RESEARCH

PTGS2 polymorphism rs689466 favors breast cancer recurrence in obese patients

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Abstract

Breast cancer is the leading cancer among women, and its increasing incidence is a challenge worldwide. Estrogen exposure is the main risk factor, but obesity among postmenopausal women has been shown to favor disease onset and progression. The link between obesity and mammary carcinogenesis involves elevated estrogen production and proinflammatory stimuli within the adipose tissue, with activation of the cyclooxygenase-2 pathway. Here, we evaluate the impact of the four most common cyclooxygenase-2 gene polymorphisms (rs689465, rs689466, rs20417 and rs20417), in combination with obesity, on the risk of breast cancer progression in a cohort of Brazilian breast cancer patients ($N = 1038$). Disease-free survival was evaluated using Kaplan–Meier curves, with multivariate Cox proportional hazards regression models for calculation of adjusted hazard ratios (HR_{adj}). Obesity did not affect disease progression, whereas rs689466 variant genotypes increased the recurrence risk among obese patients ($HR_{adj} = 2.5$; 95% CI = 1.4–4.3), either for luminal ($HR_{adj} = 2.2$; 95% CI = 1.1–4.2) or HER2-like and triple-negative tumors ($HR_{adj} = 3.2$; 95% CI = 1.2–8.5). Likewise, the haplotype *4, which contains variant rs689466, was associated with shorter disease-free survival among obese patients ($HR_{adj} = 3.3$; 95% CI = 1.8–6.0), either in luminal ($HR_{adj} = 3.5$; 95% CI = 1.6–7.3) or HER2-like and triple-negative ($HR_{adj} = 3.1$; 95% CI = 1.1–8.9) tumors. Such deleterious impact of variant rs689466 on disease-free survival of obese breast cancer patients was restricted to postmenopausal women. In conclusion, cyclooxygenase-2 genotyping may add to the prognostic evaluation of obese breast cancer patients.

Key Words

- breast cancer
- *PTGS2*
- COX2
- gene polymorphisms
- disease-free survival

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Introduction

Breast cancer is the leading cancer (Torre *et al.* 2017) and the first cause of death by cancer among women worldwide (Ferlay *et al.* 2010). It is a very heterogeneous disease, with diverse morphological and molecular presentations (Dai *et al.* 2016), resulting in great variability of clinical outcomes, even among early-stage tumors

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(Early Breast Cancer Trialists' Collaborative Group 2005). Although the advances in tumor classification and personalized treatment have contributed to reduce the global mortality of breast cancer, the prognostic evaluation of newly diagnosed tumors remains a clinical challenge, which justifies the continuous search for new biomarkers and staging models.

An additional challenge in the clinical and epidemiological approach of breast cancer is its increasing incidence, both in developed and under-developed countries, which is attributed to changes in life style (Torre *et al.* 2017). For example, the use of hormonal contraceptives and of menopausal hormone therapy contributes for higher exposure to estrogen, a major risk factor for breast cancer development (Chlebowski *et al.* 2013). More recently, weight gain after age 18 years and excess body weight among postmenopausal women have also been implicated as breast cancer risk factors (Torre *et al.* 2017). In addition to favoring breast cancer development, obesity also contributes for worse prognosis, including increased recurrence risk and shorter disease-free and overall survival, independent of tumor stage at diagnosis (Pajares *et al.* 2013, Chan *et al.* 2014). The link between excess weight or obesity and breast cancer appears to involve altered expression of hormones, especially estrogen, as well as growth factors, inflammatory cytokines and adipokines, which promote cancer cell survival, metastasis, angiogenesis and decreased cancer cell apoptosis (Crespi *et al.* 2016).

The main source of estrogen in postmenopausal women is the adipose tissue, the primary site of aromatase expression following menopause (Iyengar *et al.* 2015). Accordingly, elevated aromatase expression has been reported in the breast tissue of overweight and obese women, where aromatase levels correlate with local inflammation, characterized by the presence of crown-like structures, consisting of necrotic adipocytes surrounded by macrophages (Morris *et al.* 2011). Crown-like structures are considered as inflammatory foci, as they release several proinflammatory mediators (Mullooly *et al.* 2017), including the cyclooxygenase-2 (COX2)-derived prostaglandin E2 (PGE2). PGE2 stimulates all of the key features of mammary carcinogenesis including mutagenesis, mitogenesis, angiogenesis, metastasis, inhibition of apoptosis and immunosuppression (Harris 2014). PGE2 is also a potent stimulant of aromatase expression in preadipocytes, the predominant site of aromatase expression within adipose tissue (Morris *et al.* 2011). This obesity–inflammation–aromatase axis may be a significant contributor to increased mortality rate observed in obese postmenopausal patients, with COX2 pathway apparently playing a central role (Bowers *et al.* 2014).

Although COX2 has been associated with worse prognosis of breast cancer, its expression in breast tumors presents great variability (Glover *et al.* 2011). For instance, in invasive breast carcinoma, the frequencies of COX2 overexpression range from 17% to 84% (Glover *et al.* 2011). The mechanisms underlying the regulation of COX2 expression, and the reasons for the great interindividual variability are not yet fully understood.

COX2 is encoded by the prostaglandin-endoperoxidase synthase 2 gene (*PTGS2*), whose promoter region (PR) encloses several potential regulatory elements (RE) (Tanabe & Tohnai 2002). Single-nucleotide polymorphisms (SNP) have been described next to these RE (rs689465, rs689466, rs20417) and appear to affect gene transcription (Papafili 2002, Zhang *et al.* 2005, Sakaki *et al.* 2010, Pereira *et al.* 2014). The 3′-untranslated region (3′-UTR) of *PTGS2* also presents potential RE (Appleby *et al.* 1994), which generate consensus-binding sequences ('AUUUA') for proteins that maintain the stability (Young *et al.* 2009) or trigger the degradation of mRNA (Dixon 2000). A SNP located in the 3′-UTR of the *PTGS2* gene (rs5275) has been shown to favor mRNA stability and gene expression (Moore *et al.* 2012). Many studies explored the role of *PTGS2* SNPs on the susceptibility to breast cancer, and a meta-analysis suggests an increased risk associated with rs20417 (Li *et al.* 2015). There are fewer information regarding the impact of *PTGS2* SNPs on breast cancer outcomes, and all the available reports explored only rs5275, with no significant results (Abraham *et al.* 2009, Gerger *et al.* 2010, Jung *et al.* 2010, Knechtel *et al.* 2010). Here, we investigate the potential contribution of the four most common *PTGS2* SNPs and their haplotypes, in combination with obesity, as prognostic predictors of disease-free survival in newly diagnosed non-metastatic breast cancer.

Materials and methods

Subjects and study design

The study population consisted of an on-going prospective hospital-based cohort of Brazilian women with first diagnosis of unilateral breast carcinoma and no identification of distant metastases. Patients were recruited when assigned for curative surgery ($N=713$, either mastectomy or segmentectomy) or neoadjuvant chemotherapy ($N=325$), as their first therapeutic approach at the Brazilian National Cancer Institute (INCA), during

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the period from February 2009 to April 2013. The study was conducted following the international precepts of ethics in research, including the 1964 Helsinki declaration and its later amendments and of good clinical practice. The authors complied with the Brazilian regulation of clinical research. The study protocol was approved by the Ethics Committees of the Brazilian National Cancer Institute (INCA #129/08) and of the National School of Public Health (FIOCRUZ/CAAE 55929416.8.0000.5240), and all patients gave written consent to participate. The REMARK guidelines (REporting recommendations for tumor MARKer prognostic studies) were followed (McShane *et al*. 2005). A description of this breast cancer cohort has already been published (Vieira-Monteiro *et al*. 2016), but survival data were updated.

Clinical conducts

Treatments were chosen by the institutional medical staff, according to local standard protocols. In brief, the first therapeutic approach could be curative surgery ($N=713$, either mastectomy or segmentectomy), which was recommended for all cases of resectable tumor or neoadjuvant chemotherapy ($N=325$), for patients with locally advanced disease.

Patients initially submitted to curative surgery could be further treated with adjuvant chemotherapy (61.6%) or radiotherapy (16.4%), whereas those with *in situ* or small tumors (<2 cm) and no lymph node metastasis received hormonal therapy alone (12.9) or were clinically followed with no secondary intervention (9.1%).

Patients who initiated treatment with neoadjuvant chemotherapy could be subsequently assigned for curative surgery ($N=306$, consisting of mastectomy) or for palliative treatment ($N=19$, comprising cases of inoperable tumors or disease progression during neoadjuvant chemotherapy).

The standard chemotherapeutic protocol for both adjuvant and neoadjuvant chemotherapy was CAF-T (3 cycles of cyclophosphamide, doxorubicin and 5-fluorouracyl, followed by 3 cycles of docetaxel), which accounted for 65.8% of adjuvant chemotherapy and 90.5% of neoadjuvant chemotherapy. Alternatively to CAF-T, patients could also be treated with CAF (6 cycles; 13.2% in adjuvant chemotherapy or 2.5% in neoadjuvant chemotherapy), CA (6 cycles; 14.1% in adjuvant chemotherapy) or C-T (3 cycles each; 3.4% in adjuvant chemotherapy or 2.5% in neoadjuvant chemotherapy). Other protocol options accounted for less than 5% of either adjuvant or neoadjuvant chemotherapy. Cardiac function and blood cell counts were evaluated prior to chemotherapy selection and monitored during clinical follow-up.

All patients with HER2+ tumors also received trastuzumab, which was initiated in combination with docetaxel or after completion of chemotherapy. Subsequent hormonal therapy was also prescribed for patients with luminal tumors.

Collection of clinical and histopathological data

Patients were interviewed to provide information on their clinical history and life-style habits. The variables considered for clinical history were age at diagnosis, menopausal status and comorbidities, including any pre-existing chronic condition under medical treatment. Obesity was the only exception, being defined based on the body mass index (BMI), which was calculated as the weight (kg) divided by the square of height (m^2). Patients were classified in three groups according to their BMI, as follows: under or normal weight ($BMI \leq 24.9$), overweight ($25 \leq BMI \leq 29.9$) and obese ($BMI \geq 30$) (WHO Expert Committee 1995).

Histopathological characterization of breast tumors was performed with biopsies obtained for diagnostic purposes and was based on the 3rd edition of the WHO Classification of Tumors (Ellis *et al*. 2003) and on the Elston–Ellis histological grading system (Elston & Ellis 1991). The data on hormone receptors and HER2 status of breast tumors, according to immunohistochemical and fluorescence *in situ* hybridization analyses were used for surrogate classification of tumor subtypes (Huober *et al*. 2010). In brief, four subtypes were considered: Luminal A, positive for both estrogen receptor (ER) and progesterone receptor (PR), but negative for HER2; Luminal B, positive for either ER or PR, regardless of HER2 status or positive for the three receptors; HER2-like, negative for both ER and PR, but positive for HER2; and triple-negative, when negative for all the three receptors.

Genotyping analyses

Peripheral blood samples (3 mL) were collected from all subjects, and DNA was extracted using the Blood Genomic Prep Mini Spin Kit (GE Healthcare), following the procedures recommended by the manufacturer.

Patients were genotyped for rs689465 (*–1290 AG*) and rs20417 (*–765 GC*) using PCR-RFLP (PCR restriction fragment length polymorphism assay), as described previously (Piranda *et al*. 2010), and for rs689466

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(*–1195 AG*) and rs5275 (*8473 TC*) by allelic discrimination using TaqMan SNP Genotyping Assays (Applied Biosystems), as described previously (Festa-Vasconcellos *et al.* 2012).

Survival outcomes

Survival analyses were performed for all patients submitted to curative surgery, either as their first therapeutic approach ($N=713$) or following neoadjuvant chemotherapy ($N=306$). Total patient follow-up was 77,002 person-months, with a median follow-up time per person of 78 months. Disease-free survival was defined as the primary clinical endpoint of the study. Disease progression was characterized by the occurrence of loco-regional or contralateral recurrence of breast cancer or by any distant metastasis. The time to event (TTE) was calculated as the period between the date of surgery and the date of relapse detection, i.e. imaging diagnosis or histopathological characterization of disease progression. Patients were considered disease-free if they had no suggestive clinical symptoms or imaging diagnosis of disease progression until their last medical consult. New primary cancer lesions or deaths by causes unrelated to disease progression were censored in survival analysis. Patients achieving five years of follow-up were also censored.

Statistical analyses

A descriptive study of the cohort was conducted, presenting relative frequencies for each categorical variable. Individual features were dichotomized according to better- or worse-expected prognostic values and evaluated for their association with *PTGS2* genotypes and for their impact on survival outcomes.

Allelic and genotypic frequencies of *PTGS2* were derived by gene counting, and the adherence to the Hardy–Weinberg principle was evaluated by the chi-square test for goodness of fit. Haplotype patterns were inferred using Haploview 4.2 (Haploview internet version), based on the algorithm of expectation and maximization (Barrett *et al.* 2005). Individual diplotypes were inferred using the Haplo Stats software, version 1.3 (Schaid *et al.* 2002). The distribution of *PTGS2* genotypes according to clinical and histopathological features was evaluated using the chi-square or Fisher's exact tests.

Disease-free survival curves were estimated using the Kaplan–Meier product-limit method, with the influence of individual variables on the mean time to disease progression being evaluated with the two-sided log-rank test. The impact of individual variables on disease-free survival rates was estimated by calculation of their hazard ratios (HR) and 95% confidence intervals (95% CI). Variables that significantly affected disease-free survival in the general cohort were included as covariates in multivariate Cox proportional hazards regression models to calculate the adjusted HR (HR_{adj}) and respective 95% CI of new potential prognostic factors of breast cancer progression.

All statistical analyses were conducted using SPSS 13.0 for Windows (SPSS). The adequacy of sample sizes for statistical power analyses was evaluated using the online calculators at the website of the University of California San Francisco (http://www.sample-size.net/sample-size-survival-analysis/).

Results

Table 1 presents the main clinical and histopathological characteristics of the study cohort ($N=1038$), with the distribution of *PTGS2* genotypes being evaluated for patients with available DNA ($N=959$). Haplotypes ($N=1852$) were inferred from samples with successful *PTGS2* genotyping ($N=926$), the minimum rate of successful genotyping being 93.5% for rs5275. A previous description of this cohort formation and clinical characteristics has been already published (Vieira-Monteiro *et al.* 2016). Here, we added information concerning the frequency of comorbidities, hypertension being the most prevalent, followed by obesity and diabetes. Besides being highly prevalent in the cohort (only 30% of patients had no pre-diagnosed conditions), comorbidities were also frequently coexisting, with 21.1% of patients presenting at least two concomitant conditions. Regarding BMI, most patients were above normal weight (70%), with 30% being obese. All *PTGS2* SNPs were in Hardy–Weinberg equilibrium, with minor allele frequencies of 0.15 for rs689465 (*–1290 G*), 0.14 for rs689466 (*–1195 G*), 0.25 rs20417 (*–765 C*) and 0.37 for rs5275 (*8473 C*). The four SNPs showed strong linkage disequilibrium, forming a single haploblock with 8 haplotypes, the first 5 summing 93.8% of the cohort variability.

In order to explore the potential interaction between obesity and *PTGS2* SNPs in breast cancer presentation and outcomes, we first evaluated the distribution of clinical features and of *PTGS2* SNPs according to obesity (Table 2). Significant differences were found only for age at diagnosis, with obese patients presenting an approximate 7% decrease in the proportion of patients younger than

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Table 1 Description of the study cohort ($N = 1038$).

Features	*N*	%	Genotypes	*N*	%
Histopathological type			**rs689465**		
Ductal invasive	902	86.9	*AA*	666	72.1
Lobular invasive	58	5.6	*AG*	230	24.9
In situ (ductal or lobular)	47	4.7	*GG*	28	3.0
Others	29	2.8	Missing	35	
Tumor stage (TNM)			**rs689466**		
≤IIA	529	52.2	*AA*	680	73.7
≥IIB	484	47.8	*AG*	220	23.8
Missing	25		*GG*	23	2.5
			Missing	36	
Tumor grade (G)			**rs20417**		
G1	96	11.1	*GG*	529	57.1
G2	403	46.5	*GC*	326	35.2
G3	367	42.4	*CC*	71	7.7
Missing	172		Missing	33	
Tumor subtype			**rs5275**		
Luminal A	537	57.2	*TT*	344	38.3
Luminal B	210	22.3	*TC*	434	48.4
HER2-like	63	6.7	*CC*	119	13.3
Triple-negative	130	13.8	Missing	62	
Missing	98				
Comorbidities[a]			**Haplotype**[b]		
None	341	32.8	*1 *AAGT*	917	44.2
Hypertension	516	50.2	*2 *AAGC*	287	13.8
Obesity	282	30.0	*3 *GACC*	243	11.7
Diabetes *mellitus*	137	13.4	*4 *AGGT*	299	14.4
BMI			*5 *AACC*	201	9.7
≤18.4–24.9	275	29.3	*6 *AACT*	56	2.7
25–29.9	382	40.7	*7 *GACT*	23	1.1
More than 30.0	282	30.0	*8 *GAGC*	50	2.4
Missing	99				

[a]Patients may present two or more comorbidities concomitantly; [b]*PTGS2* haplotypes composed by rs689465, rs6894656, rs20417, rs5275.
BMI, body mass index; HER2, human epidermal growth factor receptor 2.

45 years and a 10% increase within 45–59 years, which was the age range with the highest prevalence of obesity (35%). Such increase in age according to obesity, however, was not maintained for patients older than 50 years.

Second, we evaluated the distribution of *PTGS2* SNPs according to histopathological features of breast tumors. Significant differences were found only for rs5275, whose variant genotypes (*TC*+*CC*) were significantly associated with positive lymph node status (OR = 1.33; 95% CI = 1.01–1.74). Nevertheless, the haplotypic distribution showed no significant differences according to histopathological characteristics (data not shown).

Next, we evaluated the influence of clinical and histopathological characteristics, as well as of *PTGS2* genotypes and haplotypes, on the risk of disease progression. Loco-regional or contralateral recurrence affected 47 patients, distant metastasis was observed in 165 cases and 135 deaths were recorded. Table 3 shows the results regarding the evaluation of disease-free survival in the complete cohort and according to obesity status. High tumor stage, defined by TNM ≥ IIB, neoadjuvant treatment and HER2-like or triple-negative subtypes were significantly associated with shorter disease-free survival for all breast cancer patients in the cohort, regardless of the obesity status. High histological grade (G2+G3) and age <45 years were also good predictors of breast cancer progression in the total cohort, as well as among non-obese patients. Obesity was not associated with disease progression in the present cohort (data not shown). Regarding *PTGS2* SNPs, only rs689466 (*–1195 G* variant) was associated with a significant reduction in disease-free survival, exclusively among obese patients. Accordingly, patients carrying the haplotype *4, which varies in relation to the wild-type sequence of *PTGS2* only at rs689466, presented the shortest time to breast cancer progression within *PTGS2* diplotypes. All other *PTGS2* haplotypes had no significant effects on disease-free survival, regardless of the obesity status.

Figure 1 shows the disease-free survival curves according to rs689466 genotypes in obese patients.

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Table 2 Distribution of clinical features and of PTGS2 SNPs according to obesity among breast cancer patients.

	Obesity				
	No (*N* = 657)		Yes (*N* = 282)		
Features	*N*	%	*N*	%	Px2
Age at diagnosis (years)					
<45	135	20.6	39	13.8	
45–59	271	41.2	147	52.1	
60–74	199	30.3	82	29.1	
≥75	52	7.9	14	4.9	0.006
Menopausal status					
Premenopausal	209	32.4	75	26.9	
Postmenopausal	437	67.6	204	73.1	0.09
Missing	11		3		
Tumor stage (TNM)					
≤IIA	334	52.1	129	46.6	
≥IIB	307	47.9	148	53.4	0.12
Missing	16		5		
Tumor grade (G)					
G1	58	10.6	22	8.9	
G2	250	45.5	119	48.4	
G3	241	43.9	105	42.7	0.70
Missing	108		36		
Tumor subtype					
Luminal A	320	54.2	158	59.8	
Luminal B	137	23.2	57	21.6	
HER2	43	7.3	17	6.4	
Triple negative	90	15.3	32	12.1	0.44
Missing	67		18		

	Obesity				
	No (*N* = 657)		Yes (*N* = 282)		
Genotypes	*N*	%	*N*	%	Px2
rs689465					
AA	422	72.4	185	72.8	
AG + *GG*	161	27.6	69	27.2	0.90
Missing	74		28		
rs689466					
AA	435	74.6	179	70.2	
AG + *GG*	148	25.4	76	29.8	0.20
Missing	74		27		
rs20417					
GG	332	56.9	151	59.2	
GC + *CC*	251	43.1	104	40.8	0.50
Missing	74		27		
rs5275					
TT	219	38.5	94	37.5	
TC + *CC*	350	61.5	157	62.5	0.78
Missing	88		31		
Haplotypes[a]					
*1 *AAGT*	586	44.6	238	42.1	
*2 *AAGC*	185	14.1	82	14.6	
*3 *GACC*	162	12.3	58	10.2	
*4 *AGGT*	180	13.7	94	16.6	
*5 *AACC*	122	9.3	52	9.3	
*6 *AACT*	38	2.9	14	2.6	
*7 *GACT*	16	1.2	6	1.1	
*8 *GAGC*	25	1.9	20	3.5	0.24

[a]*PTGS2* haplotypes composed by rs689465, rs6894656, rs20417, rs5275.
HER2, human epidermal growth factor receptor 2.

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Table 3 Influence of individual features on the 5-year disease-free survival of breast cancer patients.

Variables	**Total cohort** (*N* = 1019)			**Non-obese** (*N* = 645)			**Obese** (*N* = 275)		
	TTE (months)	95% CI	*P* log-rank	TTE (months)	95% CI	*P* log-rank	TTE (months)	95% CI	*P* log-rank
Age at diagnosis (years)									
<45	50.2	47.4–52.9		50.1	46.9–53.4		51.3	45.5–57.1	
≥45	55.3	54.3–56.2	**≤0.0001**	55.6	54.3–56.8	**0.001**	53.9	51.9–55.9	0.44
Tumor stage (TNM)									
≤IIA	58.6	57.8–59.4		58.4	57.4–59.4		57.9	56.1–59.6	
≥IIB	49.7	48.0–51.4	**≤0.0001**	50.2	48.0–52.3	**≤0.0001**	49.5	46.4–52.6	**≤0.0001**
Tumor subtype									
Luminal	55.8	54.9–56.8		55.8	54.6–57.0		55.3	53.5–57.1	
HER2+TN	47.7	44.7–50.6	**≤0.0001**	48.8	45.3–52.2	**≤0.0001**	45.6	39.6–51.7	**≤0.0001**
Treatment									
Surgery+adjuvancy	58.1	57.4–58.8		57.9	56.9–58.8		57.8	56.2–59.3	
Neoadjuvancy+surgery	45.7	43.3–48.1	**≤0.0001**	46.3	43.3–49.3	**≤0.0001**	46.4	42.4–50.4	**≤0.0001**
Tumor grade (G)									
G1	59.7	58.2–61.1		59.1	56.9–61.4		60.2	58.7–61.7	
G2+G3	53.1	52.0–54.3	**≤0.0001**	53.2	51.7–54.7	**0.007**	52.9	50.7–55.1	0.091
rs689465									
AA	54.5	53.3–55.7		54.9	53.4–56.3		53.4	51.0–55.7	
AG+GG	54.0	52.0–55.9	0.99	53.1	50.5–55.7	0.33	54.2	50.6–57.9	0.45
rs689466									
AA	55.0	53.8–56.1		54.5	53.1–56.0		55.8	53.7–57.8	
AG+GG	52.7	50.5–54.8	0.07	53.9	51.3–56.5	0.90	48.4	44.1–52.8	**0.001**
rs20417									
GG	54.6	53.3–55.9		55.1	53.5–56.6		52.7	49.9–55.4	
GC+CC	54.1	52.5–55.6	0.92	53.5	51.4–55.5	0.33	55.1	52.4–57.8	0.29
rs5275									
TT	54.9	53.3–56.5		56.0	54.2–57.8		50.8	46.9–54.6	
TC+CC	54.3	53.0–55.6	0.25	53.6	51.9–55.3	0.083	55.6	53.5–57.7	0.24
Diplotypes									
*1*1	55.7	53.6–57.7		55.1	52.6–57.7		55.7	51.1–60.3	
*1*2+*2*2	56.1	53.9–58.3	0.25	54.7	51.5–57.9	0.46	59.7	58.3–61.2	0.92
*1*3+*3*3	53.9	50.9–57.0	0.33	52.4	48.3–56.5	0.23	56.0	50.6–61.4	0.89
*1*4+*4*4	52.5	49.5–55.4	0.07	56.4	53.6–59.2	0.49	42.4	35.6–49.1	**0.001**
*1*5+*5*5	51.7	47.9–55.4	0.043	52.7	48.1–57.2	0.41	51.3	44.6–58.1	0.064

Statistically significant differences are presented in bold characters.
TTE, time to event, either recurrence or distant metastasis.

The deleterious impact of variant rs689466 genotypes (*AG*+*GG*) on the risk of disease progression was maintained after adjustment for the other significant covariates, even when patients were stratified according to tumor subtypes into luminal or HER2 like and triple negative. Similarly, Fig. 2 shows that the presence of haplotype *4 (in diplotypes *1*4 or *4*4) was also responsible for a significant reduction in disease-free survival among obese patients. Again, the effect was maintained when luminal tumors or HER2-like and triple-negative tumors were evaluated separately. Table 4 presents the results of the Cox logistic regression models considering either the rs689466 genotypes or the presence of haplotype *4 and the other covariates that were associated in univariate analysis (Table 3) with the five-year disease-free survival among obese breast cancer patients in the study cohort. Tumor subtype was maintained in the multivariate model with haplotype *4 although it did not reach statistical significance in this analysis.

Next, the effects of the variant rs689466 genotypes or haplotype *4 were evaluated according to menopausal status (Fig. 3). No significant differences were detected in premenopausal women (panels A and C), whereas the reduction in disease-free survival associated with either rs689466 variant genotypes or haplotype *4 (diplotypes *1*4 or *4*4) were confirmed among postmenopausal obese breast cancer patients (panels B and D). Table 5 shows the results of the Cox logistic regression models for the five-year disease-free survival among postmenopausal obese breast cancer patients. The significant impact of rs689466 genotypes or haplotype *4 on the risk of breast cancer progression was still detectable when luminal or HER2-like and triple-negative tumors were evaluated separately (Fig. 4).

Discussion

The present study aimed to evaluate the contribution of *PTGS2* SNPs, in combination with obesity, as potential prognostic factors of breast cancer outcomes. The study population consisted of a prospective single-institution cohort of breast cancer patients from Brazil with no distant metastasis at diagnosis. Individual clinical histories were fully available, and medical treatments and follow-up routines were standardized, which contributed to minimize heterogeneity and reduce uncontrolled confounding factors. However, a potential disadvantage of this single-institution design, as compared to multi-institutional studies, is the risk of demographic homogeneity, which might lead to results that cannot

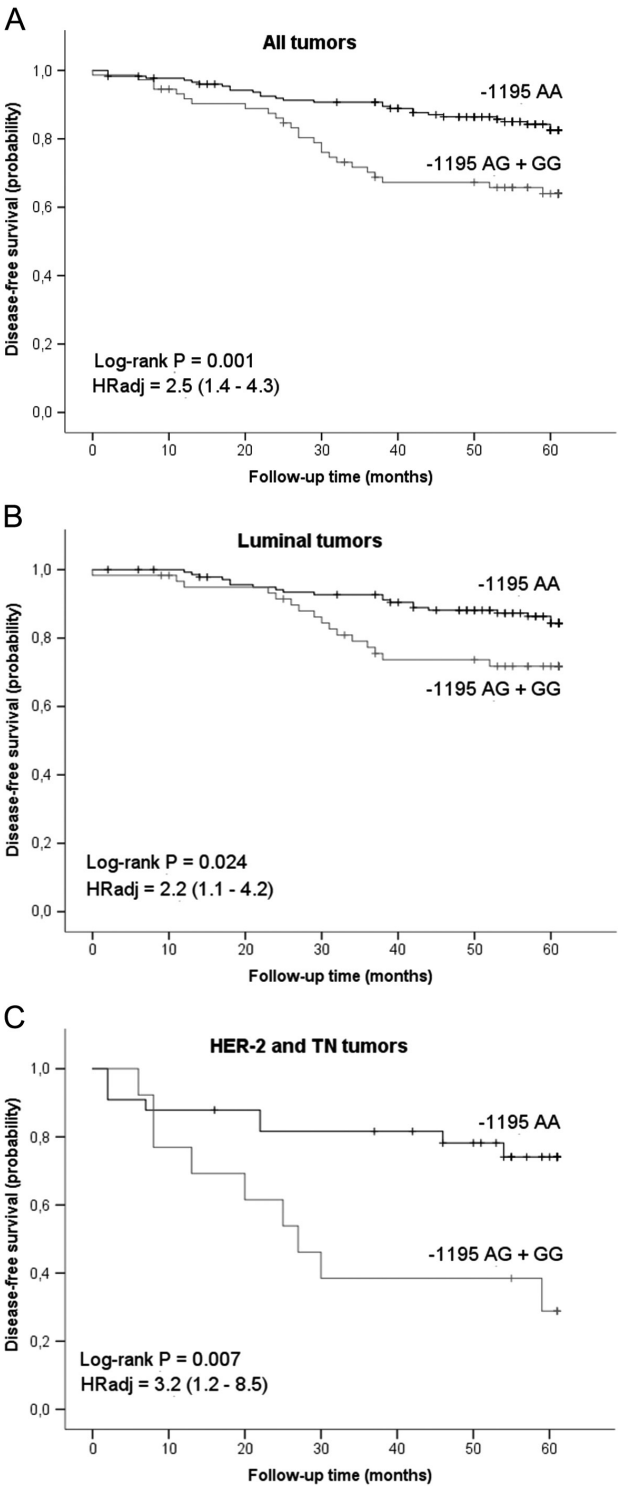

Figure 1
Disease-free survival curves in obese breast cancer patients according to *PTGS2* polymorphism rs689466 (*−1195 A>G*). Variant genotypes −1195 AG+GG were compared to the wild-type genotype AA in all types of tumor (A), in luminal tumors only (B) and in HER2 and triple-negative tumors (C). Multivariate Cox proportional hazards regression was used to calculate the hazard ratios (HR_{adj}), with adjustment for tumor subtype (A), first therapeutic approach and tumor stage based on TNM (A, B and C).

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be further extrapolated. Although we acknowledge this possibility, we expect this risk to be low in our study. INCA is a reference institution for cancer treatment in Brazil, and patients were from different cities of the state of Rio de Janeiro (16.7 million inhabitants). In addition, the Brazilian population is characterized by a high degree of genetic diversity due to intense admixture, especially in the southeast of Brazil, where Rio de Janeiro is located (Parra *et al.* 2003).

Regarding sample size, recruitment was planned for 1000 subjects, in order to have at least 100 cases of triple-negative tumors, and at least 200 cases of each variant genotype. Such figures were expected to allow robust comparisons of the five-year disease-free survival between genotypes. Because significant results were obtained only among obese patients, who represent 30% of the cohort (N=275), the obtained data were used to recalculate recommended sample sizes for enough statistical power in the survival analysis (http://www.sample-size.net/sample-size-survival-analysis/). The required sample sizes for a planned follow-up of five years were calculated to be 245 cases for rs689466 or 247 cases for haplotype *4. Both figures are below the actual number of obese patients under analysis, suggesting that the present results can be considered to be within fair levels of confidence.

The evaluation of clinical data indicates a high prevalence of comorbidities, which were often superposed. Hypertension was the most frequent comorbidity, and the figures are similar to those reported for Brazilian breast cancer patients (Lagares *et al.* 2013), as well as for adult Brazilian women of similar age (Cipullo *et al.* 2010). The prevalence of diabetes also matched previously reported data involving breast cancer patients from Brazil (Lagares *et al.* 2013) or adult Brazilian women of similar age (Cipullo *et al.* 2010). In contrast, the prevalence of obesity in the study cohort was higher than expected for women between 45 and 64 years according to the data of the Brazilian Census (IBGE 2010). Indeed, obesity is a recognized risk factor for the development of breast cancer, especially among postmenopausal women (Yung & Ligibel 2016).

The evaluation of prognostic impacts of clinical characteristics indicates no significant association between obesity and breast cancer progression in the present cohort. This finding contrasts with previous reports linking obesity with shorter disease-free survival (de Azambuja *et al.* 2010, Sparano *et al.* 2012, Pajares *et al.* 2013, Widschwendter *et al.* 2015), as well as with increased mortality (de Azambuja *et al.* 2010, Protani *et al.* 2010, Sparano *et al.* 2012, Chan *et al.* 2014, Widschwendter *et al.* 2015).

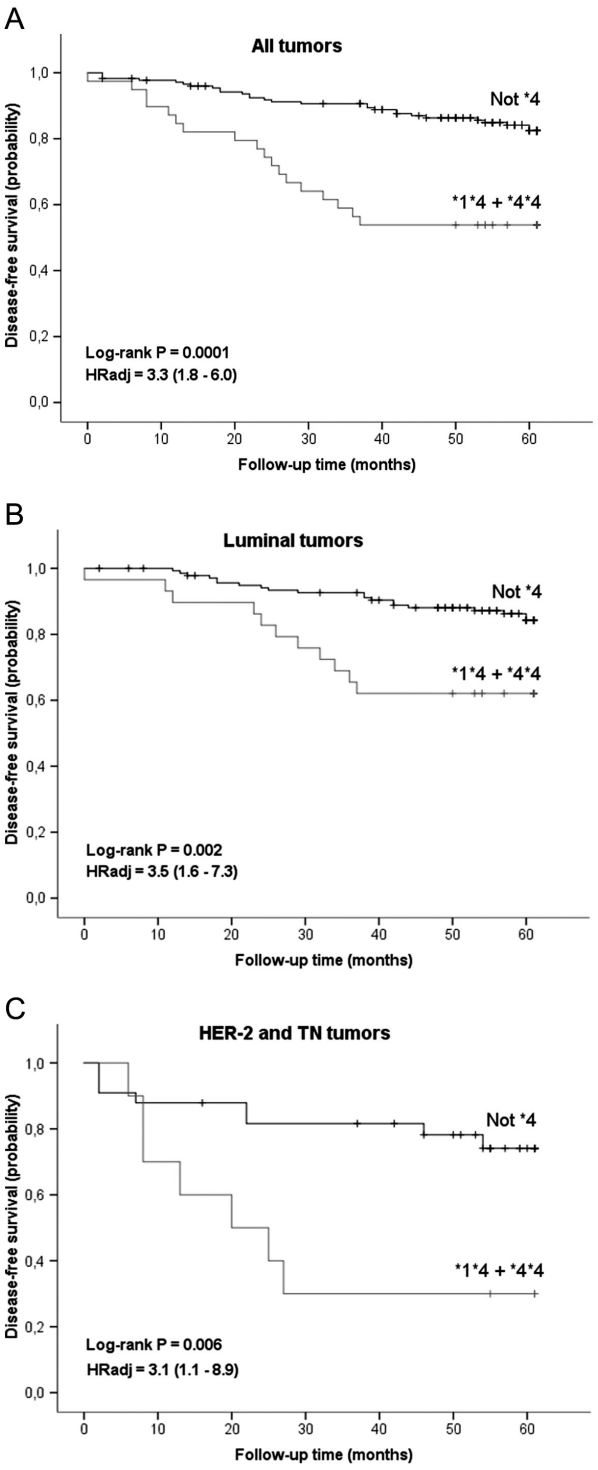

Figure 2
Disease-free survival curves in obese breast cancer patients according to *PTGS2* haplotype *4. Diplotypes *1*4+ *4*4 were compared to all other diplotypes not containing haplotype *4 in all types of tumor (A), in luminal tumors only (B) and in HER2 and triple-negative tumors (C). Multivariate Cox proportional hazards regression was used to calculate the hazard ratios (HR_{adj}), with adjustment for the first therapeutic approach (A, B and C), tumor stage based on TNM (A and B) and tumor subtype (A).

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Table 4 Logistic regression models for the five-year disease-free survival in obese breast cancer patients.

Variables	Coefficient	Default error	*P* value	Hazard ratio	95% confidence interval
Treatment	0.941	0.334	0.005	2.563	1.331–4.936
TNM	0.873	0.360	0.015	2.394	1.182–4.848
Tumor subtype	0.834	0.308	0.007	2.303	1.259–4.211
A-1195G	0.909	0.277	0.001	2.481	1.441–4.273
Treatment	1.093	0.368	0.003	2.984	1.451–6.136
TNM	0.802	0.384	0.037	2.231	1.051–4.734
Tumor subtype	0.551	0.332	0.097	1.735	0.905–3.325
(*1*4 + *4*4) vs not *4	1.187	0.307	0.000	3.278	1.795–5.986

Nevertheless, Pajares *et al.* (2013) found a significant deleterious effect on breast cancer outcomes only for patients with BMI ≥ 35 when compared to normal or underweight patients. More recently, Widschwendter *et al.* (2015) confirmed an increase in the rates of breast cancer recurrence and mortality for patients with severe obesity (BMI ≥ 40), but not for those with moderate or slight obesity (BMI 30–39.9). In the present cohort, severe obesity was detected in 24 patients (2.4%), whereas 68 (6.7%) presented moderate obesity (BMI 35–39.9), and 190 (20.2%) had slight obesity (BMI 30–34.9). Although such proportion is similar to those reported by Pajares *et al.* (2013) or Widschwendter *et al.* (2015), the limited number of individuals with severe obesity in the present cohort may have limited the detection of increased recurrence risk. Also, although the histopathological features and treatment conditions were also similar between our study and those by Pajares *et al.* (2013) or Widschwendter *et al.* (2015), the latter two involved only patients from clinical trials, and trastuzumab was not included in the therapeutic protocols.

Regarding other clinical and histopathological characteristics, large tumor size, positive lymph node status, high histological grade and negative status for hormone receptors were good predictors of breast cancer progression, as it could be expected based on previous epidemiological studies (Fragomeni *et al.* 2018). Treatment conducts also affected disease-free survival in the present cohort, with poorer outcomes being observed among patients who received neoadjuvant treatment, as compared to those who were initially treated with curative surgery. Such finding is not in agreement with more recent studies, which indicate therapeutic equivalence between neoadjuvant and adjuvant approaches in breast cancer

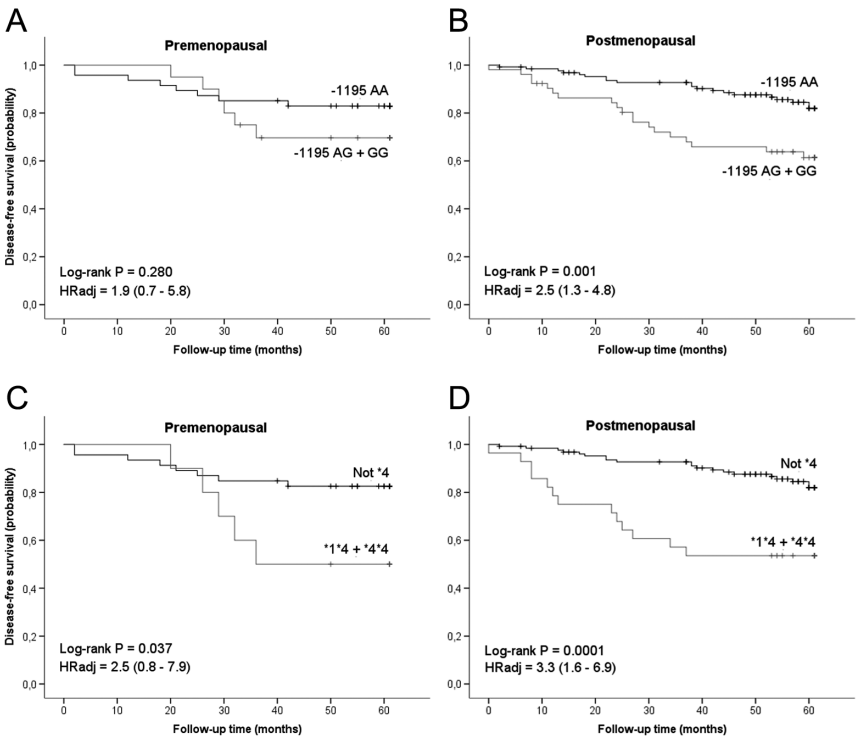

Figure 3
Disease-free survival curves in obese breast cancer patients according to menopausal status and to *PTGS2* polymorphism rs689466 (*–1195 A > G*) or haplotype *4. Premenopausal (A and C) and postmenopausal patients (B and D) were evaluated according to genotypes –1195 AG + GG vs AA (A and B) or according to diplotypes *1*4 + *4*4 vs other diplotypes not containing *4 (C and D). Multivariate Cox proportional hazards regression was used to calculate the hazard ratios (HR_{adj}), with adjustment for first therapeutic approach, tumor stage based on TNM and tumor subtype (A, B, C and D).

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Table 5 Logistic regression models for the five-year disease-free survival in postmenopausal obese breast cancer patients.

Variables	Coefficient	Default error	*P* value	Hazard ratio	95% confidence interval
Treatment	0.762	0.380	0.045	2.143	1.017–4.514
TNM	0.886	0.419	0.034	2.424	1.067–5.507
Tumor subtype	0.999	0.354	0.005	2.717	1.357–5.438
A-1195G	0.931	0.324	0.004	2.537	1.344–4.790
Treatment	0.972	0.429	0.023	2.644	1.141–6.130
TNM	0.832	0.452	0.066	2.298	0.947–5.575
Tumor subtype	0.706	0.387	0.068	2.027	0.950–4.325
(*1*4+*4*4) vs not *4	1.205	0.370	0.001	3.336	1.616–6.888

treatment (Apuri 2017) and reflects a selective indication of neoadjuvant chemotherapy for patients with locally advanced tumors. In fact, INCA's standard therapeutic conduct for breast cancer recommends to initiate treatment with curative surgery (either segmentectomy or mastectomy) if the tumor is clinically evaluated as resectable. Therefore, the worse disease-free survival associated with neoadjuvant treatment is actually due to worse tumor presentation at diagnosis. The distribution of *PTGS2* SNPs in the study cohort, regarding their allele frequencies and haplotypic pattern, was similar to those previously described for a different set of breast cancer patients from Brazil (Piranda *et al.* 2010) and was not affected by obesity status. The distribution of *PTGS2* SNPs according to histopathological features suggested an association between rs5275 variant genotypes (TC+CC) and positive lymph node status, which had not been detected in a previous study that included some patients of the present cohort (Festa-Vasconcellos *et al.* 2012). Nevertheless, such association was not confirmed when *PTGS2* haplotypes were evaluated. Unfortunately, there are no other reports in the literature exploring the association of these four *PTGS2* SNPs, or their haplotypes, with histopathological features of breast cancer.

The prognostic evaluation of *PTGS2* SNPs or their haplotypes on breast cancer outcomes indicated that the variant rs689466 genotypes, as well as the rs689466-containing haplotype *4, were significantly associated with shorter disease-free survival. Such deleterious effect of rs689466 on prognosis, however, was valid only for obese patients, suggesting that its action may depend on the availability of stimulating factors, probably released within the obesity-related inflammatory process. Accordingly, obesity has been associated with increased circulating levels of several growth factors, cytokines and adipokines, including interleukin-6, an inflammatory

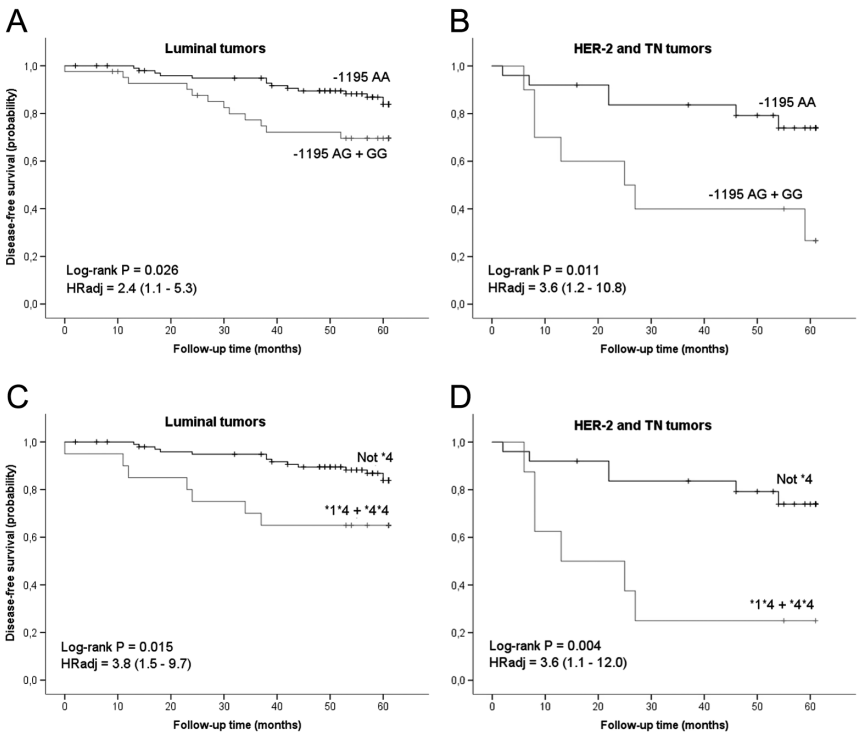

Figure 4
Disease-free survival curves in postmenopausal obese breast cancer patients according to tumor subtype and *PTGS2* polymorphism rs689466 (*–1195 A>G*) or haplotype *4. Luminal tumors (A and C) or HER2 and triple-negative tumors (B and D) were evaluated according to genotypes *–1195 AG+GG* vs *AA* (A and B) or according to diplotypes *1*4+*4*4 vs other diplotypes not containing *4 (C and D). Multivariate Cox proportional hazards regression was used to calculate the hazard ratios (HR_{adj}), with adjustment for the first therapeutic approach (A, B, C and D).

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cytokine secreted by both immune cells and adipocytes, which have been shown to promote PGE2 production via its effects on COX2, resulting in elevated aromatase levels and estrogen production in the breast tissue (Bowers *et al.* 2015). Moreover, the negative impacts of rs689466 variant genotypes or haplotype *4 on disease-free survival of breast cancer patients were dependent on the menopausal status, being detected only in postmenopausal women. Such findings appear to reinforce the role of local aromatase induction and the consequent estradiol production by breast adipose tissue after menopause, which can be induced by elevated COX2 and PGE2 in inflamed breast tissue of obese women (Bowers & deGraffenried 2015).

Interestingly, the deleterious effect of rs689466 variant genotypes or haplotype *4 on prognosis was observed irrespective of tumor subtype. Although luminal tumors have lower progression rates, they are considered more likely to have its prognosis affected by obesity in postmenopausal women, possibly because they express ER and might be more sensitive to increased aromatase expression and local estradiol release (Bowers & deGraffenried 2015). Nevertheless, triple-negative tumors have been shown to express high levels of COX2, which correlate with poor survival outcomes (Tian *et al.* 2017). Therefore, the negative impact of COX2 enzymatic activity in triple-negative tumors appears to be independent from the aromatase–estradiol axis and might be related to stimulation of self-renewal of breast cancer stem cells, as proposed by Tian *et al.* (2017).

Regarding the potential functional effect of *PTGS2* SNP rs689466 on gene transcription, three independent studies used gene-reporter assays to compare the luciferase activity driven by constructs enclosing promoter variants. Zhang *et al.* (2005), using HeLa cells, showed a 5–6-fold increase in the luciferase activity of constructs containing rs689466 *A* as compared to those with the *G* variant. This finding, however, was not corroborated in two subsequent studies. Thus, Sakaki *et al.* (2010), who also used HeLa cells, and Pereira *et al.* (2014), who used two colon cancer cell lines (HCA-7 and HCT-116), indicated higher transcriptional activity associated with the rs689466 *G* variant. Taken together, these results from *in vitro* approaches suggest that the variant genotypes of rs689466 may favor COX2 production and PGE2 synthesis in tumor microenvironment, which might contribute to increase the risk of disease progression. Unfortunately, there are no available data of *in vivo* or *ex vivo* studies to corroborate this hypothesis.

The substitution of *–1195 A* by *G* (rs689466) apparently eliminates a MYB-binding site (Zhang *et al.* 2005, Agundez *et al.* 2014) and creates an E-box motif (Pereira *et al.* 2014), which recognizes the upstream stimulatory factor (USF), a ubiquitous transcription factor involved on embryonic development, fertility, stress, growth and lipid and carbohydrate metabolisms (Horbach *et al.* 2015). There are two isoforms of USF: USF1 and USF2, the two proteins apparently occurring *in vivo* mainly as USF1/USF2 heterodimers (Viollet *et al.* 1996). USF1 was initially reported as part of the general cellular transcription machinery, but later recognized as a regulator of lipid and glucose metabolism, being linked with familial combined hyperlipidemia (Shi *et al.* 2008). Genetic variants of USF1 have also been associated with obesity (Choquette *et al.* 2007). Recently, Laurila *et al.* (2016) showed that knocking out the orthologous *USF1* gene (*Usf1*$^{-/-}$) favors lower weight gain and reduced adiposity in mice, either with regular or high-fat diet. Regarding USF2, it has been shown to induce elevated aromatase expression in ectopic endometrium from endometriosis women (Castro *et al.* 2015).

We hypothesize that obesity, either via its metabolic or inflammatory profile, segregates with elevated USF1 and/or USF2, which stimulate *PTGS2* gene transcription in individuals with the rs689466 *G* variant, thereby leading to increased COX2 production and PGE2 synthesis in tumor cells microenvironment, ultimately favoring disease progression. Such mechanistic hypothesis, however, still needs experimental validation.

In conclusion, the present results suggest a potential contribution of *PTGS2* genotyping for additional prognostic evaluation of breast cancer outcomes, especially among obese patients. Such approach, however, also needs to be validated in multi-institutional cohorts with longer clinical follow-ups. Because the present cohort is quite recent, since the study began in 2009, the total follow-up time is yet limited. In view of this time restraint, overall survival was not evaluated. Also, even for the analysis of disease-free survival, the five-year follow-up is still quite short for patients with early stage tumors, who have lower relapse rates, or for those with luminal breast cancer, who have a natural history of late relapses. It is possible that this relatively short time-frame for survival analyses may have affected the results concerning rs689466 in luminal vs HER2 or triple-negative tumors or compromised the detection of significant effects for the other *PTGS2* SNPs. Unfortunately, until the present time, we could not find sources of information on breast cancer cohorts with publically available data on *PTGS2* genotypes, clinical parameters including BMI and clinical follow-up, with survival analyses.

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Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement
D R F-A recruited patients, collected clinical and histopathological data, characterized genotypes and haplotypes, performed statistical analyses, generated tables and figures and drafted the manuscript. H A V-M recruited patients, collected clinical and histopathological data and helped with statistical analyses. D N P set the genotyping assays. M S L and T S L S recruited patients and collected clinical and histopathological data. A B conceived the epidemiological design. S S V and J A P contributed to data analysis and interpretation. R V J designed and coordinated the study, analyzed the data, wrote and revised the manuscript. All authors read and approved the final manuscript.

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