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

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 [ORadj] = 0.24; 95% confidence interval [CI] = 0.13–0.45), lymph node status < pN2 (ORadj = 0.27; 95% CI = 0.10–0.71), tumor size > 2 cm (ORadj = 0.55; 95% CI = 0.32–0.93), and hypertensive status (ORadj = 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 In addition, there seems to be great interpatient variability.6 One possible reason for such
variability is that the \textit{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.\textsuperscript{7,8} Finally, it has been reported that the \textit{ABCB1} expression may also be modulated by plasma aldosterone\textsuperscript{9} or cortisol,\textsuperscript{10} as well as by dietary salt and dehydration.\textsuperscript{9}

In this study, a systematic evaluation of \textit{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. \textit{ABCB1} expression was estimated by immunohistochemistry with three previously validated antibodies, and its association with clinical and histopathological variables, including the genetic profile regarding \textit{ABCB1} SNP, was evaluated. Finally, the impact of \textit{ABCB1} expression on short-time (2-year) disease-free survival of patients with breast cancer was also investigated.

\textbf{Methods}

\textbf{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 Cáncer (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 biomarkers\textsuperscript{11} and the international precepts of ethics in research and of good clinical practice were followed.

\textbf{Clinical and histopathological characterization}

A description of this study cohort has been published previously.\textsuperscript{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.\textsuperscript{13} The lifestyle variables were alcohol drinking, defined according to the frequency of consumption, and smoking, classified as current, previous,
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 Tumors and on the Elston–Ellis histological grading system. 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.

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. 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-PGL Ym, 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, validation experiments were conducted in this study, as recommended by the consensus for ABCB1 detection, using specimens from different nonmalignant human tissues. Liver, adrenal, and kidney proximal and distal tubules were used as positive controls, whereas tonsils, epididymis, and kidney glomeruli were used as negative controls. Because ABCB1 was expected to be detected in the biliary canaliculi, 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-reactivity toward ABCB4 (formerly known as MDR3).

Proximal and distal kidney tubules, which express ABCB1, showed positive staining (Figure 2G and H), whereas kidney glomeruli (arrows in Figure 2G), which express only ABCB4, 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) and a categorical score, both of which consider the percentage of immunostained cells and the intensity of the reaction.

The CS was calculated as follows: $CS = (\%_{\text{weak}} \times 1) + (\%_{\text{moderate}} \times 2) + (\%_{\text{strong}} \times 3).$ 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: $IRS = (\text{positivity score}) \times (\text{intensity score}).$ The positivity score was assigned a value of 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.

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
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 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 FAM™), 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: RQ = 2^(-ΔΔCt), where ΔΔCt = ΔCtABCB1 − ΔCtHMG, with ΔCtABCB1 = CtABCB1 − CtPPIA and ΔCtHMG = CtHMG − CtPPIA. 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 χ² test. Individual variables were tested for linear-by-linear associations, with calculation of trend significances (p<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 χ² test was used to identify independent predictors (p<0.05), which were used for the calculation of the corresponding adjusted ORs (ORadj). 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 patients, and the Gene Expression 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 ≥4), and 71% presented high immunostaining scores (IRS ≥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  |   |         |                           |   |         |
| 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       | 108| 15.2    | Lobular in situ           | 3  | 0.4     |
| ≥65 years         | 226| 31.7    | Others                    | 27 | 3.8     |
| Color             |   |         |                           |   |         |
| White             | 320| 45.7    | Tumor size (pT)           | 48 | 6.7     |
| Brown             | 268| 38.3    | pTis (in situ)            | 407| 56.5    |
| Black             | 88 | 12.6    | pT1 (≤2 cm)               | 312| 45.1    |
| Others            | 24 | 3.4     | pT2 (2–5 cm)              | 309| 43.4    |
| Missing           | 12 | 1.7     | pT3 (>5 cm)               | 21 | 2.9     |
| Menopausal status |   |         |                           |   |         |
| Premenopausal     | 179| 25.6    | Lymph node status (pN)    | 421| 59.6    |
| Postmenopausal    | 521| 74.4    | pN0 (none)                | 177| 25.1    |
| Missing           | 12 | 1.7     | pN1 (≤3 positive nodes    | 66 | 9.3     |
| Smoking status    |   |         |                           |   |         |
| Nonsmoker         | 464| 66.2    | pN2 (4–9 positive nodes)  | 42 | 5.9     |
| Former smoker     | 173| 24.7    | Missing                   | 6  | 0.8     |
| Current smoker    | 64 | 9.1     | Missing                   | 6  | 0.8     |
| Missing           | 11 | 1.6     | Stage grouping            | 46 | 6.6     |
| Alcohol consumption|  |         |                           |   |         |
| Never             | 384| 56.0    | ER status                 | 227| 35.2    |
| Rare (<3 days/week)| 212| 30.9    |                           | 312| 44.7    |
| Frequent (>3 days/week)| 90 | 13.1    | Missing                   | 58 | 8.6     |
| Comorbidities     |   |         |                           |   |         |
| None              | 91 | 12.8    | G1                        | 227| 35.2    |
| Hypertension      | 377| 53.3    | G2                        | 264| 39.9    |
| Obesity           | 159| 21.7    | G3                        | 312| 44.7    |
| Diabetes mellitus | 104| 14.9    | Missing                   | 111| 16.2    |
| BMI (Kg/m²)       |   |         | ER status                 |   |         |
| <18.4             | 6  | 1.0     | Negative                  | 575| 83.8    |
| 18.5–24.9         | 164| 23.8    | Positive                  | 26 | 3.6     |
| 25–29.9           | 248| 34.3    | Missing                   | 159| 21.7    |
| >30.0             | 159| 11.3    | Negative                  | 197| 27.8    |
| Missing           | 135| 18.5    | Positive                  | 488| 71.2    |
| ABCB1 genotypes   |   |         | HER-2 status              |   |         |
| rs1128503 (C1236T)|   |         |                           |   |         |
| CC                | 222| 39.7    | Negative                  | 515| 81.1    |
| CT                | 269| 48.1    | Positive                  | 103| 16.2    |
| TT                | 68 | 12.2    | Undetermined              | 17 | 2.7     |
| Missing           | 77 |         | Missing                   | 77 |         |
| rs1045642 (C3435T)|   |         | Tumor subtype             |   |         |
| CC                | 227| 39.3    | Luminal A                 | 395| 63.2    |
| CT                | 257| 44.5    | Luminal B                 | 130| 20.8    |
| TT                | 94 | 16.3    | 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.
The results indicated that the loss of \textit{ABCB1} 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 \textit{ABCB1} expression on the rates of breast cancer progression was maintained after adjustment for other prognostic factors, that is, \textit{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 \textit{ABCB1} expression on the 2-year disease-free survival of patients with breast cancer was also evaluated, considering \textit{ABCB1} mRNA levels in tumors that could be fresh-frozen at the time of the excision surgery (n=154). A significant difference was detected \((p \text{ log-rank } =0.012)\), with the patients whose tumors had low \textit{ABCB1} mRNA presenting worse cumulative survival than the patients with high \textit{ABCB1} 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 \textit{ABCB1} mRNA was extended to publically available databases from breast cancer cohorts (Tables 5 and 6). The results obtained with these cohorts confirmed that low \textit{ABCB1} 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).
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 (\(p_{\text{log-rank}} = 0.57\)) or for rs1045642 (\(p_{\text{log-rank}} = 0.55\)).

**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.\(^4,2\) Fourth, two different scoring methods\(^2,29\) 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 \(\geq 100\) 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 patients\(^29\) or 66% in 177 patients.\(^48\)
Table 2 Significant associations between individual features and ABCB1 expression in breast tumors

| Individual features | IRS status | CS | pM-W |
|---------------------|------------|----|------|
|                     | OR         | 95% CI | Median | 95% CI |        |
| Hypertension        | No         | 1    | 200   | 160–250 | <0.001 |
|                     | Yes        | 0.51 | 0.32–0.82 | 180 | 103–239 |
| Tumor size (pT)     | pT1        | 1    | 200   | 135–269 | 0.003 |
|                     | pT2 + pT3  | 0.59 | 0.37–0.95 | 185 | 155–250 |
| Lymph node status (pN) | pN0 + pN1 | 1    | 190   | 123–294 | 0.001 |
|                     | pN2 + pN3  | 3.64 | 1.44–9.19 | 202.5 | 120–240 |
| Stage grouping      | I + II     | 1    | 190   | 120–240 | <0.001 |
|                     | III        | 3.15 | 1.34–7.43 | 210 | 170–270 |
| ER status           | Positive   | 1    | 195   | 145–250 | <0.001 |
|                     | Negative   | 0.38 | 0.23–0.65 | 160 | 70–220 |
| PR status           | Positive   | 1    | 200   | 150–250 | <0.001 |
|                     | Negative   | 0.47 | 0.30–0.76 | 177.5 | 90–220 |
| 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 |

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. \( p \)-value of Kruskal–Wallis test.

Abbreviations: ABCB1, 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 ABCB1 positivity according to clinical and histopathological features of breast tumors

| Individual features | Adjusted OR | Adjusted 95% CI | Wald coefficient | pH-L |
|---------------------|-------------|-----------------|------------------|------|
| Triple-negative     | 0.24        | 0.13–0.45       | 19.6             |      |
| Hypertension        | 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: ABCB1, 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 ABCB1 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 ABCB1 immunoreactivity in normal ductal epithelia using four independent antibodies. Likewise, Scala et al51 showed that >80% of normal breast ductal epithelium stained positively for ABCB1, with staining being confined to the luminal surface.51 Considering that most breast tumors have a ductal origin, it seems that ABCB1 detection in breast carcinoma is a consequence of constitutive ABCB1 expression in the breast, rather than an acquired or rare phenotype. However, Zhu et al52 found higher ABCB1 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 ABCB1 expression in breast tumors, mRNA levels and ABCB1 genotypes were evaluated. ABCB1 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 ABCB1 protein levels or activity in acute myeloid leukemia.53 With regard to rs1045642, previous studies suggest that the TT genotype might lead to decreased ABCB1 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
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 metastases29,52,57,58 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 4 Influence of hypertension severity and treatment on ABCB1 positivity in breast tumors

| Individual features                        | IRS status | CS | pM-W |
|-------------------------------------------|------------|----|------|
| Hypertension treatment                    |            |    |      |
| Normotensive                               | 1          | 200| 160–250|<.001* |
| Monotherapy                                | 0.76       | 180| 125–243|
| Combined therapy                           | 0.58       | 188| 110–236|
| Resistant hypertension*                    | 0.17       | 160| 45–225 |
| Diuretics (n)                              |            |    |      |
| No (168)                                   | 1          | 180| 103–240|0.890 |
| Yes (180)                                  | 0.57       | 185| 103–230|
| ACE inhibitors (n)                         |            |    |      |
| No (190)                                   | 1          | 185| 108–245|0.432 |
| Yes (154)                                  | 1.09       | 180| 100–226|
| Beta-blockers (n)                          |            |    |      |
| No (227)                                   | 1          | 185| 110–240|0.234 |
| Yes (117)                                  | 0.68       | 180| 85–230 |
| ATR1 antagonists (n)                       |            |    |      |
| No (257)                                   | 1          | 180| 110–230|0.967 |
| Yes (87)                                   | 0.87       | 180| 90–250  |
| Calcium channel blockers (n)               |            |    |      |
| No (278)                                   | 1          | 180| 100–236|0.657 |
| Yes (66)                                   | 1.36       | 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. *p-value of Kruskal–Wallis test; *Classified according to Daughert.

Abbreviations: ABCB1, adenosine 5'-triphosphate–binding cassette subfamily B member 1; ACE, angiotensin converting enzyme; ATR1, angiotensin II receptor 1; CI, confidence interval; CS, continuous scale; IRS, immunoreaction score; OR, odds ratio; pM-W, p-value for the Mann–Whitney test.
With regard to the association of decreased ABCB1 expression with hypertension, an experimental model of spontaneously hypertensive rats suggested lower ABCB1 activity in the kidneys and in peripheral mononuclear cells. In addition, ABCB1 protein levels were also diminished in the kidneys of rats submitted to high-sodium diet or to adrenalectomy. Because a mineralocorticoid receptor and a tissue renin–angiotensin system have been identified in normal and malignant breast tissues, it is possible that this pathway may modulate ABCB1 expression in breast cancers and that a disruption of the renin–angiotensin system in the malignant tissue may contribute to lower ABCB1 expression in some cases.

Finally, the analysis of breast cancer outcomes suggested that the loss of ABCB1 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 ABCB1 in breast cancer would favor cell survival, proliferation, migration, or invasion. Instead, the recognized actions of ABCB1 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. It is believed that the loss of ABCB1 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 ABCB1 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. Taken together, the current results indicated that negative or low ABCB1 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.

**Table 5** Influence of ABCB1 mRNA status (low) on the disease-free survival of breast cancer cohorts available at ProgGeneV2.0

| Study ID          | n   | Follow-up (years) | DFS HR (p-value) | OS HR (p-value) | Adjusting factor | Reference |
|-------------------|-----|-------------------|------------------|----------------|-----------------|-----------|
| NKI               | 295 | 16                | <0.0001; HR = 1.5 (1.2–1.8) | <0.0002; HR = 1.6 (1.2–1.9) | ER | 57 |
| GSE1121           | 200 | 16                | <0.01; HR = 3.8 (1.4–10.0) | N/A | None | 58 |
| GSE4922_U133A     | 249 | 11                | 0.003; HR = 2.8 (1.4–5.6) | N/A | ER | 59 |
| GSE22219          | 215 | 1                 | 0.003; HR = 3.8 (1.6–9.1) | N/A | ER and grade | 60 |
| GSE17705          | 298 | 16                | 0.001; HR = 9.1 (2.3–33.3) | N/A | None | 61 |
| GSE2034           | 246 | 13                | 0.6; HR = 1.0 (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: ABCB1, 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-value for log-rank analysis; PR, progesterone receptor.

**Table 6** Influence of ABCB1 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                      | 1.4–16 | 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 ABCB1 mRNA levels were set to the median.

Abbreviations: ABCB1, adenosine 5'-triphosphate–binding cassette subfamily B member 1; DFS, disease-free survival; ER, estrogen receptor; GEO, the Gene Expression Omnibus; OS, overall survival.

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.

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