

Loss of constitutive *ABCB1* expression in breast cancer associated with worse prognosis

João Marcos de Azevedo Delou¹⁻³
 Giselle Maria Vignal⁴
 Vanessa Índio-do-Brasil^{2,5}
 Maria Theresa de Souza Accioly⁴
 Taiana Sousa Lopes da Silva²
 Diogo Nascimento Piranda^{2,3}
 Marcelo Sobral-Leite^{2,6}
 Marcelo Alex de Carvalho^{2,7}
 Márcia Alves Marques Capella^{1,8}
 Rosane Vianna-Jorge^{2,3,5}

¹Programa de Bioquímica e Biologia Celular, Instituto de Bioquímica Médica, Universidade Federal do Rio de Janeiro, ²Programa de Farmacologia, Coordenação de Pesquisa, Instituto Nacional de Câncer, ³Instituto de Ciências Biomédicas, Universidade Federal do Rio de Janeiro, ⁴Divisão de Patologia, Instituto Nacional de Câncer, ⁵Escola Nacional de Saúde Pública – FIOCRUZ, Rio de Janeiro, Brazil; ⁶Department of Molecular Pathology, Netherlands Cancer Institute, Amsterdam, The Netherlands; ⁷Instituto Federal do Rio de Janeiro, ⁸Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil

Correspondence: Rosane Vianna-Jorge
 Programa de Farmacologia, Coordenação de Pesquisa – Instituto Nacional de Câncer Rua André Cavalcanti, 37, 3º andar
 CEP: 20231-050, Rio de Janeiro, Brazil
 Tel +55 21 3207 6526
 Email farmaco@inca.gov.br

Abstract: *ABCB1* gene encodes an adenosine 5'-triphosphate-binding cassette transporter, which not only confers multidrug resistance phenotype in malignant cells, but is also present in several nonmalignant tissues. For the last thirty years, *ABCB1* expression in breast cancer has been described by many authors, but the extent of expression differs among the studies, and there is no consensus regarding its potential role in carcinogenesis or in the tumor response to antineoplastic drugs. This study aimed to characterize the expression of *ABCB1* in breast tumors as a function of genetic, clinical, and histopathological variables. The *ABCB1* expression was also evaluated in nonmalignant mammary tissues adjacent to tumors and in benign lesions. The detection of ABCB1 protein was performed by immunohistochemistry in tissue specimens of excised breasts obtained from a prospective cohort of Brazilian women with breast cancer. The association of ABCB1 protein levels with *ABCB1* mRNA, gene polymorphisms, and clinical and histopathological variables was also evaluated. The Kaplan–Meier curves and multivariate Cox regression analyses were conducted to identify independent predictors of disease-free survival of patients with breast cancer. ABCB1 was detected in 86.3% (656) of breast tumors, 98.8% (606) of nonmalignant mammary tissue adjacent to tumors, and 100% (28) of benign lesions. Reduced ABCB1 protein levels in breast tumors was associated with triple-negative subtype (adjusted odds ratio [OR_{adj}]=0.24; 95% confidence interval [CI]=0.13–0.45), lymph node status < pN2 (OR_{adj}=0.27; 95% CI=0.10–0.71), tumor size >2 cm (OR_{adj}=0.55; 95% CI=0.32–0.93), and hypertensive status (OR_{adj}=0.42; 95% CI=0.24–0.73), and it was significantly associated with shorter disease-free survival, either for all breast cancer patients (*p* log-rank =0.012; hazard ratio [HR]=3.46; 95% CI=1.21–9.91) or for those with triple-negative tumors (*p* log-rank =0.007; HR=11.41; 95% CI=1.29–100.67). The loss of constitutive *ABCB1* expression in breast cancer, especially in triple-negative tumors, seems to indicate a subgroup of worse prognosis.

Keywords: multidrug resistance, single-nucleotide polymorphisms, immunohistochemistry, disease-free survival, triple-negative breast cancer, hypertension

Introduction

Efflux of cytotoxic drugs by the adenosine 5'-triphosphate-binding cassette transporter subfamily B member 1 (ABCB1 aka multidrug resistance protein 1 [MDR1]/P-glycoprotein) is considered a potential mechanism of acquired chemoresistance to antineoplastics.¹ However, there is a controversy regarding the role of *ABCB1* expression in breast cancer.^{2,3} Literature discrepancies may involve the lack of standardized methods for the detection and quantification of ABCB1 in solid tumors.⁴ For instance, there is a long-lasting notion of uncertainty regarding ABCB1 detection due to the lack of sensitivity and/or specificity of several commercial ABCB1 antibodies.^{2,5,6} In addition, there seems to be great interpatient variability.³ One possible reason for such

variability is that the *ABCB1* gene is polymorphic, and two of its single-nucleotide polymorphisms (SNPs), rs1128503 and rs1045642, may modify the final protein conformation, compromising its membrane stability and substrate recognition.^{7,8} Finally, it has been reported that the *ABCB1* expression may also be modulated by plasma aldosterone⁹ or cortisol,¹⁰ as well as by dietary salt and dehydration.⁹

In this study, a systematic evaluation of *ABCB1* expression in the breast, encompassing benign lesions, breast tumors examined prior to any chemotherapeutic treatment and non-malignant mammary tissues adjacent to breast tumors, was done. The study was conducted in a prospective manner by using the tissue samples from a cohort of 712 Brazilian women who underwent curative mammary surgery. *ABCB1* expression was estimated by immunohistochemistry with three previously validated antibodies, and its association with clinical and histopathological variables, including the genetic profile regarding *ABCB1* SNP, was evaluated. Finally, the impact of *ABCB1* expression on short-time (2-year) disease-free survival of patients with breast cancer was also investigated.

Methods

Subjects and study design

The study population was a prospective cohort of Brazilian women who were admitted from January 2009 to December 2012 at Instituto Nacional de Câncer (INCA) for mammary surgery. The exclusion criteria were the following:

any previous oncological treatment, prior contralateral or bilateral synchronous breast cancer, and systemic metastasis at diagnosis. Figure 1 illustrates the flowchart of the study design, depicting the reasons for exclusion and the sample availability for each analysis.

The study protocol (approved by the Ethics Committee of INCA #129/08) did not interfere with the routine clinical follow-up or therapeutic choice. All the patients provided written informed consent to be enrolled in the present study. The REMARK guidelines for the characterization of biomarkers¹¹ and the international precepts of ethics in research and of good clinical practice were followed.

Clinical and histopathological characterization

A description of this study cohort has been published previously.¹² All the patients were interviewed to provide information on their clinical history and lifestyle habits. The variables considered for clinical history were age at diagnosis, menopausal status, and comorbidities, which were defined as any preexisting chronic condition under medical treatment, with the exception of obesity, which was defined based on the body mass index. Hypertension was defined according to disease severity, as inferred by the prescribed antihypertensive therapy.¹³ The lifestyle variables were alcohol drinking, defined according to the frequency of consumption, and smoking, classified as current, previous,

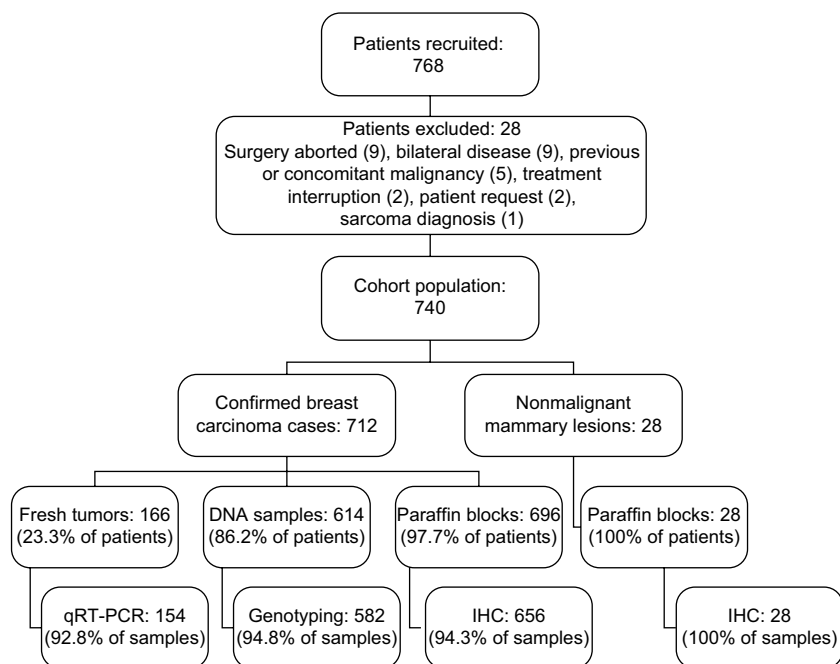


Figure 1 Flowchart of the study cohort.

Abbreviations: qRT-PCR, quantitative real-time polymerase chain reaction; IHC, immunohistochemistry.

or no habit, considering a minimum consumption of five packs (100 cigarettes).

Histopathological characterization of breast tumors was based on the 3rd edition of the World Health Organization Classification of Tumors¹⁴ and on the Elston–Ellis histological grading system.¹⁵ Data on hormone receptors and human epidermal growth factor receptor 2 (HER-2) status of breast tumors, according to immunohistochemical and fluorescence in situ hybridization analyses, were used for the surrogate classification of tumor subtypes.¹⁶

Immunohistochemistry, antibody validation, and scoring

All paraffin-embedded blocks were stained with hematoxylin and eosin to select the most representative specimen for each patient, which was processed as described previously.¹⁷ ABCB1 detection was tested with three antihuman ABCB1 monoclonal antibodies, namely sc-13131, clone G-1 (Santa Cruz Biotechnology Inc, Dallas, TX, USA.); ab3083, clone 265/F4 (Abcam, Cambridge, UK); and NCL-PGLYm, clone 5B12 (Leica Biosystems Newcastle Ltd., Newcastle, UK), and then revealed with Novolink Polymer Detection System standard protocol (Leica Biosystems Newcastle Ltd.). The most sensitive antibody among these three was sc-13131, clone G-1 (dilution 1:10,000).

Although the G-1 specificity toward ABCB1 has already been shown,^{18–21} validation experiments were conducted in this study, as recommended by the consensus for ABCB1 detection,^{2,5} using specimens from different nonmalignant human tissues. Liver, adrenal, and kidney proximal and distal tubules were used as positive controls,^{22–24} whereas tonsils, epididymis, and kidney glomeruli were used as negative controls.^{23–25} Because ABCB1 was expected to be detected in the biliary canaliculi,²³ liver samples were also stained with anti-CD10 antibody (clone 56C6; Leica Biosystems Newcastle Ltd.; diluted 1:200). Liver samples were positive for ABCB1 (Figure 2A and B), with a distribution pattern similar to that observed with the anti-CD10 antibody (Figure 2C and D). The cross-reaction against ABCB4/MDR3 was investigated using human tonsils and kidney. Tonsils and kidney glomeruli showed no immunostaining (Figure 2E–G [arrows]), indicating the absence of G-1 antibody cross-reaction toward ABCB4 (formerly known as MDR3).^{26,27} Proximal and distal kidney tubules, which express ABCB1,²⁴ showed positive staining (Figure 2G and H), whereas kidney glomeruli (arrows in Figure 2G), which express only ABCB4,²⁷ were negative (Figure 2G and H), confirming the absence of cross-reactivity against ABCB4/MDR3.

Because of the observed intratumoral variability in ABCB1 staining in breast tumors (Figure 2I and J), the individual quantification included the whole area of a representative tumor slide and was based on the two previously published scoring methods: a continuous scale (CS)²⁸ and a categorical score,²⁹ both of which consider the percentage of immunostained cells and the intensity of the reaction.

The CS was calculated as follows: $CS = (\%_{\text{weak}} \times 1) + (\%_{\text{moderate}} \times 2) + (\%_{\text{strong}} \times 3)$.²⁸ The intensity of ABCB1 staining was rated as follows: negative (complete absence of cellular reaction), weak (diffuse and mild reaction in cytoplasm, with no detectable reaction in cell membranes), moderate (detectable reaction in both cytoplasm and plasma membrane), or strong (strong in both cytoplasm and plasma membrane). Nuclear reaction was not considered.

The categorical score, or immunoreaction score (IRS) was defined by the following equation: $IRS = (\text{positivity score}) \times (\text{intensity score})$.²⁹ The positivity score was attributed 1 to 4, according to the percentage of positive cancer cells: 1 (1%–9%), 2 (10%–49%), 3 (50%–79%), or 4 (80%–100%). The intensity score ranged 0–3: negative (0), weak (1), moderate (2), or strong (3). Breast tissues were considered positive for ABCB1 when the IRS was ≥ 4 , meaning that at least 10% of the cells presented moderate staining.²⁹

All the slides were blindly evaluated by a trained PhD student (JMAD) and a pathologist (GMV).

Genotyping analyses

Genomic DNA was extracted from peripheral blood samples (3 mL) by using the Blood Genomic Prep Mini Spin Kit (GE Healthcare, Buckinghamshire, UK). Genotyping analyses were conducted by using TaqMan real-time polymerase chain reaction (RT-PCR) assays in an ABI PRISM 7500 Sequence Detector System (Applied Biosystems, Foster City, CA, USA). Each reaction contained 1 μL of genomic DNA (20 ng/ μL); 0.5 μL of probe, either C_7586662_10 for rs1128503 or C_7586657_20 for rs1045642 (Applied Biosystems); 5 μL of Genotyping Master Mix (Applied Biosystems); and 3.5 μL of water. All the experiments were carried out in 96-well plates, including a nontemplate control and at least two positive controls.

Quantification of ABCB1 mRNA

Fresh specimens of excised breast tumors were dissected by clinical pathologists, frozen in liquid N₂, and stored at Banco Nacional de Tumores (BNT/INCA). Frozen sections of breast tumors were used for RNA isolation by using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA). The

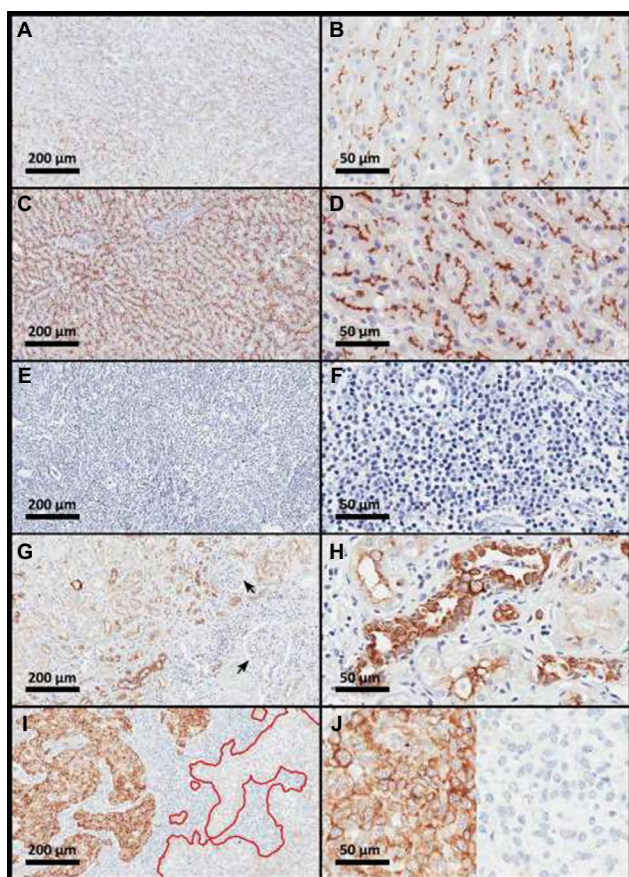


Figure 2 Immunohistochemical evaluation of ABCB1 in human tissues.

Notes: The right column indicates 100× magnification, and the left column indicates 400× magnification. (A and B) Immunostaining of ABCB1 in the liver; (C and D) immunostaining pattern control of a specific marker for biliary canalculi (CD10) in the liver; (E and F) negative controls of ABCB1 in tonsils; (G and H) controls of ABCB1 in the kidney – proximal (strong reaction) and distal (weak reaction) tubules are positive controls, and glomeruli (black arrows) are negative controls; (I and J) immunostaining of ABCB1 in a breast cancer sample, showing regions with positive (brown) and negative reactions (circumvented by a red line), as they were seen in the slide (I) or side-by-side for better visualization (J). All the sections were counterstained with hematoxylin (blue staining).

Abbreviation: ABCB1, adenosine 5'-triphosphate-binding cassette subfamily B member 1.

RNA samples were stored in RNase-free distilled water at -80°C , and the cDNA was synthesized by using 2 μg of RNA, with High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems), according to the manufacturer's instructions.

The relative quantification (RQ) of *ABCB1* transcripts was performed by using by using TaqMan quantitative real-time PCR (qRT-PCR) assays, in an ABI PRISM 7500 Sequence Detector System (Applied Biosystems). Each reaction contained cDNA templates (40 ng), 10 μL of reaction mix containing 5 μL TaqMan Gene Expression Master Mix, and TaqMan probes (Applied Biosystems), which were as follows: *ABCB1* Hs01067802_m1* (with FAMTM), peptidylprolyl isomerase A (*PPIA*) Hs00216455_m1 (with VIC[®]), used as reference gene. The thermal cycling

conditions comprised an initial denaturation step at 95°C for 10 minutes, followed by 40 cycles of 95°C denaturation for 15 seconds, and annealing at 60°C for 1 minute. The experiments were carried out in 96-well plates, including a nontemplate control and a reference sample, consisting of cDNA obtained from a commercial human mammary gland (HMG) total RNA (Clontech Laboratories, Mountain View, CA, USA). The RQ of *ABCB1* mRNA was calculated as follows: $\text{RQ} = 2^{-\Delta\Delta\text{Ct}}$, where $\Delta\Delta\text{Ct} = \Delta\text{Ct}_{\text{ABCB1}} - \Delta\text{Ct}_{\text{HMG}}$, with $\Delta\text{Ct}_{\text{ABCB1}} = \text{Ct}_{\text{ABCB1}} - \text{Ct}_{\text{PPIA}}$ and $\Delta\text{Ct}_{\text{HMG}} = \text{Ct}_{\text{HMG}} - \text{Ct}_{\text{PPIA}}$. All the data were generated in triplicates and expressed as median with their respective 95% confidence intervals (CI).

Characterization of outcomes in patients with breast cancer

Breast cancer progression was characterized by locoregional or contralateral recurrence or distant metastasis. New primary cancer lesions or deaths unrelated to breast cancer progression were censored. The patients were considered disease-free if they had no imaging diagnosis of disease progression or suggestive clinical symptoms up till their last medical consult. Because this is an ongoing cohort, the survival analysis was limited to 2 years of follow-up, which was available for all the patients.

Statistical analyses

A descriptive study was conducted. Clinical and histopathological variables were categorized and expressed as number and relative frequencies. The association between IRS and *ABCB1* genotypes and clinical or histopathological variables was evaluated by using the χ^2 test. Individual variables were tested for linear-by-linear associations, with calculation of trend significances ($p_{\text{trend}} < 0.05$) and categorized for better or worse prognosis, with the calculation of the odds ratios (ORs) and respective 95% CI. These variables were also compared for the continuous scale by using Mann–Whitney *U* test or Kruskal–Wallis test. Wald χ^2 test was used to identify independent predictors ($p < 0.05$), which were used for the calculation of the corresponding adjusted ORs (OR_{adj}). The final regression model was tested with the Hosmer–Lemeshow test. All the statistical analyses were conducted by using IBM SPSS Version 20 for Windows (IBM Corp., Armonk, NY, USA) or GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA, USA).

Disease-free survival curves were estimated by using the Kaplan–Meier product-limit method, and the impact of the individual variables was estimated by using log-rank analysis ($p < 0.05$). The significant covariates were included in multivariate Cox proportional hazards regression with the

calculation of adjusted hazard ratios (HR_{adj}) and respective 95% CIs.

Survival analyses were also performed by using *ABCB1* expression data obtained from publically available gene expression array databases. Two web platforms were evaluated: ProgGeneV2.0,³⁰ which comprises several independent breast cancer cohorts (only cohorts with at least 200 breast cancer patients were selected), and the Gene Expression Omnibus deposited data (<https://www.ncbi.nlm.nih.gov/geo/>), which was assessed via the online software KM plotter (www.kmplot.com),³¹ by using the filter for breast cancer and information regarding *ABCB1*. In both the platforms, the median value of *ABCB1* mRNA was used as a cutoff value to categorize *ABCB1* tumoral expression as “low” or “high”.

Results

Characterization of patients with breast cancer

Table 1 presents the main clinical and histopathological characteristics of the breast cancer patients ($n=712$) and the allelic and genotypic distribution of rs1128503 (*ABCB1* C1236T) and rs1045642 (*ABCB1* C3435T).

After surgery, most patients received adjuvant protocols, including chemotherapy (61.4%), hormonal therapy (12.9%), hormonal therapy plus radiotherapy (10.8%), or radiotherapy alone (5.8%). The remaining 9.1% of the patients were clinically followed up with no secondary intervention. The main protocol for adjuvant chemotherapy, comprising 89% of cases, was three cycles of cyclophosphamide, doxorubicin, and 5-fluorouracil, followed by three cycles of docetaxel.^{32,33}

Immunohistochemical characterization of ABCB1 in breast cancer

Figure 3 shows *ABCB1* immunostaining in the breast, including breast tumors (Figure 3A–H) and normal mammary tissues from women without breast cancer (Figure 3I and J). Figure 3A and B illustrates fully negative reactions, whereas Figure 3C–H shows a gradation of the immunostaining, which was graded as weak (Figure 3C and D), moderate (Figure 3E and F), or strong (Figure 3G and H). Nonmalignant mammary tissue adjacent to the tumor area ($n=606$) showed positive reaction for *ABCB1* in mammary ducts and acini (98.8% of cases), even when tumor staining was negative (Figure 3A and B). The absence of *ABCB1* in both the cancer cells and adjacent non-malignant breast tissue occurred in only five patients (0.8% of all tumor samples). All the cases

of benign lesions ($n=28$) were homogeneously positive for *ABCB1*, with strong immunostaining in all mammary ducts and acini.

Figure 4 shows the distribution of *ABCB1* immunostaining scores in the cohort. Figure 4A presents a bar graph of the IRSs, whereas Figure 4B is a histogram of the CS. According to the IRSs, 86.3% of breast tumors were positive for *ABCB1* ($IRS \geq 4$), and 71% presented high immunostaining scores ($IRS \geq 8$). The histogram of the CS does not fit into a normal distribution ($p < 0.001$; D’Agostino–Pearson normality test), and 71% of tumors had high CS values (≥ 150).

Association between ABCB1 mRNA and ABCB1 protein levels in breast tumors

Figure 5 depicts the association between *ABCB1* mRNA and *ABCB1* protein levels in breast tumors. The stratification of tumors according to their IRSs, as positive or negative for *ABCB1*, indicates a significant difference in the levels of *ABCB1* mRNA (Figure 5A), which were, on average, ~2 times higher among *ABCB1* positive tumors. However, the analysis of individual data on *ABCB1* mRNA and on *ABCB1* protein levels (evaluated as CS) indicates no linear correlation (Figure 5B) and points to a large variability in the distribution of *ABCB1* mRNA in breast tumors. In order to explore the possible causes of such variability, the influence of *ABCB1* SNPs was investigated. Figure 5C indicates a significant decrease in *ABCB1* mRNA levels among carriers of the variant homozygous *ABCB1* 1236TT (rs1128503), as compared to the other two groups of genotypes. The SNP rs1045642, however, showed no influence on the amounts of *ABCB1* mRNA in breast tumors (Figure 5D).

Influence of clinical and histopathological parameters on ABCB1 expression in breast tumors

Patients with positive or negative *ABCB1* immunostaining in breast tumors were compared with regard to clinical, genetic, and histopathological variables. The conditions significantly associated with negative *ABCB1* were hypertensive status, tumor size >2 cm, low lymph node status (pN0 or pN1), early tumor stage (I or II), negative estrogen receptor (ER) status, negative progesterone receptor (PR) status, and triple-negative subtype (Table 2).

According to the final multivariate model (Table 3), hypertensive status, large tumor size, low lymph node status, and triple-negative subtype were independently associated with *ABCB1* negativity.

Table 1 Description of individual features in breast cancer patients

Clinical features	n	%	Histopathological features	n	%
Age at diagnosis			Histological type		
24–34 years	15	2.1	Ductal invasive	597	83.9
35–44 years	82	11.5	Lobular invasive	40	5.6
45–54 years	181	25.4	Ductal in situ	45	6.3
55–64 years	208	29.2	Lobular in situ	3	0.4
≥65 years	226	31.7	Others	27	3.8
Color			Missing	1	
White	320	45.7	Tumor size (pT)		
Brown	268	38.3	pTis (in situ)	48	6.7
Black	88	12.6	pT1 (≤2 cm)	321	45.1
Others	24	3.4	pT2 (2–5 cm)	309	43.4
Missing	12		pT3 (>5 cm)	21	2.9
Menopausal status			Missing	13	
Premenopausal	179	25.6	Lymph node status (pN)		
Postmenopausal	521	74.4	pN0 (none)	421	59.6
Missing	12		pN1 (≤3 positive nodes)	177	25.1
Smoking status			pN2 (4–9 positive nodes)	66	9.3
Nonsmoker	464	66.2	pN3 (≥10 positive nodes)	42	5.9
Former smoker	173	24.7	Missing	6	
Current smoker	64	9.1	Tumor grade (G)		
Missing	11		G1	83	12.7
Alcohol consumption			G2	261	39.9
Never	384	56.0	G3	310	47.4
Rare (<3 days/week)	212	30.9	Missing	58	
Frequent (>3 days/week)	90	13.1	Stage grouping		
Missing	26		0	46	6.6
Comorbidities			I	227	32.5
None	91	12.8	II	312	44.7
Hypertension	377	53.3	III	113	16.2
Obesity	159	28.7	Missing	14	
Diabetes mellitus	104	14.9	ER status		
BMI (Kg/m ²)			Negative	111	16.2
<18.4	6	1.0	Positive	575	83.8
18.5–24.9	164	28.4	Missing	26	
25–29.9	248	43.0	PR status		
>30.0	159	27.6	Negative	197	28.8
Missing	135		Positive	488	71.2
ABCB1 genotypes			Missing	27	
rs1128503 (C1236T)			HER-2 status		
CC	222	39.7	Negative	515	81.1
CT	269	48.1	Positive	103	16.2
TT	68	12.2	Undetermined	17	2.7
rs1045642 (C3435T)			Missing	77	
CC	227	39.3	Tumor subtype		
CT	257	44.5	Luminal A	395	63.2
TT	94	16.3	Luminal B	130	20.8
			HER-2-like	38	6.1
			Triple-negative	62	9.9
			Missing	87	

Notes: n=712. Data are expressed as the number and percentage of patients in each category.

Abbreviations: ABCB1, adenosine 5'-triphosphate-binding cassette subfamily B member 1; BMI, body mass index; ER, estrogen receptor; HER-2, human epidermal growth factor receptor 2; PR, progesterone receptor.

Because all hypertensive patients were under pharmacological treatment, the effect of antihypertensive drugs on ABCB1 immunostaining was investigated (Table 4). No significant associations were found for individual drugs or their pharmacological groups. However, ABCB1 positivity was lower in more severe cases ($p_{\text{trend}} < 0.001$).

Influence of decreased ABCB1 expression on disease-free survival of patients with breast cancer

Figure 6 shows the influence of ABCB1 expression on the 2-year disease-free survival of patients with breast cancer.

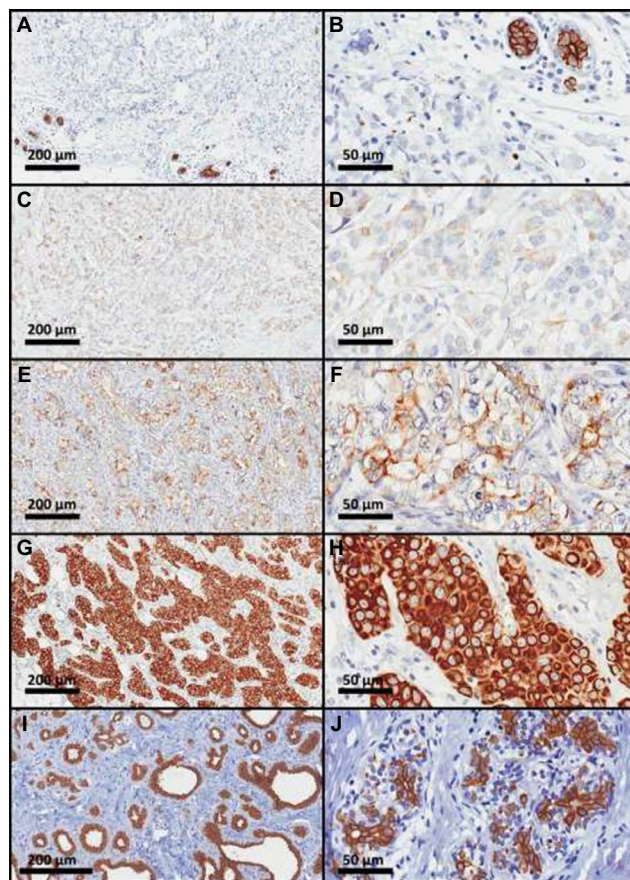


Figure 3 Photomicrographs of ABCBI immunostaining in human mammary tissue samples showing different immunoreaction intensities.

Notes: (A–H) Breast cancer samples: (A and B) negative; (C and D) weak; (E and F) moderate; (G and H) strong immunostaining; (I and J) nonmalignant mammary tissues with strong immunostaining.

Abbreviation: ABCBI, adenosine 5'-triphosphate-binding cassette subfamily B member 1.

The results indicated that the loss of *ABCBI* expression ($IRS \leq 4$) favors early-onset breast cancer progression when evaluated for all the patients in the cohort (Figure 6A) or for patients with triple-negative tumors (Figure 6B). The impact of the loss of *ABCBI* expression on the rates of breast cancer progression was maintained after adjustment for other prognostic factors, that is, *HER-2*-like or triple-negative subtypes, staging group $\geq II$, histological grade 3, and the use of chemotherapy (HR =3.5; 95% CI =1.2–9.9) when all the tumors were considered; or staging group $\geq II$ and histological grade 3 for triple-negative tumors (HR =11.4; 95% CI =1.3–100.7).

The influence of *ABCBI* expression on the 2-year disease-free survival of patients with breast cancer was also evaluated, considering *ABCBI* mRNA levels in tumors that could be fresh-frozen at the time of the excision surgery ($n=154$). A significant difference was detected (p log-rank =0.012), with the patients whose tumors had low *ABCBI* mRNA presenting worse cumulative survival than the patients with high *ABCBI* mRNA (Figure 6C). However, because of the lack of events in the group with high mRNA, the calculation of the HR was compromised. Therefore, the survival analysis based on *ABCBI* mRNA was extended to publically available databases from breast cancer cohorts (Tables 5 and 6). The results obtained with these cohorts confirmed that low *ABCBI* mRNA is associated with worse survival outcomes (both disease-free survival and overall survival), either when considered for all the patients with adjustment for ER positivity or when evaluated only among ER negative subtype or among the basal subtype (Table 6).

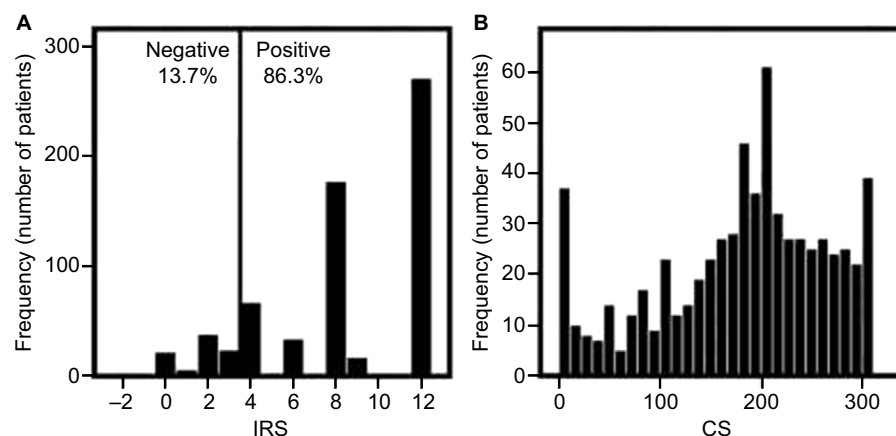


Figure 4 Distribution of ABCBI immunoreaction scores in human breast tumors.

Notes: (A) immunoreaction score (IRS); (B) continuous scale (CS).

Abbreviation: ABCBI, adenosine 5'-triphosphate-binding cassette subfamily B member 1.

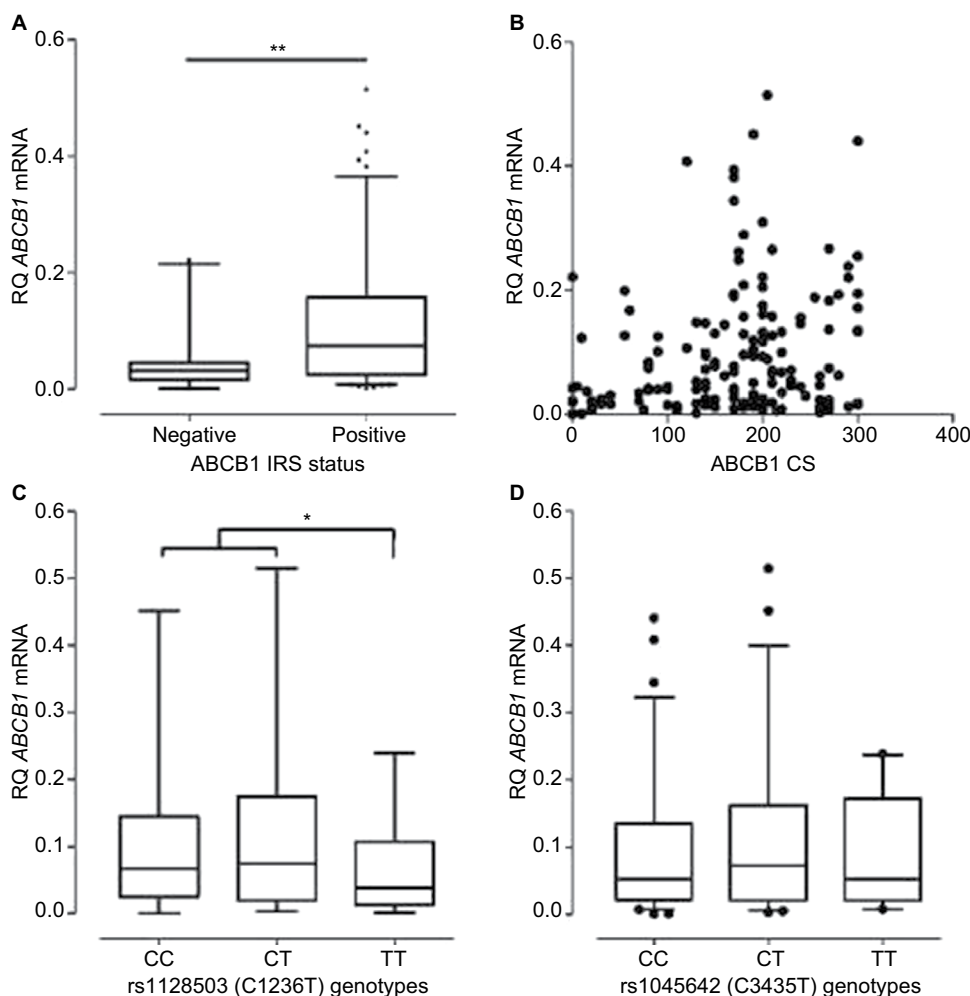


Figure 5 Distribution of *ABCB1* mRNA levels in human breast tumors.

Notes: *ABCB1* mRNA levels are shown according to the *ABCB1* IRS (**A**); *ABCB1* CS (**B**); rs1128503 genotypes (**C**); and rs1045642 genotypes (**D**). * $p < 0.05$; ** $p < 0.01$. Both in Mann-Whitney test.

Abbreviations: *ABCB1*, adenosine 5'-triphosphate-binding cassette subfamily B member 1; CS, continuous scale; IRS, immunoreaction score; RQ, relative quantification.

Finally, the influence of *ABCB1* SNPs on the 2-year disease-free survival was also evaluated, but no significant association with early-onset breast cancer progression was detected either for rs1128503 (p log-rank = 0.57) or for rs1045642 (p log-rank = 0.55).

Discussion

The present work characterized the *ABCB1* expression in breast cancer in comparison with nonmalignant breast tissue. All methodological recommendations for the detection of *ABCB1* by immunohistochemistry^{2,4} were followed. First, three different antibody clones (ie, G-1, 5B12, 265/F4) were tested. The most sensitive clone was G-1, whose specificity towards *ABCB1* was confirmed (Figure 2). Second, breast tumors were processed immediately after resection, by using buffered formalin. Third, a polymer-based kit was used to improve antibody sensitivity.⁴² Fourth, two different scoring methods^{28,29} were used. Finally, *ABCB1* mRNA

was quantified in freshly frozen tumors, testing a second parameter of *ABCB1* expression. In addition, the study was conducted in a prospective manner, by using all the available samples from a relatively large cohort of patients (656 tissue samples from 712 patients). For comparison, a literature review on *ABCB1* detection by immunohistochemistry in breast cancer retrieved only nine studies with ≥ 100 patients.^{17,29,43–49}

The results on *ABCB1* expression in breast tumors indicated high interindividual variability, considering both the percentage of immunostained cells and the intensity of the immunostaining reaction. A meta-analysis conducted by Trock et al,² comprising 31 studies, also indicated a high heterogeneity in *ABCB1* positivity, with a weighted average value of 48.5% (95% CI = 42–55.0), and a trend for higher values in more recent studies. For example, in two recent retrospective studies, the reported *ABCB1* detection was 42% in 104 patients²⁹ or 66% in 177 patients.⁴⁸

Table 2 Significant associations between individual features and ABCBI expression in breast tumors

Individual features	IRS status		CS		pM-W
	OR	95% CI	Median	95% CI	
Hypertension					
No	1		200	160–250	
Yes	0.51	0.32–0.82	180	103–239	<0.001
Tumor size (pT)					
pT1	1		200	135–269	
pT2 + pT3	0.59	0.37–0.95	185	155–250	0.003
Lymph node status (pN)					
pN0 + pN1	1		190	123–294	
pN2 + pN3	3.64	1.44–9.19	202.5	120–240	0.001
Stage grouping					
I + II	1		190	120–240	
III	3.15	1.34–7.43	210	170–270	<0.001
ER status					
Positive	1		195	145–250	
Negative	0.38	0.23–0.65	160	70–220	<0.001
PR status					
Positive	1		200	150–250	
Negative	0.47	0.30–0.76	177.5	90–220	<0.001
Tumor subtype					
Luminal A	1		195	150–250	
Luminal B	0.86	0.46–1.62	190	143–245	
HER-2-like	0.82	0.27–2.47	195	140–238	
Triple-negative	0.21	0.11–0.39	125	20–193	<0.001^a

Notes: n=656. Data are expressed as the OR, based on the IRS status, or as the median value of the CS, for each category, with the respective 95% CIs. Statistically significant differences are presented in bold characters. ^ap-value of Kruskal–Wallis test.

Abbreviations: ABCBI, adenosine 5'-triphosphate-binding cassette subfamily B member 1; CI, confidence interval; CS, continuous scale; ER, estrogen receptor; IRS, immunoreaction score; HER-2, human epidermal growth factor receptor 2; OR, odds ratio; pM-W, p-value for the Mann–Whitney test; PR, progesterone receptor.

Table 3 Multivariate model of ABCBI positivity according to clinical and histopathological features of breast tumors

Individual features	Adjusted OR	Adjusted 95% CI	Wald coefficient	pH-L
Triple-negative				
Positive	0.24	0.13–0.45	19.6	
Hypertension				
Yes	0.42	0.24–0.73	9.7	0.298
Lymph node status (pN)				
pN0 + pN1	0.27	0.10–0.71	7.1	0.552
Tumor size (pT)				
pT2 + pT3	0.55	0.32–0.93	4.9	0.139

Abbreviations: ABCBI, adenosine 5'-triphosphate-binding cassette subfamily B member 1; CI, confidence interval; OR, odds ratio; pH-L, p-value for the Hosmer–Lemeshow test.

The evaluation of nonmalignant mammary tissues indicated the presence of ABCBI in all cases of benign breast lesions, as well as in the normal ducts and acini of 98.8% of breast cancer samples. Such results are in agreement with the findings of Pavelic et al,⁵⁰ who described ABCBI immunoreactivity in normal ductal epithelia using four independent antibodies. Likewise, Scala et al⁵¹ showed that >80% of normal breast ductal epithelium stained positively for ABCBI, with

staining being confined to the luminal surface.⁵¹ Considering that most breast tumors have a ductal origin, it seems that ABCBI detection in breast carcinoma is a consequence of constitutive *ABCBI* expression in the breast, rather than an acquired or rare phenotype. However, Zhu et al⁵² found higher *ABCBI* expression in breast cancer tissues (57.3%) compared with adjacent noncancerous tissues (5.0%).

As an attempt to investigate the individual aspects that may modulate *ABCBI* expression in breast tumors, mRNA levels and *ABCBI* genotypes were evaluated. *ABCBI* mRNA levels were reduced in tumors from patients carrying the TT genotype of rs1128503, although no significant effect was detected in the final protein levels. There are no previous reports on the effect of rs1128503 in breast tumors, but a recent review indicated no effect on ABCBI protein levels or activity in acute myeloid leukemia.⁵³ With regard to rs1045642, previous studies suggest that the TT genotype might lead to decreased *ABCBI* mRNA levels in mammary carcinoma cell lines,⁵⁴ or in breast tumor,⁵⁵ which was not confirmed by the present study. Taken together, these results highlight the risks of using mRNA quantification to infer protein levels or activity. Accordingly, there seems to be a

Table 4 Influence of hypertension severity and treatment on ABCB1 positivity in breast tumors

Individual features	IRS status		CS		
	OR	95% CI	Median	95% CI	pM-W
Hypertension treatment					
Normotensive	1		200	160–250	<0.001 ^a
Monotherapy	0.76	0.40–1.43	180	125–243	
Combined therapy	0.58	0.33–1.03	188	110–236	
Resistant hypertension ^b	0.17	0.08–0.35	160	45–225	
Diuretics (n)					
No (168)	1		180	103–240	0.890
Yes (180)	0.57	0.312–1.02	185	103–230	
ACE inhibitors (n)					
No (190)	1		185	108–245	0.432
Yes (154)	1.09	0.61–1.92	180	100–226	
Beta-blockers (n)					
No (227)	1		185	110–240	0.234
Yes (117)	0.68	0.38–1.22	180	85–230	
ATRI antagonists (n)					
No (257)	1		180	110–230	0.967
Yes (87)	0.87	0.46–1.64	180	90–250	
Calcium channel blockers (n)					
No (278)	1		180	100–236	0.657
Yes (66)	1.36	0.63–2.92	187.5	130–240	

Notes: n=344. Data are expressed as the OR, based on the IRS status, or as the median value of the CS, for each category, with the respective 95% CIs. Statistically significant differences are presented in bold characters. The number of patients (n) per group are given in parentheses. ^ap-value of Kruskal–Wallis test; ^bClassified according to Daughtert. **Abbreviations:** ABCB1, adenosine 5'-triphosphate-binding cassette subfamily B member 1; ACE, angiotensin converting enzyme; ATRI, angiotensin II receptor I; CI, confidence interval; CS, continuous scale; IRS, immunoreaction score; OR, odds ratio; pM-W, p-value for the Mann–Whitney test.

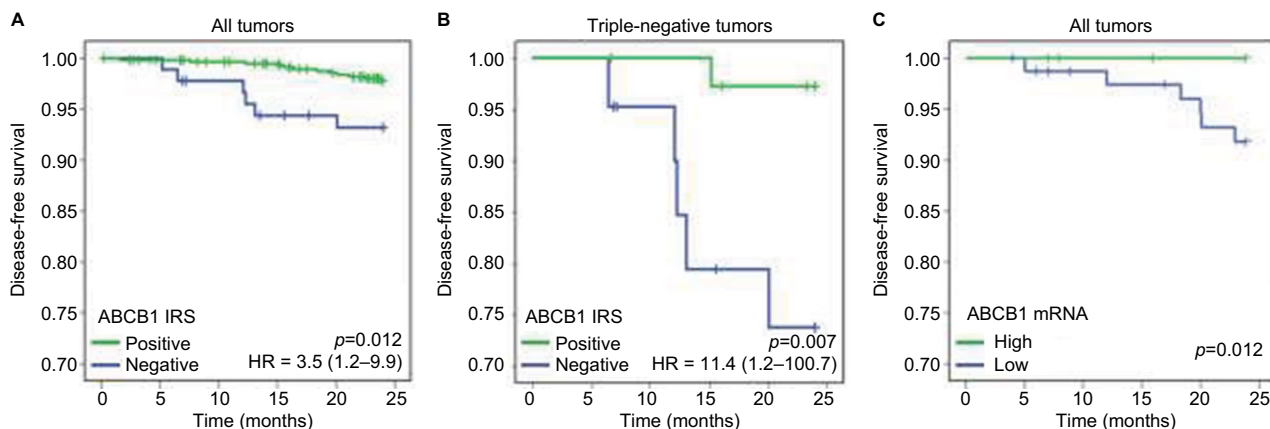


Figure 6 Two-year disease-free survival curves of patients with breast cancer based on either ABCB1 protein levels (IRS) or dichotomized mRNA expression. **Notes:** (A) IRS on all tumors; (B) IRS on triple-negative tumors; (C) mRNA levels on all tumors. p log-rank and HR with respective 95% CIs are provided for the comparison between positive IRS (green on A and B) and negative IRS (blue on A and B) or between mRNA levels above the median (high in green on C) or below the median (low in blue on C). **Abbreviations:** ABCB1, adenosine 5'-triphosphate-binding cassette subfamily B member 1; CI, confidence interval; CS, continuous scale; HR, hazard ratio; IRS, immunoreaction score.

posttranscriptional regulation of *ABCB1* expression, possibly mediated by microRNAs.⁵⁶

The evaluation of ABCB1 immunostaining according to histopathological variables indicated lower expression among tumors with large size, low lymph node status, or triple-negative subtype. Some other authors also found positive associations between ABCB1 in breast tumors and lymph node metastases^{29,52,57,58} or positive ER status.^{43,45} However, Kuroda et al⁴⁹ found higher ABCB1

immunostaining with the reduction or loss of ER, PR, and HER-2. They identified ABCB1 in 29 (59.2%) of 49 basal-like carcinomas, characterized by the presence of cytokeratins 5/6, 14, or 17, as compared to 85 (30.6%) among 278 non-basal-like carcinomas. In the present study, the expression of cytokeratins was not evaluated. Thus, *ABCB1* expression according to the luminal or basal origin of breast carcinoma or among the triple-negative basal-subtypes could not be inferred.

Table 5 Influence of ABCBI mRNA status (low) on the disease-free survival of breast cancer cohorts available at ProgGeneV2.0

Study ID	n	Follow-up (years)	DFS	OS	Adjusting factor	Reference
NKI	295	16	$p < 0.0001$; HR = 1.5 (1.2–1.8)	$p < 0.0002$; HR = 1.6 (1.2–1.9)	ER	57
GSE11121	200	16	$p < 0.01$; HR = 3.8 (1.4–10.0)	N/A	None	58
GSE4922_U133A	249	11	$p = 0.003$; HR = 2.8 (1.4–5.6)	N/A	ER	59
GSE22219	215	1	$p = 0.003$; HR = 3.8 (1.6–9.1)	N/A	ER and grade	60
GSE17705	298	16	$p = 0.001$; HR = 9.1 (2.3–33.3)	N/A	None	61
GSE2034	246	13	$p = 0.6$; HR = 1.1 (0.7–2.0)	N/A	ER	62
GSE3494_U133A	236	11	N/A	$p = 0.006$; HR = 3.6 (1.4–8.3)	ER and PR	63
GSE21653	247	13	N/A	$p = 0.004$; HR = 1.7 (1.2–2.4)	ER	64
TCGA-BRCA	594	13	N/A	$p = 0.2$; HR = 1.12 (0.7–1.1)	ER and PR	65

Notes: Data were obtained from survival plots generated on ProgGeneV2.0 for cohorts with at least 200 patients.

Abbreviations: ABCBI, adenosine 5'-triphosphate-binding cassette subfamily B member 1; DFS, disease-free survival (relapse-free or metastases-free, as available for each study); ER, estrogen receptor; HR, hazard ratio; N/A, not available; OS, overall survival; p , p -value for log-rank analysis; PR, progesterone receptor.

Table 6 Influence of ABCBI mRNA status (low) on the 5-year disease-free survival and overall survival of breast cancer cohorts available at the GEO database

Subset of breast cancer patients	DFS	OS
All		
n	3,951	1,402
p log-rank	$1e-16$	0.00025
Hazard ratio	1.89 (1.67–2.13)	1.64 (1.25–2.13)
ER positive		
n	2,061	548
p log-rank	0.0018	0.095
Hazard ratio	1.35 (1.12–1.64)	1.47 (0.93–2.33)
ER negative		
n	801	251
p log-rank	0.00071	0.027
Hazard ratio	1.54 (1.19–1.92)	1.82 (1.06–3.13)
Basal		
n	618	241
p log-rank	$2.4e-07$	0.0081
Hazard ratio	2.00 (1.54–2.63)	2.17 (1.2–3.85)

Notes: Data were obtained from survival plots generated on KM plotter by using the filter for breast cancer. Cutoff values for low and high ABCBI mRNA levels were set to the median.

Abbreviations: ABCBI, adenosine 5'-triphosphate-binding cassette subfamily B member 1; DFS, disease-free survival; ER, estrogen receptor; GEO, the Gene Expression Omnibus; OS, overall survival.

With regard to the association of decreased *ABCBI* expression with hypertension, an experimental model of spontaneously hypertensive rats suggested lower *ABCBI* activity in the kidneys and in peripheral blood mononuclear cells.⁵⁹ In addition, *ABCBI* protein levels were also diminished in the kidneys of rats submitted to high-sodium diet^{9,60} or to adrenalectomy.⁹ Because a mineralocorticoid receptor⁶¹ and a tissue renin–angiotensin system⁶² have been identified in normal and malignant breast tissues, it is possible that this pathway may modulate *ABCBI* expression in breast cancers and that a disruption of the renin–angiotensin system in the

malignant tissue⁶² may contribute to lower *ABCBI* expression in some cases.

Finally, the analysis of breast cancer outcomes suggested that the loss of *ABCBI* expression is associated with early-onset disease progression, especially among patients with triple-negative tumors. There is no presumed causal mechanism to justify how the loss of *ABCBI* in breast cancer would favor cell survival, proliferation, migration, or invasion. Instead, the recognized actions of *ABCBI* as a physiological regulator of cell cycle and apoptosis and of the lipid turnover and maintenance of membrane structure are expected to protect cancer cells from death.⁶³ It is believed that the loss of *ABCBI* in breast cancer tissue might be a consequence of the genomic instability in cancer cells, especially among triple-negative tumors, which also lack other regulatory proteins, including the ER and HER-2, rather than a specific adaptation leading to a more aggressive phenotype.

The results obtained with publically available databases corroborated the findings of the present study and suggested that low *ABCBI* mRNA may predict shorter disease-free survival and overall survival even when considering only patients with triple-negative clusters, such as the basal-type, which poses the greatest challenge for treatment because of its aggressive clinical course and lack of targeted therapy.⁶⁴ Taken together, the current results indicated that negative or low *ABCBI* expression in breast tumors, rather than being beneficial due to a possible reduction of drug efflux, seems to indicate a more aggressive phenotype, which may occur in luminal tumors, but is more frequent among triple-negative tumors.

Conclusion

In conclusion, the study results point to important paradigm changes in the concept of chemoresistance in breast cancer:

1) *ABCB1* seems to be constitutively expressed in the normal mammary tissue, being maintained, rather than acquired, in nonmalignant lesions and in most cases of breast cancer; 2) *ABCB1* protein levels might be downregulated in some breast cancers, especially in triple-negative tumors, or as a consequence of systemic arterial hypertension, although the prognostic impact of such association is yet to be determined; and 3) the absence of *ABCB1* in triple-negative tumors might contribute to identify a subgroup with worse prognosis.

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Author contributions

JMAD recruited patients, collected clinical and histopathological data, characterized genotypes and haplotypes, helped evaluating patients' blocks and slides, conducted immunohistochemical and mRNA quantification assays, performed all statistical analyses, generated tables and figures. GMV evaluated surgery resections, selected blocks, and evaluated patients' slides. VI-do-B recruited patients, collected clinical information, and helped with the statistical analyses. MTSA coordinated the immunohistochemical analyses. TSLs helped recruiting patients and set the genotyping assays. DNP set and performed mRNA expression assays. MSL performed mRNA expression assays and collected histopathological data. MAMC and MAC coordinated mRNA expression assays. RVJ conceived, designed, and coordinated the study; analyzed the data; all authors contributed toward data analysis, drafting and revising the paper and agree to be accountable for all aspects of the work.

Disclosure

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