

Combined effect of *CYP1B1*, *COMT*, *GSTP1*, and *MnSOD* genotypes and risk of postmenopausal breast cancer

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Objective: Estrogen plays a key role in breast cancer development and functionally relevant genetic variants within the estrogen metabolic pathway are prime candidates for a possible association with breast cancer risk. We investigated the independent and the combined effects of commonly occurring polymorphisms in four genes encoding key proteins of estrogen metabolic pathway on their potential contribution to breast cancer risk.

Methods: We studied 530 breast cancer cases and 270 controls of the same age and ethnicity participating in a case-control study of postmenopausal women. Genotyping was conducted for *CYP1B1* (rs1056836), *COMT* (rs4680), *GSTP1* (rs1695), and *MnSOD* (rs4880) polymorphisms by polymerase chain reaction based restriction fragment length polymorphism and TaqMan allelic discrimination method. Adjusted ORs and 95% CIs were calculated using logistic regression.

Results: None of the 4 genetic variants examined contributed to breast cancer risk individually. When the combined effects of the risk genotypes were investigated, significant associations were observed among women with two high-risk genotypes in *CYP1B1* and *COMT* (OR, 2.0; 95% CI, 1.1 to 3.5) and two high-risk genotypes in *COMT* and *MnSOD* (OR, 2.0; 95% CI, 1.0 to 3.8), compared to those with low-risk genotypes.

Conclusion: Our results suggest that individual susceptibility to breast cancer incidence may be increased by combined effects of the high-risk genotypes in *CYP1B1*, *COMT*, and *MnSOD* estrogen metabolic genes.

Keywords: Breast neoplasms, Estrogen metabolism, Combined polymorphisms, Association study

INTRODUCTION

Since the completion of the Human Genome Project, one of the biggest challenges in genetic research has been the identification of inherited genetic variants that alter susceptibility to multifactorial and polygenic diseases such as cancer [1]. The polygenic model for cancer susceptibility presumes that the combined effects of variants in many genes, each conferring a

small to modest increase in cancer risk, cumulatively account for a substantial fraction of this heritable component of risk [2]. These genetic variants might function through interactions with different behavioral, environmental, or other external risk factors.

The weight of evidence indicates that cumulative, excessive exposure to estrogen across a woman's life span contributes to and may be a causal factor for breast cancer [3]. Several endocrine related risk factors that are associated with an increased risk of breast cancer in postmenopausal women have been found consistently in many studies [4-7]. The mechanisms of carcinogenesis in the breast caused by estrogen include two different but complementary pathways. In addition to the mitogenic properties of estrogen, the

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metabolites of estrogen within the catechol estrogen (CE) pathway (semiquinones, quinones) damage DNA via the formation of superoxide radicals and depurinating DNA adducts [8-10]. Because estrogen metabolites have short half-lives and are difficult to measure *in vivo*, assessing the associations of breast cancer risk with functional genetic variants that alter metabolite concentrations might be the most direct approach possible [11].

Candidate genes include *CYP1A1* and *CYP1B1*, which encode phase I enzymes that lead to increased levels of estrogen metabolites and *COMT*, *UGT1A1*, *SULT1A1*, *GSTPs*, and *SODs* which are involved in phase II metabolism that leads to protective conjugation of estrogen metabolites or detoxify reactive oxygen species (ROS) formed in these reactions (Fig. 1) [12]. *CYP1B1* appears to be the main CYP responsible for the extrahepatic 4-hydroxylation [13]. The 4-hydroxyestrogen (4-CE) to 2-hydroxyestrogen (2-CE) concentration ratio has been reported to be 4:1 in a human breast cancer extract, thus a greater role in carcinogenesis has been suggested for 4-CE [14]. Furthermore, quinones, the further oxidized metabolites of 4-CE, may react with purine bases of DNA to form depurinating adducts that generates highly mutagenic apurinic sites. In contrast, quinones of 2-CE produce less harmful, stable DNA adducts [9]. *COMT* enzyme is involved in methylating (and thereby inactivating) CEs. This is a quantitatively most active conjugation pathway for CEs, although they can also be conjugated by glucuronidation and sulfation [15]. Members of the GST family are thought to play a role in the conjugation of quinones and *GSTP1* is the major GST expressed consistently in both normal and tumor breast tissue [16]. Finally, *SODs* catalyze the conversion of superoxide anion (O_2^-) into hydrogen peroxide (H_2O_2) and molecular oxygen, and thus protect cells from the damage induced by free radicals emerging in estrogen metabolism. As mitochondria consume over 90% of cell oxygen, mitochondrial *MnSOD* is considered of particular importance for cellular defense against oxidative damage [17].

Several genetic polymorphisms within estrogen metabolic

genes have been shown to have functional effects on the catalytic properties of their corresponding enzymes. Altered activity of phase I and II enzymes may influence local hormone levels and cause variation in the extent of DNA damage [18,19]. These person-to-person differences may define subpopulations of women with higher lifetime exposure to hormone dependent growth promotion and/or to cellular damage from particular estrogen metabolites thus suggesting higher breast cancer risk. Therefore, the purpose of our study was to evaluate the independent and the combined effects of *CYP1B1*, *COMT*, *GSTP1*, and *MnSOD* genotypes on the development of breast cancer. Each of the selected genes is being highly expressed in breast tissue and involved in distinct estrogen metabolic sub-pathway.

MATERIALS AND METHODS

1. Study population

Postmenopausal women diagnosed with invasive primary breast cancer between January 1, 2006 and December 31, 2008 at the Institute of Oncology Ljubljana, who were 50-69 years old at the time of diagnosis and of Caucasian ethnic origin were eligible for inclusion in the study. The control group consisted of postmenopausal women randomly selected from the outpatient clinic records of the Department of Obstetrics and Gynecology, University Medical Center Ljubljana that were of the same age and ethnicity, and had no history of breast cancer.

2. Data collection

In addition to general information (socioeconomic status, weight, height), data on reproductive factors (age at menarche, number of pregnancies, age at first delivery, number of deliveries, breastfeeding, age at menopause), family history of breast or ovarian cancer (first-degree relatives), smoking and alcohol consumption were collected by means of a postal questionnaire. Detailed questions were asked regarding drug

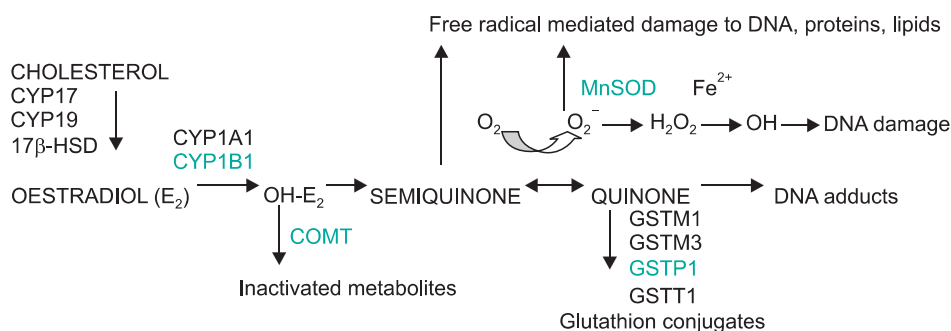


Fig. 1. A schematic presentation of enzymes with known gene polymorphisms involved in estrogen biosynthesis and metabolism. Modified from [12] with permission from Elsevier.

intake, sex hormones in particular (oral contraceptive [OC] use, hormone replacement therapy [HRT] use). A color chart displaying all preparations ever marketed in Slovenia was included in the questionnaire to aid recall. OC and HRT use for less than 1 year was considered no use. Women were assumed to be postmenopausal if they had no periods for at least 12 months before the reference date or had undergone a bilateral oophorectomy.

Estrogen receptor (ER) status was assessed by immunohistochemistry, using monoclonal rabbit ER antibody, Clone SP1 (Neomarkers, Fremont, CA, USA). Tumors were categorized as ER-positive if nuclear staining was observed in at least 10% of nuclei.

Informed written consent was obtained from all women enrolled in the study. The study protocol was approved by the National Medical Ethics Committee of the Republic of Slovenia (No. 61/06/07).

3. Specimen collection and isolation of DNA

In case patients, DNA was extracted from formalin-fixed paraffin-embedded (FFPE) normal breast tissues using HP PCR Template Preparation Kit (Roche Diagnostics GmbH, Mannheim, Germany) following the manufacturer's protocol. The control group women were invited to provide blood sample; genomic DNA was extracted from whole blood using FlexiGene DNA Kit 250 (Qiagen GmbH, Hilden, Germany) following the manufacturer's protocol.

4. Genotyping

Genotyping for the polymorphism c. 1294C>G (p. Leu432Val) in gene *CYP1B1* was done using polymerase chain reaction (PCR)-based restriction fragment length polymorphism (RFLP) method. Each PCR product was digested with restriction endonuclease Eco571 (Fermentas International Inc, Burlington, Canada) and DNA fragments were separated and visualized by electrophoresis on polyacrylamide gels. Genotyping for the polymorphisms c. 472G>A (p. Val108/158Met) in gene *COMT*, c. 313A>G (p. Ile105Val) in gene *GSTP1* and c. 47T>C (p. Val16Ala) in gene *MnSOD* was performed on 96-well plates using the fluorogenic 5'-nuclease assays on Light-Cycler 480 System (Roche Diagnostics GmbH). Each reaction mix contained genomic DNA, LightCycler 480 Probes Master (Roche Diagnostics GmbH) and Custom TaqMan SNP Genotyping Assay (Applied Biosystems, Werterstadt, Germany). All genotyping protocols (PCR reaction conditions, primers and probes) will be provided upon request from the corresponding author. Positive control samples (homozygote for wild allele, heterozygote, homozygote for variant allele) and the negative control sample were included in each batch of sam-

ples. Gels were scored by two different readers; discordant samples were repeated. Apart from *CYP1B1* 1294C>G, all polymorphisms had no samples that failed to be genotyped. For *CYP1B1* 1294C>G, 1% of the samples failed. Samples that failed to be genotyped were scored as missing. Reliability was assessed by random selection of 5% of samples in which all genotypes were confirmed by sequencing using ABI PRISM 7000 sequence detection system (Applied Biosystems). Concordance was 100% for all genotypes.

5. Statistical analyses

We used the independent t-test to compare the values of the means between cases and controls. Differences in categorical characteristics between cases and controls were assessed using chi-square tests. Observed genotype frequencies were tested for deviation from Hardy-Weinberg equilibrium (HWE) with the chi-square goodness-of-fit test. Odds ratios (ORs) for breast cancer risk and the corresponding 95% confidence intervals (CI) were calculated using logistic regression analyses. The homozygous wild-type genotype, as determined by the presence of two putatively low-risk alleles, served as a reference category, with the heterozygous genotype and homozygous variant genotype being collapsed into one category. The adjustment was made for age as a continuous variable. Additional factors (education level, body mass index, age at menarche, age at first full term pregnancy, parity, breastfeeding, OC use, age at menopause, HRT use, first degree family history of breast and/or ovarian cancer and smoking) are within the causal pathway between genetic factors and breast cancer, but could not be affecting genotypes and are thus not true confounders. Therefore, we report the results without adjustment for these factors. A $p < 0.05$ was considered statistically significant. To account for multiple testing, we used the Westfall and Young [20] permutation method, which takes into account the interdependency of the variables tested. Our focus was different combinations of two and three risk genotypes, since their effect was the main hypothesis of the article. We used 10,000 simulation runs under the null hypothesis of no effect, both to correct each p-value by itself and to calculate the probability that the significant results had occurred by chance. The statistical analyses were done using SPSS ver. 18.0 (SPSS Inc., Chicago, IL, USA) and the R statistical software ver. 2.12 (R Development Core Team, Auckland, New Zealand).

RESULTS

Overall response rates were 82.5% (825/1,000) for cases and

73.2% (732/1,000) for controls. In the 3-year period (2006-2008), we enrolled 1,493 postmenopausal women aged 50-69 years; of the 825 cases and 732 controls completing the questionnaire, complete data for all variables considered in the multivariate model were available for 78.4% (784/1,000) cases and 70.9% (709/1,000) controls. Only 38.1% of the control women agreed to provide a blood sample. The number of cases included in genotype analyses was therefore proportionally decreased by random selection to gain the 2:1 ratio in case-control comparisons. The final analysis thus included 800 postmenopausal women aged 50-69 years: 530 were diagnosed with primary breast cancer and 270 were healthy volunteers (control group). The mean age for cases and controls was 60.45 ± 5.84 and 60.10 ± 5.85 years, respectively, and did not differ significantly between the groups ($p=0.432$).

Selected characteristics of the study subjects are presented

in Table 1. Briefly, cases had significantly higher BMI at the time of diagnosis and were more likely to smoke. Control group women had a higher educational level, earlier age at menarche and were older at first delivery. Significantly more women in the control group were using both, OC and HRT. There was no significant difference between the groups in the percentage of nulliparity, number of full term pregnancies, percentage of women that breastfed, duration of breastfeeding, regimen of HRT and percentage of women having any first degree family history of breast or ovarian cancer.

There was no deviation from the HWE except for *GSTP1* ($p=0.01$). The chi-square tests for distribution revealed no significant difference between cases and controls in most of genotype frequencies except for *GSTP1* ($p=0.04$). When adjusted for age, none of the 4 genetic variants studied was, by itself, statistically significantly associated with post-

Table 1. Characteristics of study population

Variable	Cases (n=530)	Controls (n=270)	p-value
Education - highest degree obtained			
Primary school	30.7	6.3	<0.001
Secondary school	59.2	70.3	
University, PhD	10.1	23.4	
BMI (kg/m ²)*			
<25	33.6	52.2	<0.001
≥25 - <30	40.7	35.1	
≥30	25.7	12.7	
Age at menarche (yr)	13.7 ± 1.8	13.5 ± 2.1	0.021
Nulliparity	5.3	3.4	0.089
No. of full-term pregnancies [†]	1.8 ± 0.9	1.7 ± 0.9	0.127
Age at first delivery (yr) [†]	24.3 ± 4.6	24.9 ± 4.8	0.012
Women that breastfed	86.4	90.3	0.344
Duration of breastfeeding (mo) [‡]	8.1 ± 8.7	7.7 ± 7.4	0.269
OC use	42.1	54.7	0.001
Duration of OC use (yr)			
<1	57.9	45.3	0.002
≥1 - <5	14.4	22.7	
≥5 - <10	12.7	17.1	
≥10	15.0	14.9	
HRT use	29.6	65.8	<0.001
Duration of HRT use			
<1	70.4	34.1	<0.001
≥1 - <5	14.3	33.0	
≥5	15.3	33.0	
Regimen of HRT [§]			
Combined, estrogen plus progestin	71.2	67.8	0.487
Estrogen only	28.8	32.2	
First degree family history of breast and/or ovarian cancer	18.1	15.5	0.138
Smoking	20.2	15.9	0.041

Values are presented as percent (%) or mean \pm SD.

BMI, body mass index; OC, oral contraceptive; HRT, hormone replacement therapy.

*Calculated as weight in kilograms divided by height in meters squared at the age of the diagnosis. [†] Among women who had a full term pregnancy. [‡] Among those who ever breastfed. [§] Among those who ever used HRT.

menopausal breast cancer risk (Table 2).

Additionally, we examined possible combined effects of *CYP1B1*, *COMT*, *GSTP1*, and *MnSOD* genotypes on breast cancer risk by calculating adjusted ORs for all of the combinations of two and three of the risk genotypes (Tables 3 and 4). The reference group consisted of individuals with the putatively most advantageous combinations of the genotypes, low-risk genotypes, i.e., the presence of homozygous CC genotype for *CYP1B1*, GG genotype for *COMT*, AA genotype for *GSTP1* and TT genotype for *MnSOD*.

When combinations of two putative at-risk genotypes were examined (Table 3), the concurrent presence of *CYP1B1* (CG/GG) and *COMT* (GA/AA) high-risk genotypes and *COMT* (GA/AA) and *MnSOD* (TC/CC) high-risk genotypes posed a more than 2-fold risk of breast cancer (OR, 2.0; 95% CI, 1.1 to 3.5) and (OR, 2.0; 95% CI, 1.0 to 3.8), respectively. In contrast, no statistically significant effects were seen for women simultaneously carrying the *GSTP1* high-risk genotype in combination with any of the other three the risk genotypes.

When three of the putative at-risk genotypes were combined (Table 4), women with high-risk genotypes *CYP1B1* (CG/GG), *COMT* (GA/AA), *GSTP1* (AG/GG) and *CYP1B1* (CG/GG),

Table 2. Genetic variation and risk of postmenopausal breast cancer

Genotype	Cases	Controls	OR (95% CI)*	p-value
<i>CYP1B1</i>				
CC	157 (30.0)	97 (36.1)	1.0	
CG	284 (54.3)	129 (47.9)	1.4 (1.0-1.9)	0.068
GG	82 (15.7)	43 (16.0)	1.2 (0.8-1.8)	0.467
CG/GG	366 (70.0)	172 (63.9)	1.3 (1.0-1.8)	0.087
<i>COMT</i>				
GG	123 (23.2)	67 (24.8)	1.0	
GA	263 (49.6)	136 (50.4)	1.0 (0.7-1.5)	0.804
AA	144 (27.2)	67 (24.8)	1.2 (0.8-1.8)	0.481
GA/AA	407 (76.8)	203 (75.2)	1.1 (0.8-1.5)	0.642
<i>GSTP1</i>				
AA	233 (44.0)	130 (48.1)	1.0	
AG	243 (45.8)	101 (37.4)	1.3 (1.0-1.8)	0.066
GG	54 (10.2)	39 (14.4)	0.8 (0.5-1.2)	0.292
AG/GG	297 (56.0)	140 (51.8)	1.2 (0.9-1.6)	0.250
<i>MnSOD</i>				
TT	118 (22.3)	65 (24.1)	1.0	
TC	269 (50.8)	134 (49.6)	1.1 (0.8-1.6)	0.591
CC	143 (27.0)	71 (26.3)	1.1 (0.7-1.7)	0.622
TC/CC	412 (77.7)	205 (75.9)	1.1 (0.8-1.6)	0.564

Values are presented as number (%) or OR (95% CI).

OR, odds ratio; CI, confidence interval.

*Adjusted for age as a continuous variable.

GG), *COMT* (GA/AA), *MnSOD* (TC/CC) were at a 2.7-fold (95% CI, 1.1 to 6.8) and 12.2-fold (95% CI, 1.4 to 102.3) breast cancer risk, respectively, compared to those with low-risk genotypes. Similarly, clear combined effects were observed also in the other two combinations for the three at-risk genotypes, but the outcomes were not significant.

After the combinations of two and three risk genotypes were examined (Table 3 and 4), the results were further evaluated to account for multiple testing. While none of the single p-values remained significant after the correction, the overall probability that the 8 observed p-values were below 0.05 due

Table 3. Combined effects of two genotypes (*CYP1B1*, *COMT*, *GSTP1*, and *MnSOD*) and risk of breast cancer

Genotype		Cases/controls	OR (95% CI) *	p-value
<i>CYP1B1</i>	<i>COMT</i>			
CC	GG	27/26	1.0	
CC	GA/AA	130/71	1.8 (1.0-3.2)	0.071
CG/GG	GG	94/40	2.3 (1.2-4.4)	0.014
CG/GG	GA/AA	272/132	2.0 (1.1-3.5)	0.021
<i>CYP1B1</i>	<i>GSTP1</i>			
CC	AA	69/43	1.0	
CC	AG/GG	88/54	1.0 (0.6-1.7)	0.942
CG/GG	AA	161/86	1.2 (0.7-1.8)	0.518
CG/GG	AG/GG	205/86	1.5 (0.9-2.4)	0.088
<i>CYP1B1</i>	<i>MnSOD</i>			
CC	TT	33/22	1.0	
CC	TC/CC	124/75	1.1 (0.6-2.1)	0.733
CG/GG	TT	84/43	1.3 (0.7-2.5)	0.413
CG/GG	TC/CC	282/129	1.5 (0.8-2.6)	0.197
<i>COMT</i>	<i>GSTP1</i>			
GG	AA	51/27	1.0	
GG	AG/GG	72/40	1.0 (0.5-1.8)	0.896
GA/AA	AA	182/103	0.9 (0.6-1.6)	0.793
GA/AA	AG/GG	225/100	1.2 (0.7-2.0)	0.515
<i>COMT</i>	<i>MnSOD</i>			
GG	TT	19/19	1.0	
GG	TC/CC	104/48	2.2 (1.1-4.5)	0.037
GA/AA	TT	99/46	2.1 (1.0-4.4)	0.041
GA/AA	TC/CC	308/157	2.0 (1.0-3.8)	0.050
<i>GSTP1</i>	<i>MnSOD</i>			
AA	TT	44/28	1.0	
AA	TC/CC	189/102	1.2 (0.7-2.0)	0.541
AG/GG	TT	74/37	1.3 (0.7-2.4)	0.435
AG/GG	TC/CC	223/103	1.4 (0.8-2.4)	0.227

OR, odds ratio; CI, confidence interval.

*Adjusted for age as a continuous variable.

Table 4. Combined effects of three genotypes (*CYP1B1*, *COMT*, *GSTP1*, and *MnSOD*) and risk of breast cancer

Genotype			Cases/ controls	OR (95% CI)*	p-value
<i>CYP1B1</i>	<i>COMT</i>	<i>GSTP1</i>			
CC	GG	AA	10/11	1.0	
CC	GG	AG/GG			
CC	GA/AA	AA	116/62	2.1 (0.8-5.2)	0.114
CG/GG	GG	AA			
CC	GA/AA	AG/GG			
CG/GG	GG	AG/GG	246/135	2.0 (0.8-4.9)	0.118
CG/GG	GA/AA	AA			
CG/GG	GA/AA	AG/GG	151/61	2.7 (1.1-6.8)	0.029
<i>CYP1B1</i>	<i>GSTP1</i>	<i>MnSOD</i>			
CC	AA	TT	14/10	1.0	
CC	AA	TC/CC			
CC	AG/GG	TT	103/63	1.2 (0.5-2.8)	0.698
CG/GG	AA	TT			
CC	AG/GG	TC/CC			
CG/GG	AA	TC/CC	256/135	1.4 (0.6-3.2)	0.458
CG/GG	AG/GG	TT			
CG/GG	AG/GG	TC/CC	150/61	1.8 (0.8-4.2)	0.190
<i>CYP1B1</i>	<i>COMT</i>	<i>MnSOD</i>			
CC	GG	TT	1/6	1.0	
CC	GG	TC/CC			
CC	GA/AA	TT	76/49	9.4 (1.1-80.4)	0.041
CG/GG	GG	TT			
CC	GA/AA	TC/CC			
CG/GG	GG	TC/CC	240/112	13.0 (1.5-109.0)	0.018
CG/GG	GA/AA	TT			
CG/GG	GA/AA	TC/CC	206/102	12.2 (1.4-102.3)	0.022
<i>COMT</i>	<i>GSTP1</i>	<i>MnSOD</i>			
GG	AA	TT	4/5	1.0	
GG	AA	TC/CC			
GG	AG/GG	TT	102/59	2.2 (0.6-8.5)	0.256
GA/AA	AA	TT			
GG	AG/GG	TC/CC			
GA/AA	AA	TC/CC	258/129	2.5 (0.7-9.6)	0.173
GA/AA	AG/GG	TT			
GA/AA	AG/GG	TC/CC	166/77	2.7 (0.7-10.5)	0.143

OR, odds ratio; CI, confidence interval.

*Adjusted for age as a continuous variable.

to chance only was 0.01 (1%).

We were restricted from evaluating the potential combined effects of all the four genotypes because none of the cases and only one of the controls carried all of the four putatively low-risk genotypes simultaneously.

When stratified for ER status, 84.0% (n=445) of tumors were ER-positive and 16.0% (n=85) of tumors were ER-negative. Genetic variants alone or combined effects of two genotypes did not affect differently breast cancer risk according to the ER

status (data not shown).

DISCUSSION

In this case-control study of postmenopausal Caucasian women, we investigated associations of functionally relevant genetic variants in four genes (*CYP1B1*, *COMT*, *GSTP1*, *MnSOD*) encoding key proteins of the estrogen metabolic pathway with breast cancer risk. Additionally, we evaluated the potential combined effects of these genotypes on the development of breast cancer.

We focused on polymorphic genes coding for enzymes that are relevant for the given exposure, are highly expressed in breast tissue, and act sequentially in the same metabolic pathway (Fig. 1 - genes with polymorphisms included in our analysis are underlined). However, none of the 4 genetic variants in these genes contributed to breast cancer risk individually.

Several association studies in these candidate genes have been widely used to search for susceptibility alleles, but few definite associations have been established [21]. Such inconsistencies in results probably reflect the true variation in the underlying association between populations studied and the low penetrance of mutations in these multigenic pathways [22]. Although the effect of each individual single nucleotide polymorphism (SNP) was small and not significant, the genetic effect of combinations of functionally relevant SNPs may additively or synergistically contribute to increased breast cancer risk. Therefore, our a priori hypothesis specified that individual susceptibility to breast cancer may be increased by the combined effects of the risk genotypes in estrogen metabolic genes. This was confirmed by the results of our study since the concurrent presence of *CYP1B1* and *COMT* high-risk genotypes and *COMT* and *MnSOD* high-risk genotypes posed a 2-fold risk of breast cancer. Women with the three high-risk genotypes *CYP1B1*, *COMT*, *GSTP1* and *CYP1B1*, *COMT*, *MnSOD* were at a 2.7-fold and 12.2-fold breast cancer risk, respectively. This is in agreement with the only gene-gene interaction study that investigated the same gene-gene combinations [23]. They observed only marginally increased breast cancer risk with the combination of high-risk genotypes in *CYP1B1*, *COMT*, and *MnSOD* genes in women with the BMI greater than 24 kg/m² (OR, 1.4; 95% CI, 1.0 to 1.9) [23].

Several SNPs within *CYP1B1* have been shown to have functional effects on the catalytic properties of the CYP1B1 enzyme, with the 4-hydroxylase activity of the Val⁴³² variant allele displaying a 3-fold higher activity compared to Leu⁴³²

[24]. On the other hand, the *COMT* Met¹⁵⁸ variant allele has been hypothesized to produce an enzyme with a 3- to 4-fold reduced functionality compared to the wild-type Val¹⁵⁸ allele [25]. For *GSTP1* gene, a point mutation results in a single amino acid change from isoleucine (Ile) to valine (Val) at codon 105. This residue lies in close proximity to the hydrophobic binding site for electrophilic substrates, and the Val¹⁰⁵ variant allele has been demonstrated to exhibit altered specific activity and affinity for electrophilic substrates [26]. A polymorphism at codon 16 of mitochondrial targeting sequence of the *MnSOD* gene leads to a substitution from Val to Ala. This substitution alters the secondary structure of the protein, which affects the localization and transport of the enzyme into the mitochondria, where it exerts its antioxidant action [27]. Therefore, it was first expected that the Val form was likely to be associated with increased risk of cancer. However, subsequent studies revealed a controversial picture [28]. If MnSOD is inhibited to enter the mitochondrial matrix, as is the case with Val form, O₂⁻ cannot be dismutated to H₂O₂, which causes cellular damage and consequently induces apoptosis. Conversely, the Ala form of MnSOD efficiently dismutates O₂⁻ to H₂O₂, but the latter may react to yield other ROS, mostly hydroxyl radicals (·OH), which are highly detrimental to DNA. In view of these findings, it is very likely that a combination of increased production of 4-CE by Val⁴³² form of *CYP1B1* and concurrent decreased 4-CE inactivation by Met¹⁵⁸ form of *COMT* may result in elevated risk of breast cancer. Similarly, decreased 4-CE inactivation by Met¹⁵⁸ form of *COMT* leads to the production of O₂⁻, which are generated through the redox cycling between further oxidized metabolites, quinones and semiquinones. Therefore, while simultaneously carrying Ala¹⁶ form of MnSOD, excess production of H₂O₂ may result in the accumulation of DNA adducts and thus predispose to cancer. On the other hand, rather unexpectedly, the *GSTP1* Val¹⁰⁵ variant allele did not prove to influence breast cancer risk when studied separately or when potential combined effects between the two risk genotypes were examined. It has been suggested that, depending on the chemical composition of the substrate, individuals with a given *GSTP1* genotype may be at a differential risk for carcinogenesis [29]. Since *GSTP1* is polymorphic with 2 single-nucleotide substitutions in the coding region (p. Ile105Val and p. Ala114Val), and both amino acid residues lie in close proximity to the substrate-binding site, their concurrent determination will provide a clearer picture of the catalytic properties of the *GSTP1* isozyme [29]. Furthermore, as the *GST* family of enzymes are known to have overlapping substrate specificities, another explanation for the discrepancy might be that the deficiency of *GSTP1* isozyme was compensated by other isoforms (*GSTM1*, *GSTM3*,

GSTT1) [30]. A simultaneous determination of all relevant *GST* genotypes for a given exposure may therefore be a prerequisite for a reliable interpretation of the results.

Additionally, we observed a tendency of increased risk together with increased number of the putative high-risk genotypes. The ORs were more elevated in the women harboring three high-risk genotypes compared to two high-risk genotypes.

The results of the current study suggest that genetic variants without or with main effects, too small to be detected, may interact with others and confer an increased breast cancer risk. Since none of the single p-value remained significant after the correction for multiple testing, we can not point on a single combination that we expect to have an effect in the population. However, as the overall probability that the 8 observed p-values were below 0.05 due to chance was only 1%, we can claim at least some of the studied genotype combinations are associated with the increased risk of breast cancer. Gene-gene interactions among estrogen metabolic genes have been also investigated in other studies, with some studies [30-34], but not all [35-37], reporting associations between high-risk genotypes and breast cancer risk. However, with the exception of two studies reporting marginally significant increased risk of breast cancer associated with *GSTP1*, *GSTM1*, and *GSTT1* risk genotypes and our study confirming the results of Kocabas et al. [23] indicating increased risk associated with *CYP1B1*, *COMT*, and *MnSOD* risk genotypes, no specific gene-gene combination has been observed in more than one study. Inconsistencies may be at least partly explained by the differences in the populations studied and in their exposure to the agents relevant to the development of breast cancer. Furthermore, for comparable statistical power larger study sizes are needed if investigating combined effects between different genotypes. The existing reports, including ours, were limited only to the interactions of SNPs within a single cancer pathway. Yet, there is also growing evidence regarding cross talk between the risk genotypes of different cancer pathways, including DNA repair, cell cycle, immune system and others [38]. The investigation of such gene-gene interactions presents new statistical challenges as the number of potential interactions between the SNPs can be substantial.

Since the current study was retrospective in design, the data was obtained on the basis of the postal questionnaire, and a low percentage of control group women agreed to provide a blood sample, we cannot rule out a possibility of introducing biased study results. The difference in how genetic data was collected from cases and controls (i.e., FFPE normal breast tissue samples in cases and blood samples in controls) might

have raised concerns about introducing bias toward a specific allele in any of the assays. However, studies have shown that, for most of the genotypes, the use of FFPE tissue samples is a valid alternative to peripheral blood, and vice versa [39,40]. Kweekel et al. [40] compared 11 genotypes in DNA isolated from blood and FFPE colorectal cancer tissue and found none of the individual genotypes that showed a discordance between FFPE cancer tissue and blood significantly different from 0.0%, except for *GSTP1* (rs1695) with 95% CI 0.1 to 5.9. Another study analyzed 5 genotypes selected from regions commonly known to have loss of heterozygosity in breast cancer from normal breast tissues adjacent to tumors and compared them with blood genotyping [41]. They found 100% concordance and concluded that the use of adjacent normal tissues provides accurate genotyping results with high specificity [41]. Therefore, by using FFPE normal (not cancerous) breast tissue samples, which are unlikely to harbour somatic alterations associated with carcinogenesis, this might not have affected our ability to identify germ-line genetic variants. We have no logical explanation regarding the lack of HWE for the *GSTP1* (rs1695) in our study. The women in the control group were randomly enrolled from the outpatient clinic records of the Department of Obstetrics and Gynecology during their routine gynecologic exams. Genotyping was done in duplicates, using validated Custom TaqMan SNP Genotyping Assay and endpoint fluorescence scatter plots positioned the homozygotes for one allele, the heterozygotes and the homozygotes for the other allele clearly apart from each other. Furthermore, no other genetic variant appear next to this polymorphism arguing strongly against mismatch probe pairing. Additionally, *GSTP1* genotyping reliability was assessed by sequencing 5% (n=40) of samples at random, and concordance was 100%. Therefore, we believe the lack of HWE for the *GSTP1* is purely due to chance and, since it only affected the size of the groups that were compared, it might not have introduced any bias into our results. Given extensive data stratification in studying the combined effects of the three risk genotypes on breast cancer risk, the likelihood of a type I error (a false-positive result) has to be considered. Our study population was of medium size and it is possible that some interactions were not significant due to insufficient power. The strengths of the study include homogenous study population and investigation of functionally relevant genetic variants in genes, each being highly expressed in breast tissue and involved in a distinct estrogen metabolic sub-pathway. Although the determination of a priori evidence of being functionally important in the disease process is quite challenging, selecting SNPs based on their functions clearly reduces the false positive report probability [42]. The

results are consistent with biologically plausible interactions and merit further investigation of the combined effects of polymorphisms in estrogen metabolic genes on breast cancer risk.

Our focus in this study was to find gene-gene interactions, which additively or synergistically contribute to breast cancer risk. Although our pool of SNPs was small, we have managed to show that the individual susceptibility to breast cancer incidence may be increased by combined effects of *CYP1B1*, *COMT*, *GSTP1*, and *MnSOD* high-risk genotypes, especially in certain combinations. With the exception of one study reporting marginally increased breast cancer risk, we are the first to report a large increase in breast cancer risk associated with these high-risk genotypes. Replication of our results is thus needed before any firm conclusion is drawn. These findings may provide new important data on the combined effects of putative gene-gene interactions in the etiology of human breast cancer. We believe that epistasis is likely to be a ubiquitous component of the genetic architecture of common diseases, such as breast cancer. This information could be used in clinical work to identify individuals at increased risk of breast cancer, and to develop preventive strategies.

CONFLICT OF INTEREST

No potential conflict of interest relevant to this article was reported.

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