

Research Article

PON I and GLO I Gene Polymorphisms and Their Association with Breast Cancer: A Case-Control Study in a Population from Southern Italy

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Abstract

Background: Many factors may play a role in the susceptibility to the breast cancer. Oxidative stress may be one of these. Polymorphisms of genes such as paraoxonase I (PON I) and glyoxalase I (GLO I) may influence individual susceptibility to breast cancer.

In the present study, we have conducted a case-control study in order to examine the possible relation between GLO I A111E and PON I Q192R/L55M polymorphisms with the risk of breast cancer.

Methods: The three polymorphisms were characterized in 144 breast cancer postmenopausal patients and in 152 healthy women by PCR/RFLP methods using DNA from lymphocytes.

Results: Among the three polymorphisms, only PON I L55M polymorphism was associated with the patient's age and, more precisely, the heterozygous genotype that is more represented in women aged between 51-69 years. In addition, we found that individuals with the PON192 Q/R - R/R genotypes and PON55 L/M - M/M genotypes had a significantly higher risk of breast cancer compared with the other genotypes. The genotypes PON55 L/M and PON192 Q/R showed significant association with lymph nodes positivity (p < 0.001) and with a high nuclear grading (p < 0.001), respectively. Conversely the genotypes GLO I AE/EE were associated with a low nuclear grading.

Conclusions: We believe that the combination of the three polymorphisms may be a more predictive factor for the risk of this neoplasia in each single examined case.

Keywords: Breast cancer; Paraoxonase I; Glyoxalase I; Oxygen free radicals; Methylglyoxal; High-density lipoprotein

Abbreviations: BC: Breast cancer; PON I: Paraoxonase; GLO I: Glyoxalase; OFR: Oxygen free radicals; MG: Methylglyoxal; HDL: High-density lipoprotein; LDL: Low-density lipoprotein; NG: Nuclear grading; Q: Glutamine; R: Arginine; L: Leucine; M: Methionine; PCR/RFLP: Polymerase chain reaction/restriction-fragment length polymorphism; AGE: Advanced glycation end-products; ROS: Reactive oxygen species; CNS: Central nervous system; HWE: Hardy-weinberg equilibrium; OR: Odds ratio; CI: Confidence interval; SD: Standard deviation; χ^2 : Chi-square; DCIS: Ductal carcinoma *in situ* ; IDC: Invasive ductal carcinoma

Introduction

Breast cancer (BC) is a heterogeneous and complex disease that represents the most frequent malignancy in women. Many factors may play a role in the susceptibility to the breast cancer and Oxygen Free Radicals (OFR) that are continuously generated in cells exposed to an aerobic environment may be one of these [1]. Free radicals are formed in both physiological and pathological conditions in mammalian tissues [2]. In healthy conditions at cellular level, a subtle balance exists between the free radical generation and the antioxidant defense. Excess generation of these OFR and oxidants generate a phenomenon called oxidative stress which cause oxidative damage to biomolecules resulting in lipid peroxidation [3], mutagenesis and carcinogenesis [4], including breast cancer [5-8].

Therefore, it is plausible that polymorphisms of genes encoding enzymes involved in endogenous free-radical scavenging systems and anti-glycation defenses may influence individual susceptibility to BC.

There are various known antioxidant systems against oxidative stress, including Paraoxonase I (PON I) and Glyoxalase I (GLO I).

Human PON I is synthesized in liver and secreted into blood, where it is associated exclusively with High Density Lipoproteins (HDLs) [9]. In fact PON I is a High-Density Lipoprotein (HDL)-associated enzyme, preservating HDL integrity and function and has an antioxidant function for low-density lipoprotein (LDL) which is more susceptible to oxidation [10,11]. In coding region of PON I gene has been reported genetic polymorphisms which change the paraoxon hydrolysis activity among individuals [12]: Q192R which lead to glutamine \rightarrow arginine substitution at aminoacid 192 and L55M to leucine \rightarrow methionine substitution at aminoacid 55.

With respect to GLO I is a glutathione-binding protein involved in the detoxification of Methylglyoxal (MG), a by product of glycolysis. GLO I catalyzes condensation of methylglyoxal and reduced

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glutathione to form S-lactoyl-glutathione [13]. Since there is evidence that carbonyl stress and DNA/ protein glycation may play a role in breast carcinogenesis [14,15], it is plausible that polymorphism in GLO I may influence an individual's susceptibility to breast cancer. A single nucleotide polymorphism was identified in the exon 4 of GLO I gene that causes an amino acid substitution of Ala for Glu [16]. The presence of an additional acidic charge from the Glu residue was associated with a decreased enzyme activity which may cause accumulation of AGEs in cells or tissues. AGE-induced modification of biologic macromolecules such as protein, nucleotides and basic phospholipids may contribute to cell and tissue damage in vivo [17], thus increasing the risk of developing cancer. Because GLO I and PON I are involved in antioxidant activity, a reduction in GLO I or PON I enzyme activity could result in the accumulation of reactive oxygen species (ROS) that may cause oxidative stress leading to cellular and molecular damage thereby resulting in cell proliferation and malignant conversions. ROS have been implicated in many diseases, for example in multiple sclerosis [18,19], in different CNS neurodegenerative pathologies, including Alzheimer's disease [20] and Parkinson's disease [21].

So far, very few information is available about the association of GLO I A111E and Q192R/L55M polymorphisms with BC risk and only in selected populations [22-26]. However, these studies have yielded apparently conflicting results [27].

Therefore, the present study aimed to investigate whether GLOIA111E and Q192R/L55M polymorphisms are associated with breast cancer risk in a population from Southern-Italy and if there is an association between the polymorphisms and clinico-pathological parameters such as age at diagnosis, lymph nodes status and nuclear grading.

Materials and Methods

Patients

Peripheral blood samples were collected from 144 women living in Southern Italy: 110 cases were histologically pure Ductal Carcinoma In Situ (DCIS) showing no invasive components on routine pathological examination and 34 cases were Invasive Ductal Carcinoma (IDC). Before recruitment, each patient was asked to sign an informed consent form. Clinical information was obtained by review of medical records.

The patients informations included: date of birth, age at diagnosis, number of lymph nodes involved and stage at diagnosis. 115 patients (80%) underwent total mastectomy and 29 (20%), breast-conserving surgery. No adjuvant chemotherapy was administered. We have no information on treatment with radiation and or endocrine therapy. The decision to receive radiation and or endocrine therapy was made by the treating medical oncologist.

The size of the breast cancers at diagnosis (according to the criteria of the World Health Organisation) was: pT1-pT2 in 128 cases (89%) and pT3-pT4 in 16 cases (11%). These cases were classified according to Nuclear Grading (NG). We have no data on the clinical outcomes of these patients.

A total of 152 healthy women, living in the same regions of Southern Italy, who had no history or family history of any malignancy, were recruited as normal controls.

Demographic and disease data on BC patients and controls are summarized in Table 1. The healthy women were matched for age at recruitment with the breast cancer patients. These were distributed according to their age at diagnosis: ≤ 50 years (43–50 years); 51 – 69 years, and ≥ 70 years (70 – 76 years). The mean ages of breast cancer patients and normal controls were 59.2 \pm 9.7 and 60.0 \pm 9.26, respectively. No significant differences for age in two groups (p > 0.05). All DNA samples were collected after written informed consent from all individuals. Furthermore, Estrogen Receptor (ER), Progesterone Receptor (PgR) and Human Epidermal Growth Factor Receptor 2 (HER2) receptor status were assessed.

Molecular analysis

Primers and polymerase chain reaction (PCR): Heparinized peripheral blood samples were collected from breast cancer patients and healthy controls. Genomic DNA was extracted from lymphocytes by salting out method [28]. Nucleotide sequence of primers designed by us of DNA sequences is shown in Table 2. PCR amplifications were

(A) BC ^a patients (n=144)							
	Age at diagnosis						
	Range	Median	Mean ± SD⁵				
	43-76	59.5	59.2 ± 9.7				
Age group	Tumour size Nuc		Nuclear	grading	Lymph nodes		Total cases
	pT1–pT2	pT3–pT4	Low ^c	High⁴	Positive	Negative	144 (100%)
≤50	49	0	48	1	4	45	49 (34.0)
51-69	65	14	66	13	29	50	79 (54.9)
≥70	14	2	15	1	1	15	16 (11.1)
(B) Healthy controls (n=152)							
		Age					
	Range	Median	Mean ± SD				
Age group ≤50 51-69 ≥70	44-77 Controls (n) 50 77 25	60.0	60 ± 9.26				

^a BC: Breast cancer

^bSD: Standard deviation; statistical analysis was tested by the Student's *t*-test

^c Low grade is defined as better differentiated type of tumor cells, including well differentiated type and moderately differentiated type

^d High grade is defined as worse differentiated type of tumor cells, including poorly differentiated type and undifferentiated type

Table 1: Demographic and disease-related data on BC patients and control.

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Geneª	Oligonucleotides	Sequence	(bp) ^d
PONI 192Q/R	PON192F⁵ PON192R⁰	5'-TATTGTTGCTGTGGGACCTGAG-3' 5'-CACGCTAAACCCAAATACATCTC-3'	99
PONI 55L/M	PON55F PON55R	5'-GAAGAGTGATGTATAGCCCCAG-3' 5'TTTAATCCAGAGCTAATGAAAGCC3'	170
GLOI	GLO1F GLO1R	5'-TCAGAGTGTGTGATTTCGTG-3' 5'-CATGGTGAGATGGTAAGTGT-3'	713

PONI 192Q/R: Paraoxonase I Q192R; PONI 55L/M, Paraoxonase I L55M; GLOI, Glyoxalase I F: forward

^cR: reverse ^dBase pair(s)

Table 2: Oligonucleotide primer sequences used for genotyping.

	Genotype ^a	Controls (n)	(n=152)%	BC (n)	(n=144)%	OR⁵	95%Cl°	pď
	QQ	143	94.1	110	76.4	1.0		1
	QR	7	4.6	30	20.8	5.6	2.23-14.51	0.000
PONI Q192R	RR	2	1.3	4	2.8	2.6	0.40-20.83	0.259
Q	Q	0.096		0.87				
	R	0.04		0.13				
	LL	130	85.5	70	48.6	1.0		1
PONI L55M	LM	7	4.6	58	40.3	15.4	6.34-39.09	0.000
	MM	15	9.9	16	11.1	2.0	0.87-4.53	0.076
	L	0.88		0.69				
	М	0.12		0.31				
	AA	55	36.2	0	0	undefinited		1
	AE	20	13.1	56	38.9	undefinited	28.911-inf	0.000
GLOI	EE	77	50.6	88	61.1	undefinited	12.535-inf	<0.001
	А	0.43		0.2				
	E	0.57		0.8				

^aGenotypes with Q/R, L/M and A/E alleles, respectively

^bOR, odds ratios are calculated relative to subjects with the Q/Q, L/L and A/A genotypes, respectively

°CI, confidence interval

 d Two-sided $\chi 2$ test; p< 0.05 indicates statistical significance

Table 3: Allele and genotype frequencies of PONI Q192R/L55M and GLOI A111E polymorphisms and their association with BC risk.

performed in a Gene Amp PCR System 2700 (Perkin Elmer, Foster City, CA) PCR with a final volume of 50 μ l, containing 50 ng of DNA, 20 pmoles of each primer, 100 mM each of dNTP, 1.5 mM of MgCl₂, 10 mM Tris-144HCl pH 8.3, 50 mM of KCl and 1U of Taq DNA polymerase. DNA amplification was performed conventionally: initial denaturation step at 95°C for 5 minutes and then 35 cycles consisting in an denaturation at 95°C for 40 seconds, annealing for 30 seconds and extension at 72°C for 30 seconds. The final extension step at 72°C was extended to 10 minutes. The annealing temperature was optimized for each primer set.

The PCR products were resolved on 2% agarose gel and visualized by ethidium bromide staining to determine the presence of the 99 bp, 170 bp and 713 bp amplicons for PON I 192 Q/R, PON I 55 L/M and GLO I respectively.

Genotyping: The three polymorphisms were characterized in breast cancer patients and in normal controls.

Genotyping of these polymorphisms was performed using polymerase chain reaction/restriction- fragment length polymorphism (PCR/RFLP) method. The C to A substitution in GLO I exon 4, which changes Ala111Glu in the encoded protein, leads to the loss of a recognition site for the SfaNI (New England Biolabs GmbH, Germany) restriction enzyme. Single-base substitutions, which lead to the change of a glutamine residue into arginine at amino acid position 192, and a change of leucine to methionine affecting amino acid 55 of the human paraoxonase protein PON I, create recognition sites for Alw1 (PON1/192) and NlaIII (PON1/55), respectively. PCR products were digested for 3 hours at 37°C, using SfaNI, Alw1 or NlaIII, respectively (New England Biolabs GmbH). The digested product were resolved by 2% agarose gel electrophoresis and the fragments were visualized under UV light after staining with ethidium bromide to identify the single base pair change. The restriction digest reveals 99 bp fragment in the presence of Q 192 allele, and 65 bp and 34 bp fragments in the presence of R allele; 170 bp fragment in the presence of L 55 allele, and 126 bp and 44 bp fragments in the presence of M allele; 453 bp and 260 bp fragments in the presence of E allele.

Statistical analysis: Analysis of data was performed using computer software SPSS for Windows (Version 6.0.1). Comparison between mean ages at diagnosis of breast cancer patients versus healthy control age was calculated by the Student's t -test. For each group (controls and patients), allele frequencies were calculated by direct gene counting. Deviations from the Hardy-Weinberg equilibrium (HWE) were tested by the χ^2 -test on a 2 x 3 contingency table, with two degrees of freedom. Associations between the gene genotypes and the risk of breast cancer were assessed by Odds Ratios (ORs) and 95% Confidence Intervals

(CI). ORs express the relative risk (OR) of BC with a specific genotype are calculated by dividing the odds of a BC patient having a specific phenotype by the odds of a control subject having the same genotype. Estimates of statistical significance were calculated by standard χ^2 analysis for one degree of freedom, or by Fisher's exact test, where cell numbers were </5. Descriptive analysis included Student's t -test of means and the respective Standard Deviation (SD) for cases and controls. A two-sided probability value of </0.05 was considered to indicate statistical significance.

Results

Allele and genotype frequencies of PON I Q192R/L55M and GLO I A111E polymorphisms and the risk of breast cancer in BC patients and normal controls are given in Table 3.

With regard to PON I Q192R polymorphism, an increase in the frequency of Q/R genotype was observed in the BC group (20.8%) versus 4.6% in the control group (p = 0.000). Similarly, an increase was observed in the proportion of individuals with the genotype R/R in the BC group (2.8%) with respect to the normal control group (1.3%). However, this association did not reach statistical significance (p = 0.259).

Patients with the Q/R and R/R genotype had a significant increase for BC risk (OR = 5.6, 95% CI = 2.23-14.51 and OR = 2.6, 95% CI = 0.40-20.83, respectively) compared with the Q/Q genotype.

With regard to PON I L55M, the frequency of heterozygote L/M genotype was 40.3% in BC patients and 4.6% in the control group (p < 0.001), while no significant differences was observed for MM genotype among the group of patients and controls (p = 0.076).

Patients with the L/M and M/M genotype had a significant increase for BC risk (OR = 15.4, 95% CI = 6.34-39.09 and OR = 2.0, 95% CI = 0.87 - 4.53, respectively) compared with the L/L genotype.

We observed that PON I 192/55 heterozygote genotypes had a higher risk for BC than mutant PON I 192/55 homozygotes. It is likely that paraoxonase enzyme activity can be affected by other factors apart

from the genetic component [29,30].Therefore, the presence of the PON I 55M -192R alleles significantly increases the risk in BC.

However, the changing L to M amino acid (L55M) and Q to R aminoacid (Q192R) are highly dependent on which substrate is used to analyse enzyme activity.

In addition, the association between the PON 1/55 and PON 1/192 polymorphisms was estimated, and PON I haplotypes were reconstructed, but the results of this analysis are not significant (unpublished data).

With respect to GLO I, the wild type GLO I A/A genotype was found to be absent in BC patients while in control group its frequency was 36.2%.

We also found that the frequency of BC patients carrying the A/E genotype (38.9%) was three-fold higher than among the control group (13.1%). A similar trend was observed for individuals with the E/E genotype, where prevalence was found to be 1.2-fold higher among the BC patients (61.1%) compared with the control group (50.6%) (p < 0.001).

For the A111E polymorphism it was not possible to calculate the OR since the wild type GLO I A/A genotype was absent in BC patients.

We also examined the association between the three polymorphisms and clinico-pathological parameters such as age at diagnosis, lymph nodes and nuclear grading (Table 4).

PON I L55M genotype was more represented in patients' group aged between 51-69 years and was accompanied by a greater percentage of cases with positive lymph nodes (61.8%) versus 11.8% of those with L55L genotype. PON I Q192R genotype was associated with high nuclear grading (80%) versus women with Q192Q genotype and high nuclear grading (13.3%). GLO I AE/EE genotypes were associated with a low nuclear grading (43.4% and 56.6% respectively).

Furthermore, the association between cancer types (pure DCIS) and (IDC) according to lymph nodes and triple receptor status was estimated (Table 5).

Clinico-pathological	Total cases	Q192R ^a			L55M⁵			Ala111E°		
parameters	n (%)	QQ	QR	RR	LL	LM	MM	AA	AE	EE
	144 (100)									
Age at Diagnosis ^d										
≤50	49 (34.0)	40 (81.6)	7 (14.3)	2 (4.1)	26 (53.1)	17 (34.7)	6 (12.2)	0	19 (38.8)	30 (61.2)
51-69	79 (54.9)	56 (70.9)	22 (27.8)	1(12.6)	30 (38.0)	40 (50.6)	9 (11.4)	0	29 (36.7)	50 (63.3)
≥70	16 (11.1)	14 (87.5)	1(6.25)	1 (6.25)	14 (87.5)	1 (6.25)	1 (6.25)	0	8 (50.0)	8 (50.0)
		p ^e > 0.05			p = 0.008			p > 0.05		
Lymph nodes										
Positive	34 (23.6)	30 (88.2)	3 (8.8)	1 (2.9)	4 (11.8)	21 (61.8)	9 (26.5)	0	9 (26.5)	25 (73.5)
Negative	110 (76.4)	80 (72.7)	27 (24.5)	3 (27.3)	66 (60.0)	37 (33.6)	7 (6.4)	0	47 (42.7)	63 (57.3)
			p = 0.142			p < 0.001			p > 0.05	
Nuclear grading										
Low	129 (89.6)	108 (83.7)	18 (13.9)	3 (2.3)	66 (51.2)	48 (37.2)	15 (11.6)	0	56 (43.4)	73 (56.6)
High	15 (10.4)	2 (13.3)	12 (80.0)	1 (6.7)	4 (26.7)	10 (66.7)	1 (6.7)	0	0	15(10.4)
		p < 0.001			p = 0.088				p<0.001	

^aGenotypes with Q/R alleles.

^bGenotypes with L/M alleles

° Genotypes with A/E alleles

 $^{\rm d}$ The mean ± SD ages are 59.2 ± 9.7 years for cases

 $^{\circ}$ Two-sided $\chi 2$ test; p< 0.05 indicates statistical significance

Table 4: Associations between PONI Q192R/L55M and GLOI A111E genotypes according to patients' age, lymph nodes and nuclear grading.

	Total cases n= 144				
	Pure DCIS ^a <i>n</i> =110 (%)	IDC ^b n=34 (%)			
Lymph nodes positive negative	0 110(100)	34(100) 0			
ER ^c positive negative	82(74.5) 28(25.4)	19(55.8) 15(44.1)			
PgR ^d positive negative	88(80) 22(20)	21(61.7) 13(38.2)			
HER2 ^e positive negative	41(37.3) 69(62.7)	16(47) 18(53)			

^aPure DCIS, pure ductal carcinoma in situ

bIDC, invasive ductal carcinoma

°ER, estrogen receptor

^dPgR, progesterone receptor

eHER2, human epidermal growth factor receptor 2

Pure DCIS would not have potential lymph nodes. Overall, 55.8 % (19 of 34 cases) of IDC and 74.5% (82 of 110 cases) of DCIS were ER positive; 44.1 % (15 of 34 cases) of IDC and 25.4% (28 of 110 cases) of DCIS were ER negative; 61.7% (21of 34 cases) of IDC and 80% (88 of 110 cases) of DCIS were PgR positive; 38.2% (13 of 34 cases) of IDC and 20% (22 of 110 cases) of DCIS were PgR negative. ER- and PgR-positive were concordant and the percentage is more high respect ER- and PgR-negative in both IDC and DCIS.

47% (16 of 34 cases) of IDC and 37.3% (41 of 110 cases) of DCIS were HER2 positive; 53% (18 of 34 cases) of IDC and 62.7% (69 of 110 cases) of DCIS were HER2 negative. HER2 negative were concordant in both IDC and DCIS and the percentage is a few more high respect HER2 positive in IDC.

Discussion

We believe that the polymorphisms of the genes encoding for glyoxalase and paraoxonase enzymes believed yet risk factors in determining susceptibility to some neurodegenerative diseases as multiple sclerosis [31], could be also associated with the risk of breast cancer [24,32].

With regard to PON I Q192R, an increase in the frequency of PON I 192 Q/R was observed in the BC group (20.8%) versus 4.6% in the control group. Patients with Q/R and R/R genotype have a significant increase for the risk of BC (OR = 5.6, CI = 2.23 - 14.51 and OR = 2.6, CI = 0.40 - 20.83), even if this risk is higher for the heterozygous rather than for the homozygous. It is likely that, the Q to R substitution, leads to a reduction of enzyme activity, and it would be in agreement with what reported by Ferrè and Mackness [29,33].

With regard to PON I L55M, an increase in the frequency of PON55 heterozygotes L/M was observed in patients with BC (40.3%) with respect to the control group (4.6%). PON I 55 heterozygotes also had a higher risk for BC (OR = 15.4) than mutant PON I 55 homozygotes (OR = 2.0). Therefore, the presence of PON I 55M allele significantly increases the risk in BC.

The genotype M produces less enzyme but of equivalent specific activity (activity/mg protein) to the L isoform. Lower serum activity of M is due to lower concentrations.

Therefore, the paraoxonase-dependent protection against LDL

peroxidation can be compromised and this may play a specific role in BC pathogenesis. The fact that for both the polymorphisms PON I Q192R/L55M, the condition of the homozygosity is combined with a lower risk for BC Could be linked to the fact that paraoxonase enzyme activity can be affected by other factors apart from the genetic component [29,30].

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With respect to GLO I, we found that the A/E and E/E genotypes frequencies were higher among the BC patients (38.9% and 61.1% respectively) than in control group (13.1% and 50.6% respectively). However, no association was found between these genotypes and the risk of BC.

Therefore, we believe significant the association, found by us, between PON I 192/55 heterozygote genotypes and some clinicopathological parameters, fundamental for tumor prognosis: PON I 192 Q/R and 55 L/M were , in fact associated with a higher nuclear grading and a higher percentage of positive lymph nodes, respectively. Conversely the genotypes GLO I AE/EE were associated with a low nuclear grading.

With respect to associations between pure DCIS and IDC according to lymph nodes and triple receptor status, we found that pure DCIS would not have potential lymph nodes but pure DCIS does not always guarantee the absence of lymph node metastasis; they might be too small to be detected by routine pathological examination. Furthermore, with respect to triple receptor status, we found that the percentage for HER2 negative is a few more high respect HER2 positive in IDC. Probably, it doesn't have a critical role in the progression of DCIS to IDC and it would not be a valid prognostic indicator for breast cancer alone.

Conclusions

Oxidative stress, implicated in the etiology of cancer, results from an imbalance in the production of Reactive Oxygen Species (ROS) and cell's own antioxidant defenses. ROS deregulate redox homeostasis and promote tumor formation by initiating an aberrant induction of signalling networks that cause tumorigenesis [34]. Improper balance between the production of Reactive Oxygen Metabolites (ROMs) and antioxidative defense system have been defined as oxidative stress in various pathologic conditions age-dependent [35]. Furthermore, lipids, lipoproteins, antioxidative vitamins, lifestyle factors, as diet [36] have been associated with the risk of breast cancer and the elevated plasma LDL - concentration, which is more susceptible to oxidation, may result in higher lipid peroxidation [37,38] in breast cancer patients. We believe that could be an association between PON I Q192R/L55M polymorphisms and BC risk. In particular, our results suggest that PON I Q192R and L55M polymorphisms appear to be a more useful genetic marker for tumor prognosis and to identify women who might be at greater risk of developing breast cancer. Therefore, we don't believe that the analysis of a single polymorphism alone is indicative for the association to the risk of BC but the combination of them all in each single case might be a more predictive factor for the risk of this neoplasia. However, this is only a preliminary study; further studies are needed to evaluate data on activity of the examined genes.

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