

Original Article

## A STUDY ON TUMOR-INFILTRATING LYMPHOCYTES IN DIFFERENT SUBTYPES OF BREAST CANCER

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### Summary

The study aimed to investigate immune cell infiltration in different subtypes of breast cancer (BC). Retrospectively were selected 100 patients with primary BC, grouped into four molecular surrogate subtypes (Luminal A and Luminal B-like, HER2-positive and triple-negative - TN), determined by immunohistochemistry (IHC). In each patient, a percentage of stromal tumor-infiltrating lymphocytes (TILs) was determined by hematoxylin-eosin staining. IHC was performed using primary antibodies CD3, CD4, CD8, CD20, and FOXP3. Immunophenotyped lymphocytes were counted (separately intratumoral and stromal) and semi-quantitatively graded. In the studied tumors, 10% were defined as lymphocyte-predominant BC. A high count of intratumoral and stromal TILs subsets was found mainly in TN and HER2-positive BC. The stroma is the preferred localization for immune cells in all four BC subtypes. CD3+ T predominates over CD20+ B lymphocytes, with CD8+ T cytotoxic and FoxP3+ T regulatory cells dominating T subtypes. HER2 and TN are more immunogenic than Luminal A and Luminal B – like subtypes of BC. The T-cells' immune response was predominant in the studied cases of BC, with a predominance of CD8+ Tc and Foxp3+ Treg cells located mainly in the stroma.

**Keywords:** breast cancer, tumor-infiltrating lymphocytes, immunohistochemistry

### Introduction

The immune system (IS) is responsible for maintaining tissue homeostasis through coordinated activation of innate and acquired immune mechanisms. Neoplastic processes are characterized by tissue and cellular structural and functional changes that induce an immune response (IR) [1]. Tumor-infiltrating lymphocytes (TILs) are local IR directed against tumor growth and progression and are considered an independent favorable prognostic indicator in many malignancies [2,3].

Despite IR, many cases of breast cancer (BC) progress, which calls into question the immune cell infiltrate's adequate function into the tumor microenvironment in this type of neoplasm [2]. The existence of insufficiently effective antitumor immune activity suitable for immune modulation may be

suspected [4].

The role of immunosuppression in the evolution of cancer is still being studied. Many immunosuppressive factors are present in the tumor, whose presence could be a normal host response to antitumor immunity regulation and/or the presence of tumor-induced immunosuppression [4,5].

Quantification of TILs in BC itself does not reflect the dynamics and functionality of the tumor microenvironment. A better understanding of their role requires determining the lymphocyte phenotype and comparing the different TILs subtypes to BC subtypes' clinical and pathological characteristics.

## Materials and methods

### Patients

Retrospectively, 100 patients with primary BC were selected, divided into four groups of 25 patients each, according to the molecular surrogate subtypes: Luminal A, Luminal B-like, HER2-positive, and triple-negative - TN.

The patients studied were randomly selected from archival lists of the Department of General and Clinical Pathology at the Georgi Stranski University Hospital -Pleven, based on selecting tumors from archival materials. The materials contained a sufficient amount of tissue samples, and research would in no way threaten the samples with damage and exhaustion.

By the time the patients were selected, we had examined 290 cases of BC, diagnosed in the period 31.12.2014-06.01.2011. All the patients selected for the study had no evidence of inflammatory diseases or other conditions associated with an inflammatory reaction in the breast. The cases in which preoperative antitumor therapy had been given, those for which only specimens from core biopsies were available or archival materials were missing, were excluded.

Hematoxylin and eosin (HE), ER, PR, HER 2, and Ki67 staining archival slides of the patients were examined. One histological slide was selected for each patient to assess the percentage of stromal TILs (TILs %) in HE staining. We prepared slides to evaluate the TILs subtype populations in staining with immunohistochemistry (IHC) from its corresponding paraffin block.

The ethics commission at the Medical University - Pleven approved the projects. All patient data were summarized and coded, and tissue samples were used without identifying the cases studied.

Demographic data and the clinicopathological factors studied (the type of surgery, tumor stage, histological degree of differentiation (grade - G), histological variant, lymph node (LN) status, lymphovascular invasion (LVI), values for ER, PR, HER2, and Ki-67) were recorded on a questionnaire specially designed for the study.

### ***Histological examination (following the recommendations current at the time of the study)***

The cases of BC were subtyped according to the WHO histological classification [6]. The Nottingham grading system (modified Elston & Ellis scheme, 1991) was used to grade the invasive carcinomas [7]. The tumors were staged according to the 7th revision of the TNM classification of the American Joint Committee on Cancer (AJCC) and the Union for International Cancer Control (UICC) from 2010 [8]. Microscopic evaluation of ER, PR, and HER-2 / neu was performed according to the recommendations of the American Society of Clinical Oncology / College of American Pathologists Guidelines (ASCO/CAP guidelines) [9,10]. The interpretation of the IHC results for Ki67 was performed according to the recommendations of the Working Group on BC [11].

The clinically pathological surrogate subtypes of BC were defined based on the IHC results for ER, PR, HER-2 / neu, and Ki67, according to the St Gallen international expert consensus for primary treatment of early BC 2013 [12].

The microscopic evaluation of TILs in routinely stained (HE) tissue sections was based on the recommendations of the International TILs Working Group 2014, with computer-assisted scoring of the percentage of stromal TILs (TILs %) [1].

### ***Immunohistochemical examination***

From formalin fixed-paraffin embedded tissue specimens, tissue sections were made, with a thickness of 3-4µm, placed on 7109-A Silanized Microscope slides.

For the IHC study, a visualization system EnVision™ FLEX, High pH (DAKO), AutostainerLink 48 technique (DAKO), and the following primary antibodies were used: CD3 (Polyclonal, Rb, RTU, Dako, Glostrup, Denmark), CD4 (clone 4B12, Mo, RTU, Dako, Glostrup, Denmark), CD8 (clone C8 / 144B, Mo, RTU, Dako, Glostrup, Denmark), CD20 (clone L26, Mo, RTU, Dako, Glostrup, Denmark) and FoxP3 (clone 236A / E7, Mo, 1:100, Bioscience, San Diego, CA USA).

A computer-assisted scoring of positive IHC stained cells for CD3, CD4, CD8, CD20, and FOXP3 was performed independently by two pathologists who had no information about the clinical and pathological data of the studied cases.

The immunophenotyped intratumoral and stromal lymphocytes were counted separately and then were graded semi-quantitatively.

CD3 +, CD4 +, CD8 + and CD20 + TILs were scored in the tumor and stroma of five randomly selected fields, at high magnification (high power fields - HPF), x400. Results with up to 25 IHC -positive cells were considered as low, and more than 25 positive cells were considered as high TILs values.

FoxP3-positive lymphocytes were also scored semi-quantitatively in the tumor and stroma, but in at least ten fields of tumor area, at high magnification. The presence of <15 and ≥ 15 FoxP3-positive cells was reported as low or high FoxP3 expression, respectively.

### **Statistical analysis**

The obtained results were summarized and statistically analyzed by software (IBM SPSS Statistics V21).

The frequency of the studied clinicopathological characteristics and the semi-quantitatively graded intratumoral and stromal TILs subtypes was determined using the Frequency Table. Crosstab and Chi-Square Test were used to determine the correlations between BC subtypes (despite the small number of cases studied from each molecular surrogate BC subtype) and the results for the number and localization of TILs subsets. The same methods were used to search for correlations between the reported results and the clinicopathological characteristics of the individual BC

subtypes. The association between TILs subsets in the different molecular subtypes of BC was investigated by Spearman's rho test.

A value of  $p < 0.05$  was considered statistically significant.

## **Results**

### **Data on the studied patients**

Most of the studied patients were over 45 years of age (91.0%). Mastectomies accounted for the majority (76.0%) of the surgical interventions. Most of the tumors were classified as invasive ductal (no special type - NST) carcinoma (83.0%), and the remainder were defined as lobular (9.0%) or other particular morphological types of BC (8.0%). Tumors with low and moderate malignancy (G1 and G2) predominated in number (n = 54) compared to those with high grade (G3) (n = 46). LVI was found in 24 cases; 41 of the cases were with a negative status of the axillary LN, and 37 - with a positive one. The majority of patients (52%) were in the T1-T2 tumor stage.

The distribution of TILs subsets, depending on their localization and amount in all the BC cases studied (n = 100), are presented in Table 1.

The distribution of TILs subsets in the different BC molecular subtypes (n = 25) is shown in Table 2-5.

When the distribution of TILs was studied, we found that in all subtypes of BC, B-lymphocytes (CD20+) and T-lymphocytes (CD3+) were more common in the stromal than in the intratumoral area, where their concentration was high. The subtypes of T-lymphocytes (CD4+, CD8+, and FoxP3+) and their high concentration were less often found intratumorally than in the stroma in Luminal A and HER2-positive BC. The same relationship was found for T-helpers (CD4+) and T-cytotoxic cells (CD8+) in Luminal B and T-helpers (CD4+) in TNBC. There was no significant difference in the distribution of TILs (intratumoral versus stromal) as far as semi-quantitatively graded regulatory T lymphocytes (FoxP3+) in Luminal B and with CD8+ and FoxP3+ in TNBC.

It is noteworthy that the high intratumoral concentration of TILs subsets was not observed in any of the studied cases with BC of Luminal A subtype (see Table 2). High intratumoral

**Table 1.** Results for the amount and localization of TILs in the studied tumors (n = 100)

Intratumoral	n=100 (%)	Stromal	n=100 (%)	$\chi^2$	df	p
CD3				58.94	2	<0.0001
0	10 (10.0)		2 (2.0)			
Low	76 (76.0)	Low	31 (31.0)			
High	14 (14.0)	High	67 (67.0)			
CD4				69.55	2	<0.0001
0	65 (65.0)		9 (9.0)			
Low	35 (25.0)	Low	84 (84.0)			
High	0	High	7 (7.0)			
CD8				36.63	2	<0.0001
0	20 (20.0)		1 (1.0)			
Low	72 (72.0)	Low	62 (62.0)			
High	8 (8.0)	High	37 (37.0)			
FoxP3				28.49	2	<0.0001
0	25 (25.0)	0	5 (5.0)			
Low	70 (70.0)	Low	68 (68.0)			
High	5 (5.0)	high	27 (27.0)			
CD20				52.99	2	<0.0001
0	41 (41.0)	0	3 (3.0)			
Low	59 (59.0)	Low	80 (80.0)			
High	0	High	17 (17.0)			
TIL (%)						
≤ 50%			90 (90.0)			
> 50%			10 (10.0)			

**Table 2.** Results for the amount and localization of TILs in Luminal A subtype of BC (n = 25)

Intratumoral	n=25 (%)	Stromal	n=25 (%)	$\chi^2$	df	p
CD3				21.12	2	<0.0001
0	4 (16.0)					
Low	21 (84.0)	Low	11 (44.0)			
High	0 (0)	High	14 (56.0)			
CD4				24.74	2	<0.0001
0	18 (72.0)		1 (4.0)			
Low	7 (28.0)	Low	23 (92.0)			
High	0	High	1 (4.0)			
CD8				13.03	2	0.0015
0	6 (24.0)		0 (0)			
Low	19 (76.0)	Low	18 (72.0)			
High	0 (0)	High	7 (28.0)			
FoxP3				17.06	2	0.0002
0	11 (44.0)	0	0 (0)			
Low	14 (56.0)	Low	20 (80.0)			
High	0 (0)	High	5 (20.0)			
CD20				16.29	2	0.0003
0	13 (52.0)	0	1 (4.0)			
Low	12 (48.0)	Low	20 (80.0)			
High	0 (0)	High	4 (16.0)			
TIL (%)						
≤ 50%			25 (100.0)			
> 50%			0 (0)			

**Table 3.** Results for the amount and localization of TILs in Luminal B subtype of BC (n = 25)

Intratumoral	n=25 (%)	Stromal	n=25 (%)	$\chi^2$	df	p
CD3				18.79	2	<0.0001
0	2 (8.0)					
Low	20 (80.0)	Low	7 (28.0)			
High	3 (12.0)	High	18 (72.0)			
CD4				12.52	2	0.0019
0	13 (52.0)		2 (8.0)			
Low	12 (48.0)	Low	21 (84.0)			
High		High	2 (8.0)			
CD8				10.48	2	0.0053
0	6 (24.0)		0 (0)			
Low	17 (68.0)	Low	16 (64.0)			
High	2 (8.0)	High	9 (36.0)			
FoxP3				5.69	2	0.0581
0	3 (12.0)	0	0 (0)			
Low	21 (84.0)	Low	20 (80.0)			
High	1 (4.0)	High	5 (20.0)			
CD20				14.19	2	0.0008
0	12 (48.0)	0	1 (4.0)			
Low	13 (52.0)	Low	21 (84.0)			
High	0 (0)	High	3 (12.0)			
TIL (%)						
≤ 50%			24 (96.0)			
> 50%			1 (4.0)			

**Table 4.** Results for the amount and localization of TILs in HER2-positive subtype of BC (n = 25)

Intratumoral	n=25 (%)	Stromal	n=25 (%)	$\chi^2$	df	p
CD3				16.45	2	0.0003
0	1 (4.0)		1 (4.0)			
Low	20 (80.0)	Low	6 (24.0)			
High	4 (16.0)	High	18 (72.0)			
CD4				21.17	2	<0.0001
0	19 (76.0)		3 (12.0)			
Low	6 (24.0)	Low	20 (80.0)			
High	0 (0)	High	2 (8.0)			
CD8				10.29	2	0.0058
0	3 (12.0)		0 (0)			
Low	20 (80.0)	Low	14 (56.0)			
High	2 (8.0)	High	11(44.0)			
FoxP3				9.52	2	0.0086
0	4 (16.0)	0	2 (8.0)			
Low	20 (80.0)	Low	13 (52.0)			
High	1 (4.0)	High	10 (40.0)			
CD20				10.10	2	0.0064
0	6 (24.0)	0	0 (0)			
Low	19 (76.0)	Low	21 (84.0)			
High	0 (0)	High	4 (16.0)			
TIL (%)						
≤ 50%			20 (80.0)			
> 50%			5 (20.0)			

**Table 5.** Results for the amount and localization of TILs in TN subtype of BC (n = 25)

Intratumoral	n=25 (%)	Stromal	n=25 (%)	$\chi^2$	df	p
CD3				8.08	2	0.0176
0	3 (12.0)		1 (4.0)			
Low	15 (60.0)	Low	7 (28.0)			
High	7 (28.0)	High	17 (68.0)			
CD4				13.33	2	0.0013
0	15 (60.0)		3 (12.0)			
Low	10 (40.0)	Low	20 (80.0)			
High	0 (0)	High	2 (8.0)			
CD8				5.37	2	0.0682
0	5 (20.0)		1 (4.0)			
Low	16 (64.0)	Low	14 (56.0)			
High	4 (16.0)	High	10 (40.0)			
FoxP3				2.20	2	0.2019
0	7 (28.0)	0	3 (12.0)			
Low	15 (60.0)	Low	15 (60.0)			
High	3 (12.0)	High	7 (28.0)			
CD20				13.64	2	0.0011
0	10 (40.0)	0	1 (4.0)			
Low	15 (60.0)	Low	18 (72.0)			
High	0 (0)	High	6 (24.0)			
TIL (%)						
≤ 50%			21 (84.0)			
> 50%			4 (16.0)			

concentrations were found in a small number of cases in Luminal B and HER2 - positive BC (see Table 3 and 4). Of all the BC subtypes, the high concentration of TILs intratumorally was mostly established in the TN subtype (see Table 5).

High stromal concentrations of TILs subspecies were found in most BC cases with TN and HER2-positive, compared with those with Luminal A and Luminal B (see Table 2-5).

### **Predominant subsets of lymphocytes in the subtypes of BC**

In Luminal A subtype BC, CD3 + T-lymphocytes predominated over CD20 + B-lymphocytes intratumorally ( $\chi^2 = 5.70$ ; Df = 1; p = 0.0196) and stromally ( $\chi^2 = 9.17$ ; Df = 2; p = 0.0102). CD3 + T-lymphocytes predominated over CD20 + B-lymphocytes intratumorally and in Luminal B subtype ( $\chi^2 = 11.63$ ; Df = 2; p = 0.0030); HER2- positive ( $\chi^2 = 7.60$ ; Df = 2; p = 0.0224) and TN ( $\chi^2 = 10.77$ ; Df = 2; p = 0.0046) BC.

Like in Luminal A BC, CD3 + T lymphocytes predominated over CD20 + B lymphocytes in the tumor stroma in other surrogate types of BC: Luminal B type ( $\chi^2 = 18.71$ ; Df = 2; p = 0.0001); HER2 positive ( $\chi^2 = 18.24$ ; Df = 2; p = 0.0001)

and TN ( $\chi^2 = 10.10$ ; Df = 2; p = 0.0064) BC.

CD8-T cytotoxic (Tc) and FoxP3-T regulatory cells (Treg) dominated among the T-subtypes, and the domination had a statistically significant value in Luminal B, HER2, and TN subtypes of BC - Table 6.

B-cell CD20 + humoral IR was not a prominent feature in BC and is found mainly in the HER2-positive subtype, while the highest concentration of B-lymphocytes was found in the TN BC subtype (see Table 2 - 5).

### **Correlations between the subtypes of lymphocyte infiltrate**

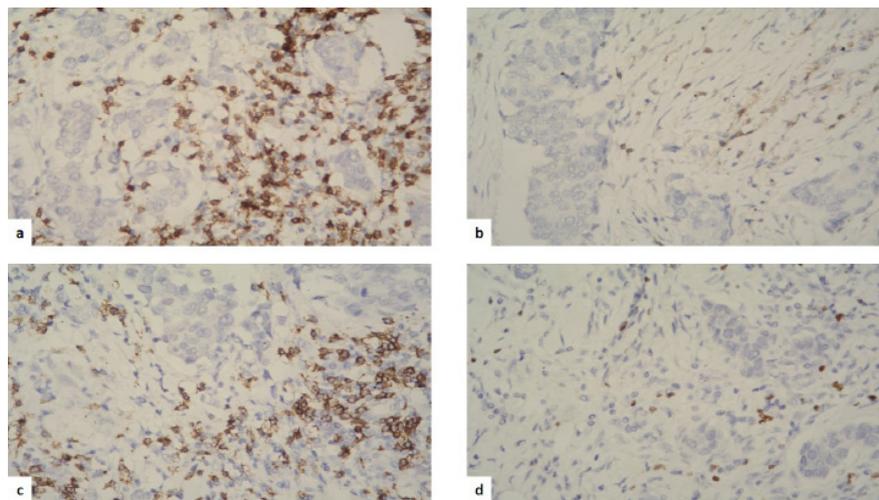
CD8 + Tc and Foxp3 + Treg cells are more commonly seen together intratumorally in Luminal B, HER2-positive, and TN BC subtypes, and stromally in the Luminal B and TN BC ones.

### **Immunogenicity of BC subtypes**

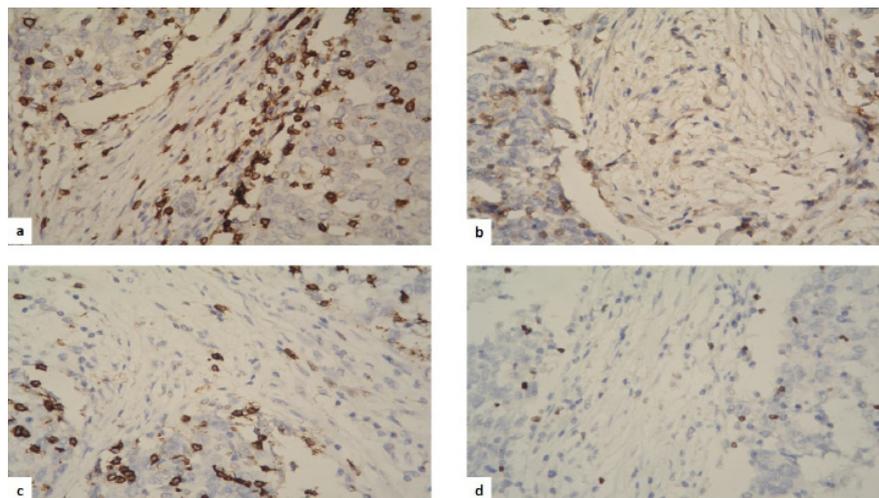
Despite the small number of cases studied from each surrogate BC subtype, it was found that a high concentration of intratumoral and stromal lymphocytes was found predominantly in TN, followed by HER2-positive BC - Figures 1 and 2.

**Table 6.** Statistically significant correlations between lymphocyte subspecies in different molecular subtypes BC

Intratumoral		BC subtype	p	Stromal		BC subtype	p
CD3	CD8			CD3	CD8		
		Luminal B	0.001			Luminal B	0.018
		TN	0.002				
CD3	FoxP3			CD3	FoxP3		
		Luminal B	0.008			HER-2	0.011
		HER-2	0.035				
		TN	0.010				
CD8	FoxP3			CD8	FoxP3		
		Luminal B	0.002			Luminal B	0.021
		HER2	0.001			TN	0.008
		TN	0.001				



**Figure 1.** Microscopic evaluation of IHC stained T-lymphocyte subtypes (a - CD3; b - CD4; c – CD8; d- FoxP3) in HER2-positive BC, x400



**Figure 2.** Microscopic evaluation of IHC stained T-lymphocyte subtypes (a - CD3; b - CD4; c – CD8; d- FoxP3) in TNBC, x400

**BC with TIL > 50% (Lymphocyte-predominant BC - LPBC)**

Of the tumors studied, 10% were defined as LPBC: HER2-positive (n = 5), TN (n = 4) and Luminal B (n = 1).

**Correlations of TIL with the clinical and pathological characteristics – see Table 6**

A statistically significant association was found between the number of stromal CD8-

Tc lymphocytes and lymphovascular invasion (LVI) in HER2-positive tumors ( $p = 0.014$ ). In this latter subtype of BC, 28% had LVI, of which 85.7% had a high count of stromal CD8 Tc lymphocytes.

A correlation was found between the number of stromal FoxP3-Treg lymphocytes and the histological grade (G) ( $p = 0.015$ ) in the Luminal B subtype. A high number of stromal FoxP3+ cells was found in 20% of the Luminal B cases, all of which (100%) were G3 BC.

No statistically significant correlation was found between the lymphocyte subspecies and any clinico-pathological parameters studied in Luminal A and TN BC.

## Discussion

Most studies have reported that stromal TILs are a better and more reproducible parameter than intratumoral TILs [1,13,14]. The main reason for this difference is that intratumoral TILs are presented less frequently, in smaller quantities, and are difficult to identify in routine staining. IHC allows for their easy visualization and enhances the information obtained in the determination of stromal TILs [15-17].

In all four molecular subtypes of BC we studied, the major subtypes of immune cells involved in cellular and humoral IR (CD3 + T- and CD20 + B-lymphocytes, respectively) were localized mainly in the stroma. The stroma is also a preferred site for T-lymphocytes (helper CD4 +, cytotoxic CD8 + and regulatory FoxP3 +) in Luminal A and HER2-positive BC subtypes, as well as for CD4 + and CD8 + in Luminal B and CD4 + in TNBC. No significant difference was found between the intratumoral and intrastromal distribution of TILs, concerning semi-quantitatively graded regulatory T lymphocytes (FoxP3 +) in Luminal B and with CD8 + and FoxP3 + in TNBC.

There is accumulated evidence supporting the clinical significance of IR in cancer. Data from clinical trials have demonstrated the potential of immunotherapy, and increased survival rates have been reported in patients with urothelial carcinoma [18], lung cancer [19], and others [1]. Immunotherapy for carcinomas is a possibility for effective treatment by overcoming the phenomenon of immunosuppression - an essential factor involved in the process of tumor

development and progression of neoplasms. Active immunotherapy aims to modify the amount or the elements comprising the subgroups of lymphocytes and reactivate antitumor IR, thus improving the outcome of the disease [20].

Clarifying the role of IS and modulation of IR promise new therapeutic opportunities for BC, including TNBC, given the lack of targeted molecular treatment in this aggressive subtype of BC [21]. Cytotoxic treatment, such as chemotherapy and radiation therapy, can stimulate and potentiate the body's immune reactivity, inducing a specific response [22] and immunogenic tumor cell death as an end-result [1,4,17,21,23]. Improved survival has been reported in patients with metastatic TNBC on immunotherapy and chemotherapy [24]. The clinical significance of the synergistic effect of combining therapy with other treatment regimens is studied [16].

The complex of cells involved in antitumor immunity is an essential functional factor [17]. It varies depending on the type and organ localization of the neoplasm [1]. For evaluating TILs subtypes in BC, the IHC method with the most commonly used markers, such as CD20, CD3, CD4, CD8, and FoxP3, identifying B, T, and T-lymphocyte subtypes is preferable [20,25].

The CD3 antigen is a receptor glycoprotein found in mature T lymphocytes. The CD4 antigen is a glycoprotein on the surface of helper T cells (Th), regulatory T cells, monocytes, and macrophages. The CD8 antigen is also a T-cell receptor glycoprotein [2] expressed in T-cytotoxic (Tc) cells. CD20 is a transmembrane protein in B-cell precursors and mature B cells, which is lost after differentiation into plasma cells [25].

Regulatory T lymphocytes (Treg) are a specific CD4 + T-cell population that suppresses the activation of other immune cells and maintains systemic immune homeostasis. Treg cells also play an essential role in suppressing tumor-associated antigen-specific immunity. Foxp3 (Forkhead Box Protein 3) - positive lymphocytes are a more noticeable subpopulation of immunosuppressive regulatory T cells. It has been suggested that FoxP3 levels may be an indicator of tumorigenesis in BC [5]. There are conflicting data on their clinical significance in patients with this neoplasm [5, 22, 26, 27].

Like in other studies [23, 25], we found that T-cell-mediated immunity was a leading factor in the antitumor response in BC. Humoral B-cell immunity was less pronounced. In all the four molecular subtypes we studied, cases with T-cell CD3 expression (intratumoral and stromal) predominated over those with CD20-positive B lymphocytes. There were more cases with a high concentration of CD3 + T-lymphocytes than cases with high CD20 + B-lymphocyte infiltration. Although they were detected in a smaller number of tumors, most cases of B-lymphocyte expression were detected in the HER2-positive BC subtype, while high concentrations of B-lymphocytes were most commonly found in TNBC.

In our study, CD8-Tc and FoxP3-Treg cells were the predominant subtypes of CD3 + T-lymphocytes in all four molecular subtypes of BC, and it was shown that these subtypes of TILs are more commonly seen together. Statistically significant values are presented in Table 6. The large number of cytotoxic CD8-Tc cells found was probably a signal of an active IS attempt to reject the tumor, which had been unsuccessful due to their suppression by FoxP3 + Treg cells or to a suppressive effect of tumor cells specific to individual molecular subspecies BC. According to data from some studies, other TILs subspecies are predominant. In the literature, there are differences in the study design (selection of BC subtypes, the study of unequal numbers and different markers for lymphocyte infiltrate, use of different TILs detection techniques and methods for immunophenotyping) [2,25,28].

Among the BC subtypes we studied, TN had the most prominent lymphocyte infiltrate, followed by HER2-positive non-luminal BC. In these two subtypes, there was a high concentration of all TILs subsets in more cases as compared to the Luminal A and Luminal B types of BC. The data we obtained corresponds to those found in other studies, according to which these molecular subtypes of BC are more immunogenic than others [13,16,17,21,29,30].

The term lymphocyte-predominant BC (LPBC) pertains to tumors in which lymphocytes occupy more than 50% -60% of the stromal area in routinely stained tissue sections [1]. In the present study, 10% of the neoplasms studied were defined as LPBC: HER2-positive (n = 5),

TN (n = 4), and Luminal B (n = 1), and no case of Luminal A was assigned to this category. Similar results were obtained by Ohtani et al., using a cut-off for TILs of 30% and 50%. According to them, 8.2% of invasive BC are LPBC, with TN ranking first, followed by HER2 – positive, and Luminal B accounted for the smallest percentage. They reported no cases of Luminal A, defined as LPBC [29].

Identification of new reliable and long-term prognostic factors in BC remains a major unresolved issue [5,16,17,22,26,31]. The classic markers determine the possibility of progression in the first five years after therapy. After this period, their importance for long-term survival decreases, especially in patients with TNBC [5].

The prognostic significance of TILs and TILs subsets remains a long-standing topic of debate [2,16,17,22,26,27]. In some studies, the degree of lymphocyte infiltration assessed by routine staining was found to have prognostic value in TN and HER2 + BC, despite the lack of detailed information on immune subpopulations in the infiltrate [1,13,16,23,32,33]. Active and persistent antitumor IR potentially identifies more immunogenic tumors. The different numbers and compositions of lymphocytes reflect the unequal cell biology of carcinomas, with different mechanisms for generating immunological memory and the ability to control residual tumors effectively [1]. It is suggested that lymphocyte subtype scoring may contribute to a better prognosis in BC [5,14,17,22,26,27]. Finding a correlation between specific characteristics of TILs with clinical results and proven prognostic indicators is part of the complex process of including a biomarker in routine practice [3,15,16,25,26,28,31,34]. Additional studies are needed before introducing changes in daily clinical practice and using TILs and TILs subtypes as a biological prognostic biomarker [13,16,31].

Our results showed that lymphovascular invasion (LVI) was associated with a high concentration of stromal CD8-Tc in the HER2-positive subtype and a low histological degree of differentiation (G3) high count of stromal FoxP3-Treg in the Luminal B subtype. Therefore, two of the major functional components of tumor-associated IR: cytotoxic CD8 + and regulatory Foxp3 + T cells agree with adverse prognostic

factors such as LVI and G3 as found in other studies [6,7,35-37].

## Conclusions

The results obtained for the amount, localization and immune phenotype of TILs in the different subtypes of BC could be used for additional studies to:

- determine their significance as an independent prognostic marker;
- establish how the influence of these parameters can be used for immunomodulation and creation of an effective antitumor immune response;
- study their role as a predictive marker in selecting patients eligible for specific therapeutic regimens.

Clinical follow-up of patients and disease outcomes should be considered so that TILs and TILs subtypes can be used as a safe and long-term prognostic marker. An in-depth study of the association between the inflammatory infiltrate, and the effect of specific therapy is required to determine the predictive value of TILs.

The mechanisms by which immune cells interact with each other and with the tumor cell population remain unclear. Further investigations on lymphocyte markers in a larger number of patients with each type of BC are necessary.

Determining the amount of different immune cell populations and their localization in the tumor and comparing them with clinical and pathological data in different types of BC may serve as a basis for an in-depth analysis of the role of lymphocyte infiltration in the tumorigenesis and progression of this neoplasm. Patients with BC could be given a new treatment option by developing future therapeutic approaches based on specific biomarkers. The immune system is a promising new target for this goal.

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