association, the meta-analysis provided useful information for the uncertainty of the estimated genetic risk; that is, the pooled OR excluded with 95% certainty the fact that 894T-carriers and 4b-carriers would have > 10 and 25% increased risk of BC, respectively. The heterogeneity between studies was, in general, low, without an indication of a differential magnitude of effect in large versus small studies.

The conclusions reached in the present analysis of GAS and meta-analysis were based on relatively small numbers of studies and participants for each gene polymorphism and, therefore, any inferences have to be cautious. Candidate-gene studies have a tendency to lack the power to detect weak genetic associations of common variants.^{17,18} For example, to achieve a power >80% to identify a modest genetic effect (OR 1.2) of a polymorphism present in 10% of the population, a sample size of 10 000 subjects or more would be needed.^{17,18,34} Therefore, the sample sizes required to detect an association have to be far beyond what is currently available and no single institution will be able to provide a reasonable number of patients.¹⁹ Meta-analysis of multiple studies has a clear role in offering an analysis with the potential for enhanced power and it has been advocated that publication of each GAS should be accompanied by an updated meta-analysis that places all available evidence in context.^{17,18} Our GAS did not detect a significant role of individual NOS3 polymorphisms and these findings were supported by the meta-analysis results.

In the meta-analysis, only the unadjusted pooled ORs were calculated, as data for possible confounding factors that influence the estimates of associations (for example, age and lifestyle) were not provided. Sampling variability and stratification in GAS could be potential confounding factors on the role of genetic variants.^{35,17} The existence of diversity of these factors across studies may result in the presence of heterogeneity. In addition, the risk effect may depend on interaction with other risk factors that collectively modulate the development of BC.⁸

It has been reported that the polymorphisms might be in LD, and interaction of the polymorphisms within haplotypes can be a major

determinant of disease susceptibility than can be the individual polymorphism.^{33,36,34} Thus, individual NOS3 genotypes might not be reliable markers of risk for developing BC. The haplotype analysis approach is expected to be more powerful than single-marker analysis, because of the ancestral structure incarcerated in the distribution of haplotypes.^{37,38} Particularly if the markers used define mutations within functional DNA, then the haplotypes composed of these markers can have more of a biological role. In our analysis, we focused on haplotypes defined by the two most commonly investigated and potentially functional NOS3 variants (instead of other tested variants or HapMap tagging polymorphisms) because we aimed to update the examined associations in the context of meta-analysis. Although a meta-analysis of NOS3 haplotypes may provide more reliable information than single polymorphisms,³⁹ such an approach was not feasible in this study, as haplotypes were not defined by all previous studies.

Interestingly, no positive association signal for the NOS3 gene or the 7q36 locus has been detected to date from the eight published GWASs for BC.¹⁶ Although the culprit genetic variation in the NOS3 gene has not been elucidated and as GWASs have largely focused on common variants, unknown rare variants or structural variation tagged by G894T or 4b/a may account for the inconsistent associations of these common variants.^{34,40} However, the available evidence from candidate-gene and hypothesis-free studies cannot support a major contributory role of G894T and 4b/a in BC pathogenesis.

In conclusion, the GAS detected an association for two NOS3 haplotypes and BC in the main analysis and the meta-analysis of individual polymorphisms showed that there is significant association between T786C polymorphism and the risk of developing BC. The results of the present individual GAS and meta-analysis should be interpreted with some degree of caution, as the numbers of studies and participants were relatively small. However, BC is a complex disease with multifactorial etiology, and therefore, a minor contributory pathogenetic role of NOS3 variants in conjunction with other genetic or environmental factors cannot be excluded.² Collaborative research in BC may help in identifying the contributing role of variants by performing GAS and GWASs with adequate power.^{33,34,17,19,20} Furthermore, the design of rigorous studies for investigating epistatic and gene-environment interactions and the use of data generated by genomic studies may help in deriving more conclusive claims about the genetics of BC.

- 1 Singletary, S. E. Rating the risk factors for breast cancer. *Ann. Surg.* **237**, 474–482 (2003).
- 2 Milne, R. L. & Benítez, J. Current strategies in the search for low penetrance genes in cancer. *Histol. Histopathol.* **23**, 507–514 (2008).
- 3 Pance, A. Nitric oxide and hormones in breast cancer: allies or enemies? *Future Oncol.* **2**, 275–288 (2006).
- 4 Gatti, G., Simsek, S., Zurrida, S., Kurne, A., Giannetti, I., Demirer, S. *et al.* Possible role of nitric oxide in the biology of breast carcinoma: review of the available literature. *Breast* **13**, 1–6 (2004).
- 5 Thomsen, L. L. & Miles, D. W. Role of nitric oxide in tumour progression: lessons from human tumours. *Cancer Metastasis Rev.* **17**, 107–118 (1998).
- 6 Casas, J. P., Cavalleri, G. L., Bautista, L. E., Smeeth, L., Humphries, S. E. & Hingorani, A. D. Endothelial nitric oxide synthase gene polymorphisms and cardiovascular disease: a HuGE review. *Am. J. Epidemiol.* **164**, 921–935 (2006).
- 7 Yang, J., Ambrosone, C. B., Hong, C. C., Ahn, J., Rodriguez, C., Thun, M. J. *et al.* Relationships between polymorphisms in NOS3 and MPO genes, cigarette smoking and risk of post-menopausal breast cancer. *Carcinogenesis* **28**, 1247–1253 (2007).
- 8 Li, Y., Ambrosone, C. B., McCullough, M. J., Ahn, J., Stevens, V. L., Thun, M. J. *et al.* Oxidative stress-related genotypes, fruit and vegetable consumption and breast cancer risk. *Carcinogenesis* **30**, 777–784 (2009).
- 9 Lee, K. M., Choi, J. Y., Lee, J. E., Noh, D. Y., Ahn, S. H., Han, W. *et al.* Genetic polymorphisms of NOS3 are associated with the risk of invasive breast cancer with lymph node involvement. *Breast Cancer Res. Treat.* **106**, 433–438 (2007).

- 10 Lu, J., Wei, Q., Bondy, M. L., Yu, T. K., Li, D., Brewster, A. *et al.* Promoter polymorphism (-786t>c) in the endothelial nitric oxide synthase gene is associated with risk of sporadic breast cancer in non-Hispanic white women age younger than 55 years. *Cancer* **107**, 2245–2253 (2006).
- 11 Hefler, L. A., Grimm, C., Lantzsch, T., Lampe, D., Koelbl, H., Lebrecht, A. *et al.* Polymorphisms of the endothelial nitric oxide synthase gene in breast cancer. *Breast Cancer Res. Treat.* **98**, 151–155 (2006).
- 12 Ghilardi, G., Biondi, M. L., Cecchini, F., DeMonti, M., Guagnellini, E. & Scorza, R. Vascular invasion in human breast cancer is correlated to T→786C polymorphism of NOS3 gene. *Nitric Oxide* **9**, 118–122 (2003).
- 13 Hong, C. C., Ambrosone, C. B., Ahn, J., Choi, J. Y., McCullough, M. L., Stevens, V. L. *et al.* Genetic variability in iron-related oxidative stress pathways (Nrf2, NQO1, NOS3, and HO-1), iron intake, and risk of postmenopausal breast cancer. *Cancer Epidemiol. Biomarkers Prev.* **16**, 1784–1794 (2007).
- 14 Royo, J. L., Moreno-Nogueira, J. A., Galan, J. J., Gonzalez-Martin, A., Ruiz, A., Gonzalez-Mancha, R. *et al.* Lack of association between NOS3 Glu298Asp and breast cancer risk: a case-control study. *Breast Cancer Res. Treat.* **100**, 331–333 (2006).
- 15 Schneider, B. P., Radovich, M., Sledge, G. W., Robarge, J. D., Li, L., Storniolo, A. M. *et al.* Association of polymorphisms of angiogenesis genes with breast cancer. *Breast Cancer Res. Treat.* **111**, 157–163 (2008).
- 16 Hindorf, L. A., Sethupathy, P., Junkins, H. A., Ramos, E. M., Mehta, J. P., Collins, F. S. *et al.* Potential etiologic and functional implications of genome-wide association loci for human diseases and traits. *Proc. Natl Acad. Sci. USA* **106**, 9362–9367 (2009).
- 17 Zintzaras, E. & Lau, J. Synthesis of genetic association studies for pertinent gene-disease associations requires appropriate methodological and statistical approaches. *J. Clin. Epidemiol.* **61**, 634–645 (2008).
- 18 Zintzaras, E. & Lau, J. Trends in meta-analysis of genetic association studies. *J. Hum. Genet.* **53**, 1–9 (2008).
- 19 Zintzaras, E. & Kitsios, G. D. Synopsis and synthesis of candidate-gene association studies in chronic lymphocytic leukemia: the CUMAGAS-CLL information system. *Am. J. Epidemiol.* **170**, 671–678 (2009).
- 20 Zintzaras, E. & Zdoukopoulos, N. A field synopsis and meta-analysis of genetic association studies in peripheral arterial disease: the CUMAGAS-PAD database. *Am. J. Epidemiol.* **170**, 1–11 (2009).
- 21 Zintzaras, E., Kitsios, G. D., Ziogas, D. C., Rodopoulou, P. & Karachalios, T. Field synopsis and synthesis of genetic association studies in osteoarthritis: the CUMAGAS-OSTEO information system. *Am. J. Epidemiol.* **171**, 851–858 (2010).
- 22 Kitsios, G. D. & Zintzaras, E. Synopsis and data synthesis of genetic association studies in hypertension for the adrenergic receptor family genes: the CUMAGAS-HYPERT database. *Am. J. Hypertens.* **23**, 305–313 (2010).
- 23 Awata, T., Neda, T., Iizuka, H., Kurihara, S., Ohkubo, T., Takata, N. *et al.* Endothelial nitric oxide synthase gene is associated with diabetic macular edema in type 2 diabetes. *Diabetes Care* **27**, 2184–2190 (2004).
- 24 Kim, J. U., Chang, H. K., Lee, S. S., Kim, J. W., Kim, K. T., Lee, S. W. *et al.* Endothelial nitric oxide synthase gene polymorphisms in Behcet's disease and rheumatic diseases with vasculitis. *Ann. Rheum. Dis.* **62**, 1083–1087 (2003).
- 25 Weir, B. S. *Genetic Data Analysis II: Methods for Discrete Population Genetic Data* 91–132 (Sinauer Associate, MA, 1996).
- 26 Stephens, M., Smith, N. J. & Donnelly, P. A new statistical method for haplotype reconstruction from population data. *Am. J. Hum. Genet.* **68**, 978–989 (2001).
- 27 Zintzaras, E. & Ioannidis, J. P. HEGESMA: genome search meta-analysis and heterogeneity testing. *Bioinformatics* **21**, 3672–3673 (2005).
- 28 Zintzaras, E. & Ioannidis, J. P. Heterogeneity testing in meta-analysis of genome searches. *Genet. Epidemiol.* **28**, 123–137 (2005).
- 29 Cochran, W. G. The combination of estimates from different experiments. *Biometrics* **10**, 101–129 (1954).
- 30 Higgins, J. P. & Thompson, S. G. Quantifying heterogeneity in a meta-analysis. *Stat. Med.* **21**, 1539–1558 (2002).
- 31 DerSimonian, R. & Laird, N. Meta-analysis in clinical trials. *Control Clin. Trials* **7**, 177–188 (1986).
- 32 Harbord, R. M., Egger, M. & Sterne, J. A. A modified test for small-study effects in meta-analyses of controlled trials with binary endpoints. *Stat. Med.* **25**, 3443–3457 (2006).
- 33 Kitsios, G. D. & Zintzaras, E. Synopsis and data synthesis of genetic association studies in hypertension for the adrenergic receptor family genes: the CUMAGAS-HYPERT database. *Am. J. Hypertens.* **23**, 305–313 (2010).
- 34 Kitsios, G. D. & Zintzaras, E. Genome-wide association studies: hypothesis-free or 'engaged'? *Transl. Res.* **154**, 161–164 (2009).
- 35 Zintzaras, E. Methylenetetrahydrofolate reductase gene and susceptibility to breast cancer: a meta-analysis. *Clin. Genet.* **69**, 327–336 (2006).
- 36 Kitsios, G. & Zintzaras, E. Genetic variation associated with ischemic heart failure: a HuGE review and meta-analysis. *Am. J. Epidemiol.* **166**, 619–633 (2007).
- 37 Zintzaras, E., Kitsios, G. & Stefanidis, I. Response to endothelial nitric oxide synthase polymorphisms and susceptibility to hypertension: genotype versus haplotype analysis. *Hypertension* **49**, E2 (2007).
- 38 Zintzaras, E., Kitsios, G. & Stefanidis, I. Endothelial NO synthase gene polymorphisms and hypertension: a meta-analysis. *Hypertension* **48**, 700–710 (2006).
- 39 Moreno Jr, H. Genetic polymorphisms and haplotypes of eNOS in breast cancer. *Breast Cancer Res. Treat.* **109**, 181–182 (2008).
- 40 Kitsios, G. D. & Zintzaras, E. Genomic convergence of genome-wide investigations for complex traits. *Ann. Hum. Genet.* **73**, 514–519 (2009).