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RESEARCH ARTICLE

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Prognostic role of SOX2 and STAT3 expression on circulating T lymphocytes and CD44+/CD24^{neg} cells in the locally advanced and metastatic breast cancer

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Abstract

Background: Breast cancer (BC) is associated with a continuous increase in incidence, with high mortality rates in several countries. CD44, STAT3, and SOX2 are related to regulating of somatic cell division, tumorigenesis, and metastasis in BC.

Methods: A cross-sectional study was carried out at the Hospital de Cancer de Pernambuco (HCP) between 2017 and 2018. Fifty-one women with locally advanced (LA) and 14 with metastatic BC were included in the study.

Results: High CD44+/CD24^{neg} and CD44+/CD24^{neg}/SOX2+ levels in Luminal B (LB), HER2+, and triple-negative breast cancer (TNBC) compared with controls ($p < 0.05$). Low CD44+/CD24^{neg}STAT3+ levels in LB, HER2+, and TNBC compared with controls ($p < 0.05$). High T lymphocytes, and low STAT3 + T, and SOX2 + T levels in BC patients ($p < 0.05$). High SOX2 + T levels in patients with axillary lymph node-negative (N0) compared with the axillary lymph node-positives (N1 and N2 groups; $p < 0.05$). High SOX2 + T levels in N1 compared to N2 ($p < 0.05$). High T lymphocytes and low SOX2 + T levels in the LA tumor compared to metastatic disease ($p = 0.0007$ and $p = 0.02$, respectively). High CD44 + /CD24^{neg}STAT3+, and T lymphocyte levels in TNBC patients with LA tumor compared to metastatic ($p < 0.05$). Low STAT3 + T levels in TBNC patients with LA tumor compared to metastatic ($p = 0.0266$).

Conclusion: SOX2 and STAT3 expression on circulating T lymphocytes and CD44 + / CD24^{neg} cells in peripheral blood have prognostic roles in breast cancer. SOX2 and STAT3 expression are potential predictive biomarkers of disease progression in breast cancer regardless of tumor subtype.

KEYWORDS

breast cancer, SOX2, STAT3

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1 | INTRODUCTION

Breast cancer (BC) is a disease characterized by the growth of heterogeneous abnormal cells that starts in the mammary glandular tissue and has an intrinsic ability to progress to other organs.[1] The BC results from the interaction of genetic factors with environmental factors that may lead to the accumulation of mutations of new oncogenes and tumor suppressor genes.[1] However, inherited genetic alterations represent 5% to 10% of the cases of BC with a family history of breast and/or ovarian tumors.[2]

For the classification of the immunophenotype of BC, the immunohistochemical technique is used for the analysis of estrogen receptor (ER) and progesterone receptor (PR) expression, human epidermal growth factor receptor-type 2 (HER2). The cell proliferation test is also carried out using the KI67 marker.[3,4] Perou et al. performed the analysis of gene expression by microarrays and described five breast tumor phenotypes: Luminal A (LA), Luminal B (LB), HER2 overexpression (HER2+), basal and normal breast-like subtype.[5] Breast tumors classified as triple negative (TN) by immunohistochemistry are those that do not express ER, PR and HER2. The triple negative breast cancer (TNBC) does not express ER/PR, HER2 and is characterized by the expression of cytokeratin (CK)-5, CK6, CK14, CK17, epidermal growth factor receptor (EGFR), E-caderin, and p63, being associated with the worst prognosis.[4,6]

The potential effects of tumor-infiltrating lymphocytes on breast cancer are numerous and complex. Most BC studies using hematoxylin-eosin (H&E) and multivariate analyses show inflammatory infiltrate is associated with better, worse, or no relationship to prognosis. However, there is evidence that the function of these inflammatory cells is often compromised with immune response.[7] Some molecules expressed in immune system cells can be used as biomarkers because they are expressed in tumor cells, which are involved in the process of tumorigenesis and metastases. The CD44 molecule is considered an important biomarker of inflammatory response and is presented in the form of cell membrane receptors and free in plasma (soluble). CD44 is present on all leukocytes and the surface of tumor stem cells.[8] Tumor stem cells (TSC) are considered to have more aggressive phenotypes and have a subpopulation of cancer cells with high regenerative and differentiating power, being involved with resistance to conventional treatments, high incidence of recurrence, and metastasis.[9]

Quanri et al. demonstrated elevated serum levels of CD44 in patients with BC and unfavorable clinical outcome, being a prognostic index in patients with HER2+ breast cancer, because it participates in the interaction between cancer cells and the tumor microenvironment, favoring the growth, invasion, and metastasis.[10] In luminal tumors, Tsunoda et al. suggested the association of CD44+/CD24− gene expression with overexpression of other genes with ALDH1 and SOX2 that are related to patients with positive lymph nodes, micrometastases and resistance to conventional treatments.[11] Honeth et al.[8] demonstrated the association of high CD44 expression in tumor tissue and especially in basal types, with 94% of hereditary BRCA1 breast cancer having surface CD44, and suggested that CD44 is a good phenotypic marker of tumor stem cells.

Another important molecule is CD24, which is expressed on the glycosylated cell surface, and its high expression is described in cancer of the ovary, breast, prostate, bladder, kidneys, among others, and is involved in cell adhesion and metastasis. The metastatic associations of CD24 increase its importance as a prognostic factor.[12]

In addition to membrane receptors such as CD44 and CD24, there are intracellular proteins called transcription factors that participate in the activation, signaling, and production of growth factors important for cell differentiation and proliferation. Transcription factors are involved in the mechanisms of carcinogenesis and tumor metastases, as they are responsible for the processes of disordered cell division and consequent formation of malignant tumors.[13]

The proteins of the signal transducers and activators of transcription (STAT) family are cytoplasmic transcription factors and comprise seven members, STAT1 through STAT4, STAT5a, STAT5b, and STAT6. The phosphorylation of tyrosine-specific residues is a step towards the activation of STAT.[14,15] STAT3 signaling is generally low and occurs after JAK activation, following phosphorylation, dimerization, nuclear translocation and binding to STAT3 DNA.[16] The activation of STAT3 in the tumor cell raises the level of BCL-1 and BcL-XL, which are antiapoptotic genes and cell cycle proteins to cyclin D1 and c-Myc, which makes the tumor resistant to chemotherapy treatments that act in the mechanisms of programmed cell death.[17]

Wei et al.[18] reported high protein expression of STAT3 in tumor tissue of 51 cases of BC compared to regions of normal breast tissue. High levels of STAT3 were reported in Stage IV tumors compared with Stage I and Stage II. In turn, when related to the presence of lymph nodes, elevated levels of STAT3 expression were observed in patients with positive lymph nodes.[18] Sonnenblick et al.[19] described high expression of STAT3 in HER2 + BC patients and resistance to trastuzumab therapy.

The Sex determining region Y-box 2 (SOX2) gene encodes the SOX2 transcription factor, which contributes significantly to cell division regulation. SOX2 participates in early embryonic development, neural differentiation, and other biological processes. The functions of SOX2 have been described in cell cycle participation and release of growth factors responsible for angiogenesis.[20]

SOX2 has been reported to be directly associated with tumorigenesis and metastasis. In embryonic stem cells, the reduction in SOX2 expression is associated with the loss of pluripotent status and a propensity for differentiation.[21] Studies involving tumorigenic cell populations have demonstrated the abnormal expression of transcription factors related to embryonic cell self-renewal, such as octamer-binding transcription factor 4 (OCT4), SOX2, and Nanog, and their involvement in the carcinogenesis of various tumors.[22]

High expression of SOX2 has been found in 30% of tumors analyzed by immunohistochemistry.[23] Other authors have demonstrated in vitro that SOX2 expression in embryonic stem cells is associated with high CD44+ expression and can form undifferentiated and metastatic tumors in tumor strains with unfavorable clinical features.[24]

Carcinogenesis is dependent on a small subset of cells within the tumor, called cancer stem cells (CSC), originating from mutations in genes associated with the control of cell proliferation and differentiation of stem cells with the capacity to initiate and maintain tumor growth.[25] Some authors have shown a relationship between membrane receptors such as CD44 activating transcription factors which are intracellular proteins that participate in the signaling and production of growth factors fundamental for cell differentiation and proliferation, these transcription factors are involved in the mechanisms of carcinogenesis and metastasis, they are responsible for the processes of disordered cell division in malignant tumors, after their activation by the phosphorylation mechanism with STAT3, directing themselves to the nucleus and promoting the transcription of proteins capable of increasing the tumor capacity of invasion and metastasis similar to SOX2.[18,26]

Liu et al.[27] observed elevated levels of SOX2+ and CD24+ in tumor stem cells in hepatocellular carcinoma. Low expression of CD24 has been shown to be related to reduction of SOX2 and STAT3 levels. CD24 and STAT3 molecules are important in the regulation of tumor stem cells in liver carcinoma.[27]

Tumor stem cells exhibit a CD44+/CD24− phenotype and express greater tumor heterogeneity and resistance to current treatments. STAT3 and SOX2 have been described as important transcription factors for tumor progression. In vitro studies have shown increased expression of SOX2 in patients with BC. Therefore, it was decided to evaluate the expression of CD44+/CD24−, STAT3 and SOX2 in peripheral blood of patients with locally advanced and metastatic BC.

2 | MATERIALS AND METHODS

2.1 | Study and subjects

A cross-sectional study was carried out at the Hospital de Cancer de Pernambuco (HCP) between 2017 and 2018. HCP is a hospital that exclusively serves users of the Unified Health System (SUS), responsible for approximately 55% of oncological care in the State of Pernambuco, Brazil. The great majority of the patients attended to at the HCP are of low income, and they arrive at the oncological service with advanced tumors. The analyzes of cell populations were carried out at the Translational Research Laboratory of the Instituto de Medicina Integral Prof. Fernando Figueira (IMIP). The institutional review board of HCP (No 51422115.3.0000.5205) approved the study protocol.

A total of 65 BC women were included, aged 18–65 years. Of these, 51 women had Stage III (locally advanced) breast cancer and axillary lymph node-positive, and 14 with metastatic disease (Stage IV).

As a control group, 24 clinically healthy women were included (18–65 years) who did not present cancer and without a family history of breast cancer were evaluated.

At the time of inclusion, a questionnaire was applied containing the eligibility criteria. The inclusion criteria were BC women, age above 18 years and with clinical staging of locally advanced tumors and metastatic disease. For controls group, the criteria were clinically healthy women over the age of 18.

2.2 | Diagnosis and clinical staging

2.2.1 | Clinical diagnosis

A clinical diagnosis with a history and physical examination was performed, plus imaging examinations. The patients who had tumors impalpable in their breasts were sent to undergo core biopsy guided by ultrasound. Patients with palpable tumors underwent ultrasound and mammography examinations, and subsequently underwent core biopsy.

2.2.2 | Pathological diagnosis

The patients performed the core biopsy procedure at the HCP anatomopathology service. The histological sections of the biological material were laid out on slides and stained by hematoxylin and eosin (HE). Histological type, nuclear grade, angiolymphatic invasion were classified according to the recommended.[28,29] The prognostic panel was then carried out and corresponds to the immunophenotyping of the patients determined using the immunohistochemical technique. The histological grade was classified according to the Nottinghan system proposed by Elston and Ellis,[30] where the tumor is classified as well differentiated (low grade or grade I), moderately differentiated (intermediate grade or grade II), or poorly differentiated (high grade or grade III). The histopathological data were obtained from the reports of the pathological anatomy service of the HCP. The main data obtained were tumor size, histological type, nuclear grade, histological grade, lymph node status, and clinical staging.

Breast tumors were classified as triple-negative when negative for estrogen, progesterone and HER2 receptors.[31] HER2+ breast cancer were considered, the tumors with intense coloration (3+) throughout the cell membrane, and in more than 30% of the cells evaluated. The interpretation of hormone receptors was based on the consensus of the American Society of Clinical Oncology/College of American Pathologists 2013.[32] All the slides of the included patients were reviewed by a second pathologist from the pathological anatomy service of the HCP.

2.2.3 | Clinical staging

Staging for malignant breast tumor was performed after confirmation for breast neoplasia, and with CT scans, abdomen, and bone scintigraphy, determining the absence or presence of tumors, and secondary lesions. The patients with breast tumors who presented secondary lesions were classified in metastatic stage. Clinical staging was performed using the American Joint Committee on Cancer (AJCC; 8th edition).[33]

2.3 | Flow cytometry

The volume of 4 mL of venous blood sample from the patients was collected in a tube with ethylenediamino tetra-acetic acid (EDTA) (BD Vacutainer®) and maintained at room temperature (24°C). The time between collection and processing was up to 60 min. Before processing, the blood sample tube was placed in the homogenizing equipment (BD Bioscience) and then analyzed by flow cytometry.

The first stage was the analysis of the proteins expressed on the cell surface (CD44, CD24, and CD3). The cells were fixed with 100 µL of Cytofix/Cytoperm™ BD preheated and incubated for 10 min at 37°C. Then, after centrifugation at 250*g* for 8 min, 5 µL of anti-CD3, anti-CD44 and anti-CD24 monoclonal antibodies were added (BD Biosciences). Monoclonal antibodies are conjugated to fluorophores such as PE, FITC, PerCP, and PECY-7, which differ from each other in color. Monoclonal antibodies have specificity for molecules expressed in the membrane and inside the cells, making it possible to characterize human leukocytes and other human cells, as well as analyze their functions. These molecules are identified as clusters of differentiation (CD).

STAT3 and SOX2 proteins are expressed inside cells (cytoplasm and nucleus, respectively). Cell permeability was performed to detect these proteins using BD™ Phosflow reagents (BD Biosciences). After incubation for 20 min at room temperature and protected from light, the cells were permeated with 750 µL of Perm Buffer III (BD Biosciences) at 4°C and incubated for 30 min submerged in ice, protected from light. Then the cells were washed with 3 mL of phosphate-buffered saline (PBS), centrifuged at 250*g* for 8 min and the supernatant was discarded.

Three washes were performed using PBS 1x concentrated (pH 7,4). Then, intracellular marking with anti-SOX2 and anti-STAT3 monoclonal antibodies was performed and incubated for 40 min protected from light. Then a new washing stage was performed, the supernatant discarded, and the cells were re-suspended at 300 µL of Stain Buffer and acquisition on the flow cytometer. After the washes, 50 000 cellular events were acquired in the FACSVERSE equipment (Becton Dickinson) and the analyzes were carried out in the FACSSUITE program (Becton Dickinson) and expressed in percentage values.

2.4 | Statistical analysis

The descriptive statistics of the categorical variables were represented in absolute and relative frequencies, while the continuous variables were presented as measures of central tendency (mean/standard deviation or median and interquartile (25%–75%). For the quantitative variables, the Shapiro-Wilk normality test was initially applied. The quantitative variables of this study were presented in values of median and interquartile range (IQR: 25%–75%). The Mann–Whitney non-parametric test was used for comparison between two groups. The Kruskal–Wallis nonparametric test was used for comparison between three or more groups. To avoid type I statistical error, the analyzes by the Kruskal–Wallis test were followed by Dunn's correction for multiple testing. The level of statistical significance of $p < 0.05$ was adopted. Statistical analysis was performed using the graphpad prism v8.0 program (Graphpad software).

3 | RESULTS

3.1 | Clinical variables

A total of 65 women with nonspecial invasive carcinoma and 24 healthy women were evaluated. There was no significant difference in the median age value between the patients (47 years; IQR 40.0–56.0) and controls (49 years; RQI 44.5–53.5; $p = 0.675$).

The predominant histological type was invasive ductal carcinoma without other specifications (98.46% SOE). Regarding the size of the tumor, most of the patients presented with a tumor > 5 cm (86.15%).

The most frequent tumor phenotype was triple negative (TN) (44.61%), followed by luminal B (29.23%) and HER2 (26.15%). Most of women with breast cancer had locally advanced tumors at Stages IIIA and III B (27.7% and 50.8%, respectively) and 21.5% had metastatic disease. Regarding lymph node status, most of the patients were found to have axillary lymph node-positive (N1 and N2) and nuclear grade III (53.8%) (Table 1).

3.2 | Analysis of CD44+/CD24^{neg} and T lymphocytes expressing SOX2 and STAT3

High CD44+/CD24^{neg} and CD44+/CD24^{neg}/SOX2+ cells levels were observed in subtypes LB, HER2+, and TN compared to controls ($p < 0.05$; Figure 1). Low CD44+/CD24^{neg}STAT3+ levels were observed in LB, HER2+ and TNBC compared with controls ($p < 0.05$; Figure 1). BC patients had high levels of T lymphocytes, and low levels of STAT3 + T lymphocytes and SOX2 + T lymphocytes ($p < 0.05$; Figure 1D–F).

No significant differences were observed in the levels of CD44 + /CD24^{neg}, CD44+/CD24^{neg}/SOX2+ and CD44+CD24^{neg}/STAT3+ according to lymph node status (Figure 2).

High levels of SOX2 + T lymphocytes were observed in the axillary lymph node-negative patients (N0) compared with the N1 and N2 groups ($p = 0.0197$ and $p = 0.0002$, respectively). BC patients with N1 had high levels of SOX2 + T lymphocytes compared to group N2 ($p = 0.03$) (Figure 2F).

High T-lymphocyte levels in patients with locally advanced tumor compared metastatic disease ($p = 0.0007$). For SOX2 + T lymphocytes, low levels were observed in locally advanced tumor patients compared to metastatic disease ($p = 0.02$) (Figure 3).

3.3 | Analysis of patients with locally advanced metastatic triple negative breast cancer (TBNC)

As the TNBC group had the largest number of patients ($n = 29$), it was possible to perform the analysis by comparing patients with locally advanced and metastatic tumors.

TABLE 1 Clinical characteristics of 65 breast cancer women.

Variables	Breast cancer N = 65
Age	
Median (IQR)	47 (40.0–56.0)
Tumor	
≤2 cm	2 (3.09)
>2 e ≤ 5 cm	7 (10.76)
>5 cm	56 (86.15)
Lymph node status	
N0	10 (15.4)
N1	35 (53.8)
N2	20 (30.8)
Molecular subtypes	
Luminal B	19 (29.23)
HER2+	17 (26.15)
Triple negative	29 (44.61)
Grade	
I	3 (13.9)
II	21 (32.3)
III	35 (53.8)
Clinical staging	
IIIA	18 (27.7)
IIIB	33 (50.8)
IV	14 (21.5)

Note: Hospital de Cancer de Pernambuco (2017–2018).
Abbreviation: IQR, interquartile.

Elevated levels of CD44+/CD24^{neg}STAT3+ cells and T lymphocytes were observed in TBNC patients with locally advanced tumor compared metastatic disease group ($p = 0.0015$ and $p = 0.0386$, respectively). Low STAT3 + T lymphocytes levels were observed in TBNC patients with locally advanced tumor compared to metastatic disease ($p = 0.0266$) (Figure 4).

4 | DISCUSSION

Despite the great advances in research over the last few decades, there is still a high incidence of the mortality of women with BC in developing countries such as Brazil. In light of the conquests in the technology of diagnostic images and medicines for target therapy, there are still many tumors that show resistance to treatment and a great power for dissemination and death. Many researchers are trying to clarify which mechanisms promote this high capacity for invasion, dissemination, and tumor metastasis, as well as the resistance of the existing therapies. Attempts are also being made to explain the relationship between tumor behavior and the host's immune response. Along these lines, the studies have evolved into the evaluation of molecules in cell cultures in animal models and in vitro, but as far as the in vivo study is concerned and mainly in the blood, there are still few articles published.

In this study, most of the patients presented with breast cancer at more advanced stages with tumors larger than 5 cm and axillary lymph node-positive at the time of diagnosis, being more difficult to control the local cancer and consequently favoring the evolution to metastatic disease. In the developing countries, late diagnosis is the main challenge to control the increase in mortality. Most of the patients studied presented a median age of 47 years and corresponds to the worldwide findings of increased incidence and mortality in women with BC in this age group.[34]

The tumors showed the most frequent triple negative subtype, and these tumors are those that show the most aggressive characteristics, with faster growth, progress easily to metastatic disease, and so far without a specific target therapy for the treatment of this tumor subtype. It is believed that specific molecule changes may justify the aggressive behavior of these tumors and in this study, we proved that STAT3 is a molecule involved in these tumors, the STAT3 pathway was presented as responsible for the treatment resistance and survival of the tumor cell, for the invasion and angiogenesis mechanisms through the phosphorylation, dimerization and nuclear translocation of STAT3 when interacting with protein kinases and other molecules.[34,35]

In this study, we showed an increase in CD44+/CD24neg-STAT3+ expression levels in locally advanced carcinomas when compared to metastatic carcinoma. It is believed that in the proliferative phase of the tumor there is an increase in this molecule and activation of this pathway to maintain tumor biology. Wang et al. have shown an increase of IL-22 in TNBC because of JAK-STAT3/MAPKS/AKT pathway activation compared to normal breast tissue. IL-22 is associated with increased migration capacity and resistance to paclitaxel, a medicinal product used in TN tumor treatments.[16]

Tumor aggressiveness is directly associated with lymph node status and in this study more than 80% had clinically axillary lymph node-positive at the time of diagnosis, independent of the tumor subtype. The association with stem cell tumors and axillary status can be seen in the studies that showed the association of positive sentinel lymph node with the expression of the molecule CD44^{+}CD24^{neg}.[36] This study showed that there was no statistical difference in the levels of CD44+/CD24^{neg}, and CD44+/CD24^{neg}-SOX2+ and CD44 + CD244+CD24^{neg}STAT3+ according to lymph node status assigning these findings to the highest percentage of locally advanced and metastatic diseases even in patients with clinically axillary lymph node-negative. In the study of 448 primary breast tumors, the authors showed the relationship between the increasing circulating CD44 expression and increasing recurrence at a distance, decreasing time-free disease. There has been an association of large tumors of high histological grade and positive lymph nodes, the increase of CD44 expression is directly related to endothelial cell adhesion and high metastatic power.[37]

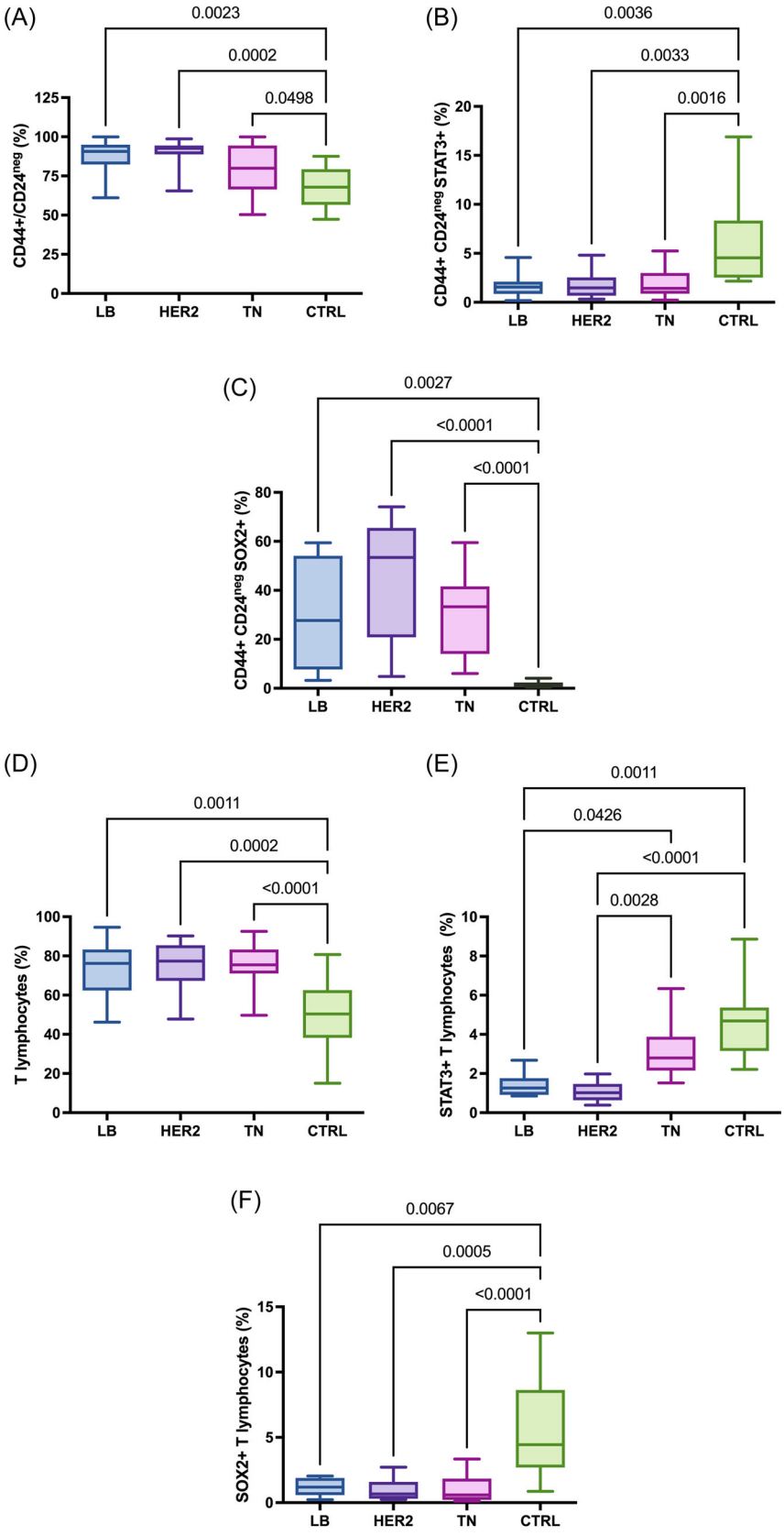

FIGURE 1 Analysis of CD44+/CD24^{neg} (A), CD44+/CD24^{neg}/SOX2+ (B), and CD44+/CD24^{neg}/STAT3+ (C), T lymphocytes (D), and STAT3 T lymphocytes (E) and SOX2 T lymphocytes (F) in peripheral blood of women with luminal B (LB; $n = 19$), HER2+ ($n = 17$), and triple-negative (TN; $n = 29$) breast cancer and healthy women (CTRL-controls; 24). The Kruskal–Wallis test was used. It was considered $p < 0.05$.

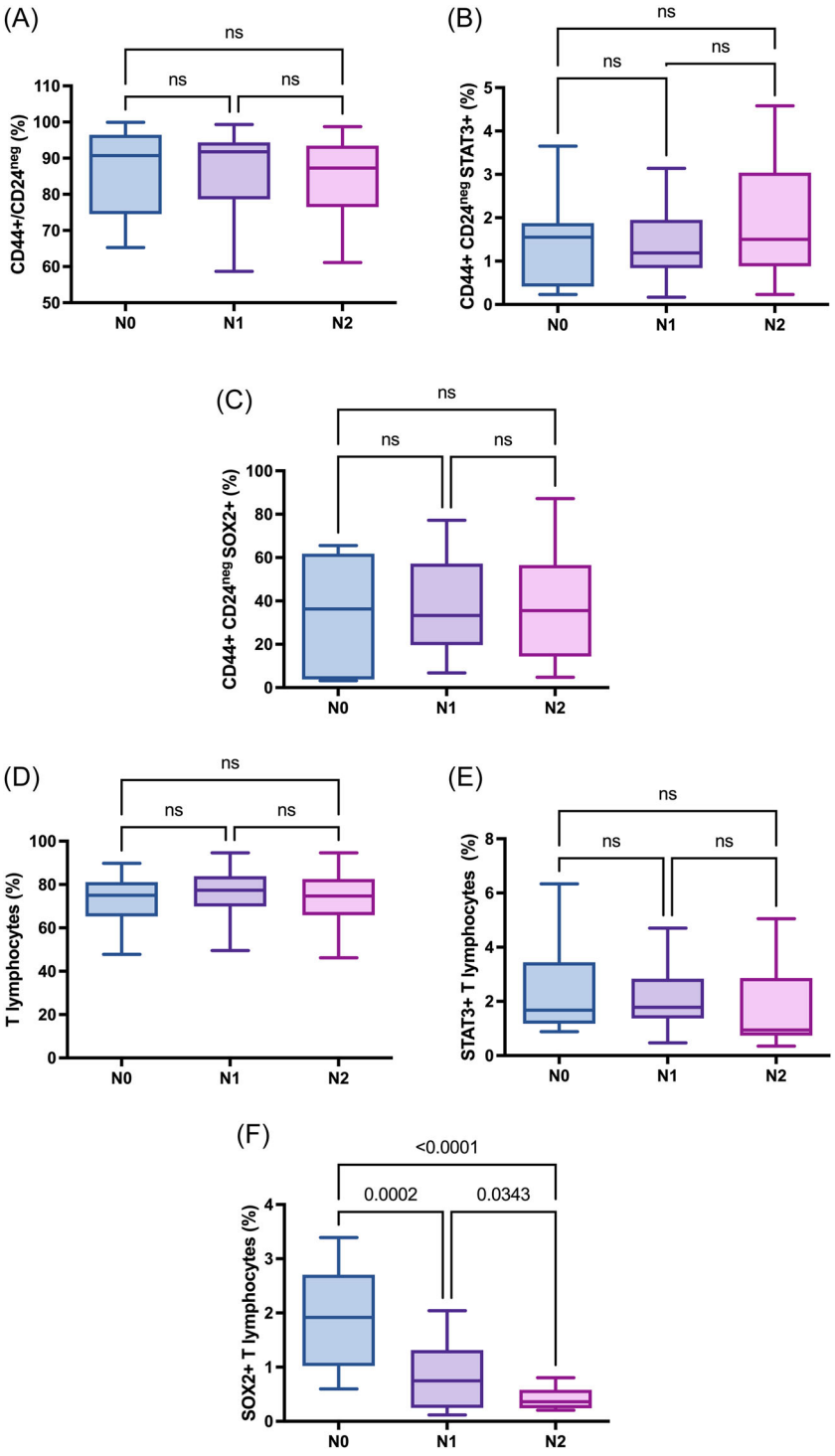

FIGURE 2 Analysis of CD44+/CD24^{neg}, CD44+/CD24^{neg}/SOX2+, and CD44+/CD24^{neg}/STAT3+, T lymphocytes, and STAT3 T lymphocytes and SOX2 T lymphocytes in peripheral blood of women with locally advanced breast cancer (LA; stage IIIA/B, $n = 51$) and metastatic (stage IV, $n = 14$). The Mann–Whitney test was used. It was considered $p < 0.05$.

When the expression of the marker of tumor stem cells is evaluated with the tumor subtypes, we found a direct relationship with the increase of CD44 + CD24^{neg} expression in all subtypes of BC compared to the control, justifying the relationship between the high potential of the biological tumor behavior and the aggressiveness of the neoplastic disease. These findings are similar to Chekhum et al.,[38] that evaluated 132 patients with BC and the expression of CD44^{+}CD24^{neg} by immunohistochemistry in the different tumor subtypes. These authors showed that 44.8% of the patients with TN had high levels of CD44+CD24^{neg} which were associated with survival and the presence of metastases. There is a direct relationship between tumor aggressiveness, independent of the stage at which the patient is found, with the TN subtype and the high potential for metastasis. This marker can be predictive of an indication of aggressiveness and justifies the relationship between the findings of metastatic diseases with the presence of stem cell markers.[22,38] A surface marker for stem cells, such as CD44+, which is already associated with more aggressive tumors, may be a predictive factor

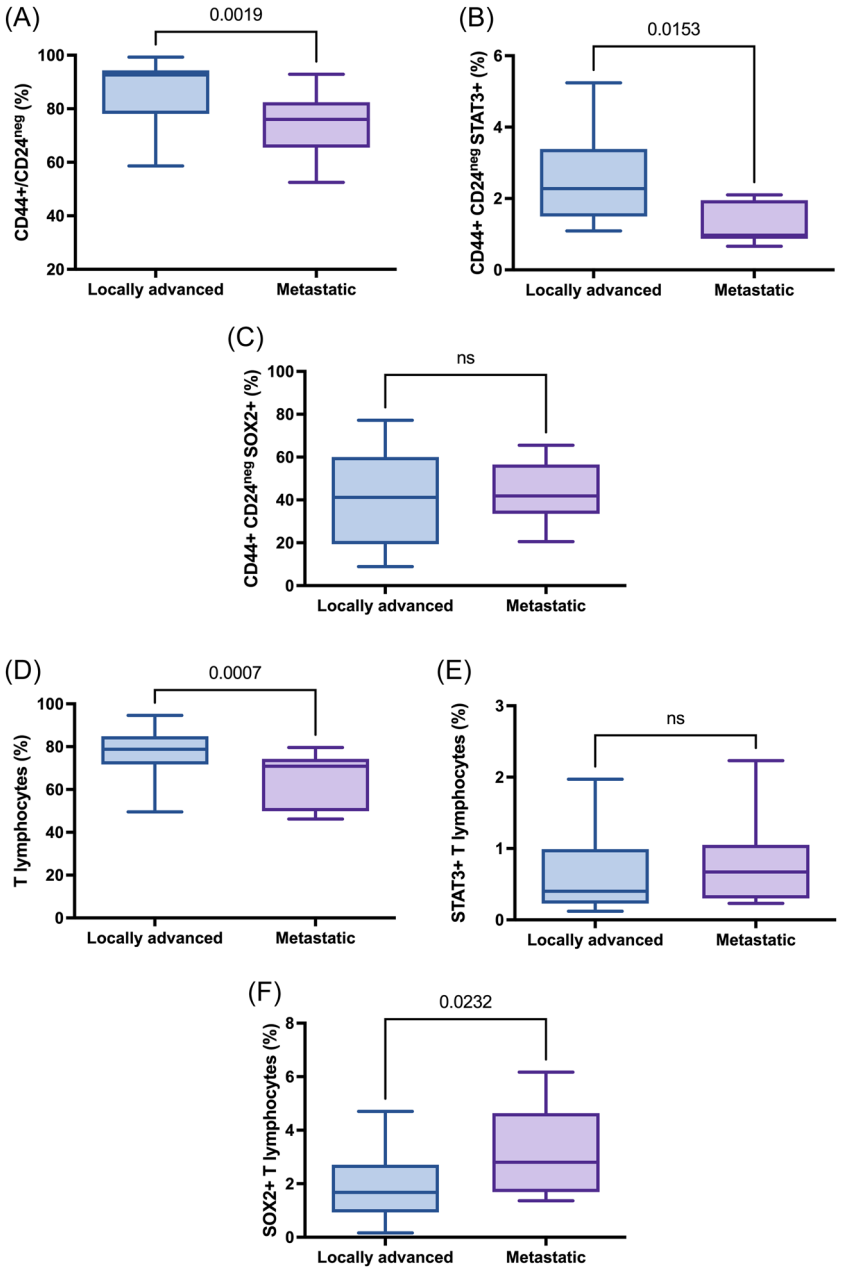

FIGURE 3 Analysis of CD44+/CD24^{neg}, CD44+/CD24^{neg}/SOX2+, and CD44+/CD24^{neg}/STAT3+, T lymphocytes, and STAT3 + T and SOX2 + T in peripheral blood of breast cancer women with axillary lymph node-negative - N0 (n = 10), and axillary lymph node-positive [N1 (n = 35) and N2 (n = 20)]. The Kruskal–Wallis test was used. It was considered $p < 0.05$.

for a worse prognosis, and it is possible that the appearance of drugs that block this molecule may serve as targeted therapy and may change the prognosis of the BC. Tumor stem cells show a CD44+/CD24^{neg} phenotype and express greater tumor heterogeneity and resistance to current treatments.[39]

Some authors have demonstrated that in the luminal subtypes resistance to hormonal treatments may occur and evolve into more aggressive clinical presentations, showing an association between the resistance to the treatment of this subtype with the high expression of tumor stem cell markers, CD44^{high} expression was related to resistance to standard treatment and advanced staging of the luminal subtypes.[40] Prospective studies with a greater number of cases, the real clinical significance of the CD44+ findings in peripheral blood can be determined. In 2007, some authors reported the relationship of CD44 to induction of hepatic metastasis and soluble expression of CD44 in animal models.[41] These authors described the pathways that activate CD44+ and promote tumor invasion in BM.[42]

To evaluate the STAT3 molecule in the BC, several authors have presented the relationship between the activation of this molecule with proliferation, survival and angiogenesis, after stimulation by several kinases and growth factors that subsequently are responsible for dimerization and activation. Through the tyrosines kinases, the STAT3 molecule can be translocated from the cytoplasm to the nucleus and at specific targets regulate gene transcription.[43]

In the evaluation of specific targeted therapies, HER2+ tumors are studied and evaluated for the activation of the STAT3 molecule and has been presented by some authors as a mechanism developed for resistance to existing drug treatment, this activation of STAT3

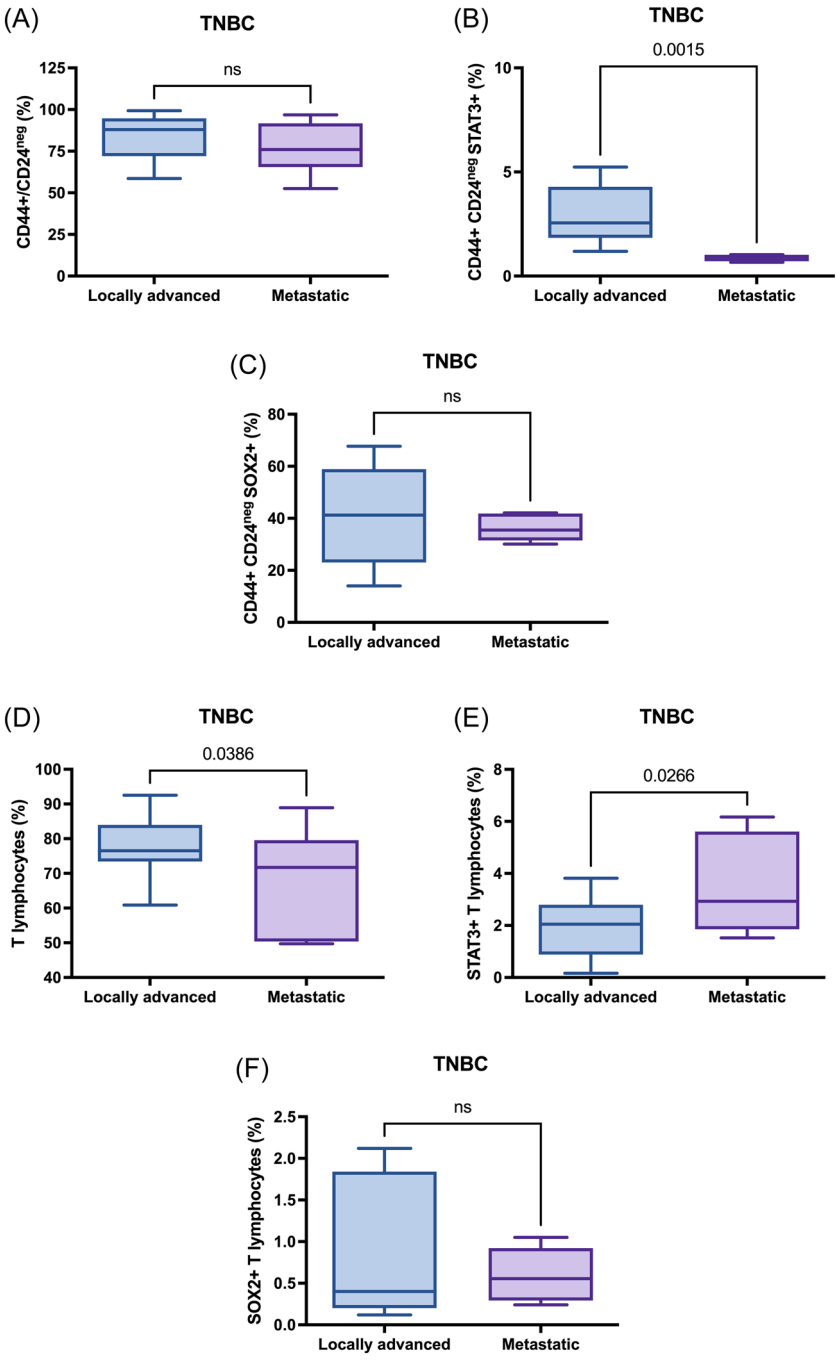

FIGURE 4 Analysis of CD44+/CD24^{neg}, CD44+/CD24^{neg}/SOX2+, and CD44+/CD24^{neg}/STAT3+, T lymphocytes, and STAT3 + T and SOX2 + T in peripheral blood of women with triple-negative breast cancer (TNBC, $n = 29$) locally advanced (LA; stage IIIA/B, $n = 20$) and metastatic (Stage IV, $n = 9$). The Mann–Whitney test was used. It was considered $p < 0.05$.

seems to work as a trigger for activating other molecules. This route may also be the subject of new treatments in the future, which will try to prevent the progression and spread of BC.[44]

In our study, we observed an increase in elevated levels of CD44+/CD24^{neg}STAT3+ cells and T lymphocytes in the blood of patients with locally advanced TNBC compared to the group with metastatic disease. It is believed that increased levels of STAT3 may be associated with the continuous process of activation of tumor mechanisms associated with the production of cell growth factors and division.[45,46]

STAT3 appears to participate as a molecule responsible for the immune response, preventing immunosuppression. The STAT3 molecule is related to the antiapoptotic and pro-proliferative expression of oncogenes, but it also participates in the modulation of the host's immune responses and all these activities regulate the process of proliferation, invasion, angiogenesis, and metastasis. This molecule participates in the process of immunosuppression. This immunosuppression is due to the potent negative regulation of T helper lymphocytes which is also an important gene activator crucial for immunosuppression.[45,46]

Yu et al.[47] have demonstrated that constitutive activation of STAT3 leads to an inhibition of the expression of mediators necessary for the activation of the immune response against tumor cells, in addition, the activity of STAT3 promotes the production of immunosuppressive factors that activate more STAT3 molecules in various subsets of

immune cells, altering gene expression programs and thereby inhibiting antitumor immune responses.[47]

Alterations in the regulation of STAT3 may result in an abnormal acute phase response, leading to elevated production of cytokines that may cause suppression of the immune response in the tumor microenvironment. This in turn can provide an ideal scenario for tumor avoidance and metastasis.[48] In this study, low STAT3 + T lymphocytes levels were observed in HER2+ and TN patients compared with controls. Feng et al. showed that STAT3 is one of the main activation pathways for differentiation in TH17 cells, being involved in tumor progression and growth of tumor-associated regulating T cells.[49]

In the analysis of the group of patients, we observed elevated levels of CD44+/CD24^{neg} STAT3+ cells and low levels of STAT3 + T lymphocytes in the locally advanced TNBC patients when metastatic disease. STAT3 is super-expressed and constitutively activated at TNBC, which is highly related to TNBC initiation, progression, metastasis, chemotherapy resistance, and unfavorable survival results. STAT3 is not only able to induce gene expression related to cancer but also interacts physically and cooperates functionally with other oncogenic transcription factors, promoting the aggressiveness of TNBC.[50]

In that study, high levels of SOX2 + T lymphocytes were observed in patients with locally advanced compared metastatic disease. The activated SOX2 molecule is present in the Treg lymphocytes in the process of tumorigenesis and promotes alterations in the antitumor mechanisms of the hosts, besides, these increased levels may signal an unfavorable evolution for these patients. SOX2 functions as a transcription factor in the nucleus to regulate oncogenic genes and when overexpressed participates in tumorigenesis, angiogenesis, and the processes of invasion and metastasis. However, there are mechanisms specific to controls that explain the self-renewal and pluripotency of embryonic stem cells that are regulated by a highly integrated network of essential transcription factors, in which SOX2 is included as the key molecule for all tumorigenesis mechanism.[51,52]

Percentage levels of CD44^{+}/CD24^{neg} and CD44+representing/CD24 neg/SOX2+ were elevated in patients with BC compared to controls. In several studies, the relationship of CD44^{+}/CD24^{neg} with tumor stem cells has been demonstrated, and SOX2 activates genes and interferes with ontogenetic mechanisms.[38,52,53]

In our study, the CD44+/CD24^{neg}/SOX2+ percentage levels were raised in the blood of patients with Luminal B, HER2+ and TN breast cancer compared to the control group. In tumor tissue, a relation of CD44 and SOX2 expression in the process of tumorigenesis, invasion and metastasis by BC were observed.[21] Huang et al.[24] compared the clinical characteristics of patients with BC and axillary metastases that had high expression of SOX2, being significant the expression of SOX2 and independent of the other common prognostic factors including degree, tumor size, RE, and status of HER2. In tumors subtype TBNC, several authors have attempted to link paclitaxel treatment to SOX2 gene silencing to promote decreased invasiveness, inhibit tumor stem cell growth and inhibit drug resistance in patients with TBNC tumors.[16] The overexpression of SOX2 is associated with the progression of breast cancer through cell proliferation and tumorigenesis, as well as recurrences and metastases.[27]

There is a search for noninvasive markers that may be used for cancer diagnosis and prognosis. STAT3 and SOX2 have been described as important transcription factors for tumor progression. These molecules in the BC may be related to the worst prognosis and are potential biomarkers in breast tumors, their use being indicative of a response to chemotherapy. According to a study by Sodja et al.,[54] the expression of SOX2 mRNA in peripheral blood was a promising marker for molecular screening of head and neck cancer and an important prognostic marker in patients with advanced disease treated with neoadjuvant chemotherapy, indicating an important role in the dissemination of circulating tumor cells and metastatic disease.

It is important to stress that studies comparing levels of markers in the blood with quantitative methodologies reduce the biases of the analysis of tumor tissue by an immunohistochemical technique because it is a semi-quantitative method. In addition, the investigation of these markers in peripheral blood has some advantages such as being less invasive, easy to access and making possible analyzes right from diagnosis, treatment, and relapse. This study expands the knowledge about the participation of SOX2+, STAT3+, CD44, and CD24 in breast cancer and shows the potential of these molecules for new therapeutic approaches.

5 | CONCLUSIONS

Based on the results of this study, we can conclude that the levels of CD44+CD24^{neg}, CD4+CD24^{neg}STAT3+and CD44+CD24^{neg} SOX2+, SOX2 + T, and STAT3 + T cells in peripheral blood of BC women are altered independent of the tumor subtype. STAT3+ and SOX2+ levels are different between locally advanced and metastatic disease, and they are potential prognostic biomarkers, and possible therapeutic targets for breast cancer.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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