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Can TILs be associated with prognostic factors and survival rates in breast cancer? A retrospective analysis

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ABSTRACT

Introduction: The relationship between the tumor inflammatory infiltrate, also known as tumor-infiltrating lymphocytes (TILs), and invasive breast carcinomas has been extensively studied in recent years to verify its association with prognosis and response to treatment. The goal of this study was to associate the presence of TILs with patient's survival time. **Methods:** We studied prognostic clinicopathological characteristics already established in the literature and their impact on overall five-year survival time of patients with invasive breast cancer treated at *Hospital Santa Casa* in Belo Horizonte, Minas Gerais, Brazil, in 2011 (n=290). This was an observational and retrospective study. **Results:** The presence of TILs was associated with tumors of no special type (p=0.018) and with younger age of the patients (p=0.042). Smaller tumor size (HR: 19.24; 95%CI 4.30–86.15; p<0.001), absence of metastasis to the axillary lymph nodes (HR: 2.80; 95%CI 1.02–7.70; p=0.002), positivity for progesterone receptor (HR: 0.39; 95%CI 0.17–0.87; p=0.022), and presence of TILs (HR: 0.23; 95%CI 0.08–0.65; p=0.005) were associated with longer survival times. **Conclusions:** This study suggests that the presence of TILs, along with other clinicopathological characteristics, is a prognostic factor in breast cancer.

KEYWORDS: survival analysis; breast cancer; immunohistochemistry; tumor-infiltrating lymphocytes; tumor biomarkers; prognostic factors.

INTRODUCTION

Breast cancer comprises a diverse group of lesions that differ in their microscopic presentation and biological behavior. Malignant breast tumors respond differently to cancer therapy[1,2].

Breast cancer is the most common malignancy among women and the leading cause of cancer-related deaths worldwide. In 2018, more than two million new cases were diagnosed, with more than six hundred thousand deaths[3]. Breast cancer surpasses lung cancer as the leading cause of cancer throughout the world in 2020, with an estimate of 2.3 million new cases, representing 11.7% of all cancer cases[3,4]. For the year 2023, 704,000 new cases of cancer were estimated in Brazil, with female breast cancer being the one that most affects women, corresponding to 30.1%, with an estimate of 73,610 new cases for 2023[5].

Ample evidence suggests that host antitumor immunity plays an important role in combating tumor cells, with recognition of tumor antigens and their immunogenicity leading to a subsequent adequate response in three phases: elimination, equilibrium, and escape[6,7]. Thus, much emphasis in clinical research has been placed on targeted therapies, such as the use of antibodies and other factors that stimulate the immune system[8]. Tumor inflammatory infiltrating is a potential mechanism for identifying patients who will benefit from immunotherapy or checkpoint inhibition[9].

The clinicopathological characteristics of tumors, such as intrinsic tumor biology, microenvironment, and stage of the disease at the time of diagnosis, contribute to the evaluation of the risk of disease relapse, and can be used to identify patients

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for whom adjuvant therapy is unnecessary[10]. Immunotherapy and specific targeted therapies have been employed with good results for certain tumor types[8]. The presence of pre-existing intra-and peritumoral lymphocytic infiltrates seems to have a positive impact on the patient's response to treatments and the prognosis of these diseases. The association between the presence of tumor-infiltrating lymphocytes (TILs) and survival rates has been widely studied in addition to that between TILs and treatment response[11]. The number of present TILs varies according to the breast cancer tumor subtype. The levels of lymphocyte subpopulations can be identified as additional strategies in patients with a low to moderate presence of TILs[12,13]. Patients with triple-negative tumors (e.g., negative for estrogen (ER) and progesterone receptors (PR) and without overexpress HER2 membrane protein), and who presented elevated levels of CD8+ and CD4+ T lymphocytes, had a greater response to systemic treatment and longer survival times. Recent studies have revealed that TILs are independent prognostic factors for triple-negative invasive breast cancer[10], and that intratumor heterogeneity is associated with less immune cell infiltration, less activation of the immune response, and worse survival rates in breast cancer[14].

The aim of this study was to evaluate the association between clinicopathological characteristics and the level of tumor-infiltrating lymphocytes (TILs) with the overall survival rate over five years of follow-up in patients diagnosed with invasive breast cancer and treated at *Hospital Santa Casa* in Belo Horizonte, a public referral hospital for the treatment of this disease in the State of Minas Gerais, Brazil, in 2011.

METHODS

Ethical procedures

The study was approved by the Ethics Committee of the Teaching and Research Institute of Santa Casa in Belo Horizonte on October 2, 2017 under number 1.958.532, and was conducted according to the Resolution of the Ministry of Health No. 466/12. Data were obtained from the records of *Hospital Santa Casa* in Belo Horizonte, and the patients were treated according to the institution's protocols. The privacy and confidentiality of the information were protected. There are no conflicts of interest to the researchers in charge of the study.

Study design and location

This retrospective and observational study was conducted at *Hospital Santa Casa* in Belo Horizonte, a public hospital of the Brazilian Unified Health System (SUS).

Population and eligibility criteria

The study population comprised patients diagnosed with invasive breast cancer in 2011, whose anatomopathological analysis was carried out in the Laboratory of Anatomical Pathology at *Hospital Santa Casa* in Belo Horizonte, and who were treated at this hospital as well.

Exclusion criteria

Patients with incomplete or missing information or absence of pathological results, and patients who underwent biopsy at Santa Casa and were treated at another hospital or who abandoned treatment were excluded (n=46, 15.9%). For the survival analysis, patients with zero follow-up time recorded or those with missing data were also excluded (n=68, 23.4%).

Variables

A breast pathologist (CBN) reviewed the anatomopathological diagnosis and immunohistochemical profile and evaluated the presence of TILs. The variables included were patient age, histological type, histological grade, estrogen (ER), progesterone (PR) receptor and HER2 protein status, T (tumor size), N (lymph nodes involved), M (distant metastases), sex (female or male), tumor inflammatory infiltrate (absent or present), and survival at the five-year follow-up visit. Estrogen and progesterone receptor status and HER2 protein expression were evaluated according to ASCO/CAP international recommendations[15,16]. Clinical staging of these patients followed the recommendations of the American Joint Committee on Cancer categories[17]. Tumors were classified and graded according to the WHO classification for breast tumors, 5th edition, published in 2019[18]. The protocols established by the breast surgery and clinical oncology services of *Hospital Santa Casa* in Belo Horizonte were followed. The standard operating procedure used to perform the immunohistochemical reaction (polymer method) followed the recommendations of the ASCO/CAP (American Society of Clinical Oncology/College of American Pathologists)[15,16]. TILs were evaluated through the microscopic analysis of the slides stained with hematoxylin and eosin, based on the recommendations of the College of American Pathologists and International Immuno-Oncology Biomarker Working Group guidelines for TILs assessment in invasive breast carcinoma[19]. We searched for mononuclear cells (mainly lymphocytes) within the stroma between the carcinoma cells (stromal TILs), and classified them as absent or present. Immune infiltrates outside the tumor borders, for example, in adjacent normal tissue or areas of DCIS, were not included. In addition, TILs in areas with crush artifacts, necrosis, and/or extensive central regressive hyalinization were not evaluated. The same evaluation method was used for all histological tumor types. Patient data were collected to generate the survival curves. Table 1 illustrates the methods used to assess HER2, ER, and PR statuses.

Data analysis

The student's t-test was used to compare differences in means for age, and categorical variables were compared using Fisher's

Table 1. Clinicopathological characteristics of patients with invasive breast cancer diagnosed and treated at *Hospital Santa Casa* in Belo Horizonte (MG), Brazil, in 2011 (n=244).

Variable		n	(%)
Gender			
Female		241	98.7
Male		3	1.3
Age in years – mean (SD)	58.4 (14.0)	244	100
Histological types – invasive tumors			
Invasive carcinoma of no special type (ductal NOS)		218	89.3
Invasive lobular carcinoma		14	5.7
Other special types		12	4.9
Histological grade			
I		16	6.5
II		139	57.0
III		89	36.5
Tumor size (according to pathological staging)			
T1 (up to 2 cm)		103	42.2
T2 (>2 cm and up to 5 cm)		118	48.4
T3 (>5 cm)		15	6.1
T4 (any size, extension to chest wall or skin)		5	2.1
No information		3	1.2
Lymph nodes (according to pathological staging)			
0 (no positive lymph nodes)		120	49.2
1 (up to 3 positive lymph nodes)		85	34.8
2 (4–9 positive lymph nodes)		28	11.5
3 (10 or more positive lymph nodes)		7	2.9
No information		4	1.6
Estrogen receptor (ER)			
Negative		53	21.7
Positive		191	78.2
Progesterone receptor (PR)			
Negative		91	37.2
Positive		153	62.7
HER2 status			
0/1+ (negative)		213	87.3
2+ (equivocal)		7	2.9
3+ (positive)		22	9
No information		2	0.8
Pathological stage			
I		103	42.2
II		118	48.4
III		15	6.1
IV		5	2
No information		3	1.2
Presence of TILs			
Absent		34	13.9
Present		207	86.9

SD: standard deviation; TILs: tumor-infiltrating lymphocytes.

exact test. Statistical significance was set at $p<0.05$. A statistical analysis was performed to associate the presence of inflammatory cells with clinicopathological factors already established in the literature. Additionally, patient survival was evaluated in the follow-up years. Kaplan-Meier curves were constructed and compared using the log-rank test. The Cox model was used for univariate and multivariate analyses with SPSS software version 21 (Statistical Package for Social Sciences) for Mac. Variables with a p-value <0.25 in the univariate analysis were included in the multivariate model. Hazard ratios (HRs) and 95% confidence intervals (CIs) were calculated for the univariate and multivariate analyses. For survival analysis, only overall survival was considered, and calculated as the time between the date of diagnosis and the date of death due to breast cancer (this was the event of interest) or the date of the last available medical record information for the patients who survived.

RESULTS

The results are presented in the following two sections. First, the clinicopathological characteristics of patients diagnosed with invasive breast cancer treated at *Hospital Santa Casa* in Belo Horizonte in 2011 (n=244) and the association between these characteristics and tumor inflammatory infiltrate (Tables 1 and 2) are shown.

Secondly, the survival data are shown, illustrating the association between the tumor inflammatory infiltrate and the clinicopathological characteristics of patients diagnosed with invasive breast cancer treated at *Hospital Santa Casa* in Belo Horizonte in 2011 (n=222) (Tables 3 and 4; Figure 1).

Characteristics of patients and tumors

Of 290 patients, 46 (15.9%) were excluded due to lack of complete data. Two hundred forty-one patients (98.7%) were female, and three (1.3%) were male, with a mean age of 58.2 (standard deviation±13.8 years). The predominant histological type was invasive carcinoma with no special type (ductal NOS), which corresponded to 218/244 (89.3%) patients, and the predominant histological grade was II, which represented 139/244 (57.0%) patients. The tumors were positive for estrogen and progesterone receptors in 191/244 (78.2%) and 153/244 (62.7%) patients, respectively. There were 213/244 (87.3%) HER2-negative cases, of 22/244 (9.0%) HER2-positive cases, and of 7/244 (2.9%) cases with equivocal HER2 status. Most patients were classified as stage II (118/244 patients, 48.4%).

TILs were present in 86% of the primary tumors studied, and were absent in 14% (Tables 1 and 2). The histological type was associated with the presence of TILs (p=0.018); 192/218 (88.1%) cases of invasive breast cancer with no special type (ductal NOS) had TILs, whereas TILs were present in only 9/14 cases (64.3%) of invasive lobular carcinomas. The presence of TILs was associated

Table 2. Association between the clinicopathological characteristics of patients with invasive breast cancer diagnosed at *Hospital Santa Casa* in Belo Horizonte (MG), Brazil, in 2011 and the tumor inflammatory infiltrate (n=244).

Variable	n	TILs absent (n = 34)	(%)	TILs present (n = 210)	(%)	p
Gender						
Female	241	34	14.1	207	85.9	1.000
Male	3	0	0	3	100	
Age in years – mean (SD)		62.9 (13.8)		57.7 (13.9)		**0.041**
Histological types						
Invasive carcinoma with no special type (ductal NOS)	218	26	11.9	192	88.1	
Invasive lobular carcinoma	14	5	37.5	9	64.3	**0.018**
Other special types	12	3	25	9	75	
Histological grade						
I	16	3	18.8	13	81.3	
II	139	24	17.3	115	82.7	**0.058**
III	89	7	7.9	82	92.1	
Tumor size pathological						
1	103	17	16.5	86	83.5	
2	118	15	12.7	103	87.3	
3	15	1	6.7	14	93.3	0.825
4	5	1	20.0	4	80.0	
No information		0		3		
Lymph nodes (according to pathological staging)						
0	120	19	15.8	101	84.2	
1	85	10	11.8	75	88.2	
2	28	3	10.7	25	89.3	0.589
3	7	1	14.3	6	85.7	
No information		1		3		
Estrogen receptor (ER)						
Negative	53	29	15.2	162	84.8	0.372
Positive	191	5	9.4	48	90.6	
Progesterone receptor (PR)						
Negative	91	25	16.3	128	83.7	0.184
Positive	153	9	9.9	82	90.1	
HER2						
0/1+	213	33	15.5	180	84.5	
2+	7	0	0	7	100	0.073
3+	22	0	0	22	100	
No information	2	2	0.87			
Clinical stage						
I	103	17	16.5	86	83.5	
II	118	15	12.7	103	87.3	
III	15	1	6.7	14	93.3	0.500
IV	5	1	20	4	80	
No information	3	3				

SD: standard deviation; TILs: tumor-infiltrating lymphocytes; p<0,05 are in bold.

Table 3. Univariate analysis (Cox model) – Survival of patients with invasive breast cancer treated at *Hospital Santa Casa* in Belo Horizonte (MG), Brazil, in 2011 (n=222).

Variable	Hazard ratio	p
Tumor size		
T1*	1	<0.001
T2	4.68 (1.36–16.18)	0.015
T3	20.52 (5.11–82.40)	<0.001
T4	12.74 (2.12–76.56)	0.005
Presence of TILs	0.57 (0.23–1.41)	0.222
Histological type		
Invasive carcinoma with no special type (ductal NOS)	1	0.270
Invasive lobular carcinoma	2.45 (0.83–7.30)	0.106
Other special types	1.24 (0.168–9.17)	0.835
Histological grade		
Grade I*	1	0.020
Grade II	1.41 (0.18–11.13)	0.744
Grade III	3.97 (0.52–30.36)	0.184
Axillary status		
N0	1	0.008
N1	3.26 (1.22–8.69)	0.018
N2	4.93 (1.59–15.29)	0.006
N3	10.25 (2.04–51.46)	0.005
Stage		
Stage I*	1	<0.001
Stage II	2.74 (0.6–12.49)	0.194
Stage III	10.80 (2.41–48.30)	0.002
Stage IV	20.46 (2.86–146.30)	0.003
Hormone receptors		
Positivity for estrogen receptor	0.64 (0.27–1.52)	0.316
Positivity for progesterone receptor	0.35 (0.16–0.73)	0.005
HER2		
0 or 1+*	1	0.283
2+	3.21 (0.76–13.62)	0.114
3+	0.98 (0.23–4.14)	0.973

*Reference category (i.e., used for comparison with other categories). TILs: tumor-infiltrating lymphocytes.

with a younger age (mean age of patients with TILs present, 57.7 years, and 62.9 years for patients without TILs, p=0.041). All tumors with HER2 overexpression (3+) and equivocal cases (2+) showed the presence of TILs, corresponding to 100% of these patients (29/29) (p=0.073).

Patients with tumors of a higher histological grade had more TILs, although the diference was not statistically significant (p=0.058), corresponding to 82/89 cases (92.1%) of grade III tumors (Table 2). Tumor size, lymph node positivity, and hormone receptor status were not associated with the presence of TILs.

Table 4. Multivariate analysis (Cox model) - Survival of patients with invasive breast cancer treated at *Hospital Santa Casa* in Belo Horizonte (MG), Brazil, in 2011 (n=222).

Variable	Category	Hazard ratio	p
Tumoral size			
T1*		1	0.001
T2		4.63 (1.27–16.87)	0.020
T3		19.24 (4.30–86.15)	< 0.001
T4		6.97 (1.00–48.68)	0.050
Histological grade			
Grade I*		1	0.920
Grade II		0.81 (0.10–6.96)	0.846
Grade III		0.95 (0.11–8.56)	0.967
Progesterone receptor (PR)			
PR negative*		1	0.004
RP positive		0.39 (0,17–0.87)	0.022
TILs			
Absent*		1	0.200
Present		0.23 (0.08–0.65)	0.005
Axillary status			
No positive nodes*		1	0.002
At least one positive node		2.80 (1.02–7.70)	0.046

*Reference category. TILs: tumor-infiltrating lymphocytes.

Survival analysis

The median follow-up time was 63.5 (1-84.2) months. In univariate analysis, tumor size, stage, progesterone receptor positivity, and negative axilla were associated with a longer survival time (Table 3). The overall survival rate of the entire cohort in the follow-up years was 85.2%. The presence of TILs was not associated with survival time (p=0.222; HR: 0.57; 95%CI 0.23–1.41).

In the multivariate analysis, when tumor and patient characteristics were added to the model, smaller tumor size (HR, for T3 versus T1, 19.24; 95%CI 4.30–86.15); p<0.001), absence of metastasis to the axillary lymph nodes (having a positive axilla versus no positive axillary nodes), (HR 2.80; 95%CI 1.02–7.70; p=0.002), positivity for progesterone receptor (HR: 0.39; 95%CI 0.17–0.87; p=0.022), and presence of TILs (HR: 0.23; 95%CI 0.08–0.65; p=0.002) were associated with longer survival times (Table 4, Figure 1).

DISCUSSION

In this study, we showed the relationship between TILs and the clinicopathological characteristics of patients with invasive breast cancers diagnosed and treated at *Hospital Santa Casa* in Belo Horizonte in 2011, and the five-year survival rate. A high frequency of tumors with TILs was identified, corresponding to

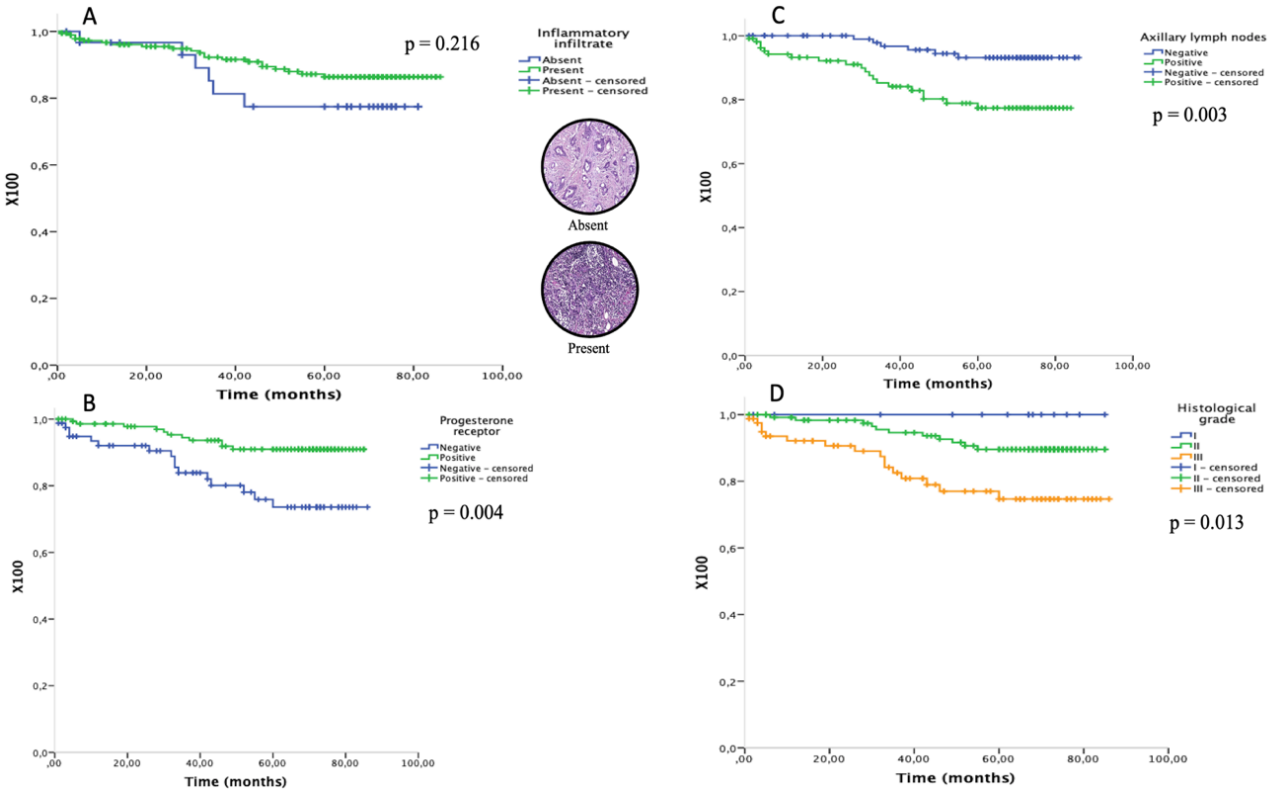

Figure 1. Overall survival curve of patients diagnosed with invasive breast tumors treated at *Hospital Santa Casa* in Belo Horizonte (MG), Brazil – 2011: (**A**) associated tumor inflammatory, infiltrate absent or present, magnification 400x, invasive carcinomas NST, (**B**) associated progesterone receptor, negative or positive, (**C**) associated with axillary lymph nodes, negative or positive, and (**D**) associated with histological grade I, II or III. p-values refer to the log-rank test.

207/244 (85.9%) patients. Additionally, the presence of TILs was associated with the tumor type, especially invasive carcinoma of no special type (ductal NOS), tumors of a higher histological grade, and younger age, corroborating the results described in the medical literature[20,21]. All tumors with HER2 overexpression (3+) and equivocal cases (2+) showed the presence of TILs, corresponding to 100% of these patients (29/29). Most hormone receptor positive tumors also show the presence of TILs. The characteristics of the patients and their tumors were like those reported in the literature[22], with a predominance of invasive carcinoma of no special type (ductal NOS), followed by invasive lobular carcinoma and histological grade II. Furthermore, survival time is associated with classic prognostic factors, such as tumor size and grade, positivity of regional lymph nodes, and PR positivity[17].

The association between inflammatory infiltrates and survival time is mediated by factors related to both patients and tumors[23]. TILs have a potential role in predicting the improved survival benefits achieved with several therapies, and the quantification of TILs is feasible on H&E-stained tissue sections during diagnostic procedures[9,17]. In our study, patients with TILs had longer survival times in multivariate analysis, which suggests that the presence of TILs is an independent prognostic factor in breast cancer. Unfortunately, detailed information on treatment strategies was only available for approximately 20% of our cohort, making the evaluation of different therapies unreliable.

Previous studies have revealed that the presence of TILs is associated with longer overall survival times in triple negative and HER2-positive cancers but shorter time in luminal HER2-negative breast cancer[24,25]. HER2-overexpressing and triple-negative tumors are more immunogenic, suggesting that an immunosuppressive mechanism could explain the shorter overall survival time observed in some of these patients, as described by some authors.[25,26] In some previous studies, on ER-positive and HER2-negative tumors, no significant association was found between TILs and survival rates. We believe that this could be explained by the substantial heterogeneity of the disease and the fact that patients with these subtypes usually already have long survival times[24,27]. In contrast, patients with HER2-negative tumors and a higher concentration of TILs usually have a worse prognosis and shorter disease-free and overall survival times, suggesting diverse biological behaviors for TILs and the microenvironment in different tumor types[8,23,28].

The complexity of the immune response to tumors is likely oversimplified in current measurement models[29]. In our study,

TILs were not stratified into subpopulations; only the presence or absence of TILs was evaluated through the microscopic analysis of the slides stained by H&E used for the anatomopathological diagnosis of the patients, which is a limitation. No immunohistochemical study has been performed to verify the type of inflammatory cells, as was the case in other studies[8,11,20]. International collaborative efforts are standardizing the histopathologic reporting of immune infiltrates to allow the application of these parameters in clinical and research settings[24]. The recognition of the prognostic value of the immune infiltrate has been the basis for establishing a breast cancer immunological grade[17,24,29].

Immunotherapy associated with chemotherapy and/or hormone therapy shows promising results for patients with metastasis or residual disease after treatment, especially for patients with triple-negative tumors. TILs can be used as predictors of response to chemotherapy and immunotherapy. Understanding tumor immunobiology and TILs is a huge challenge for science, and through gaining this knowledge, new diagnostic and therapeutic approaches for cancer patients can be validated[13,30,31].

Several studies have shown that the response to conventional antitumor agents (chemotherapy, radiotherapy, and target-specific therapy) appears to be mediated in part by their effects on the immune system, both in stimulating tumor immunogenicity and modulating the immune system and its microenvironment within the tumor[12,30,31]. The interaction between the signaling pathways of the estrogen and progesterone receptors and the immunological tumor microenvironment is largely unknown and needs to be studied in more detail[9].

One of the strengths of this study is the analysis of all patients admitted over the course of one year for diagnosis and treatment of their disease at a reference service for breast cancer in a public hospital of the Brazilian Unified Health System (SUS). All patients underwent their diagnosis, tumor excision, and therapy protocol performed by the same surgeons, pathologists, and oncologists, leading to a more homogeneous group for comparative studies. Unfortunately, in 2011, equivocal HER2 cases (2+) were not retested for HER2 gene amplification (FISH), because this test was not available in our public health system. Furthermore, anti-HER2 therapy (trastuzumab) was not available at our hospital at that time; thus, patients with HER2-overexpressing tumors did not receive anti-HER2 therapy.

Another possible limitation was the follow-up period. The patients' follow-up time for the survival analysis was limited to five years, which is a short period for the evaluation of the overall survival rate of patients diagnosed with invasive breast cancer; however, significant differences were demonstrated. Perhaps, a greater difference in survival times could be found with a 10- or 15-year follow-up period. The low socioeconomic status of most participants, the social stigma associated with cancer, and the delay in obtaining complementary examinations by the public health system, even though patients were admitted to a referral hospital, could be possible factors responsible for the considerable number of patients who were lost to follow-up. Additionally, there was some difficulty in accessing data because, in our country, most hospitals that treat patients within the public health system do not have computerized charts with integrated data on the evolution and treatment of these patients.

TILs can be easily identified by pathologists through H&E slides, and they can be used as prognostic markers as well as predictive markers of response to treatment in conjunction with other markers already established in the literature and by other molecular analyses. The presence of TILs could contribute to the classification and staging of tumors, as well as to determining the immunological profile of the disease at different times over the course of treatment. In our study, not only were TILs associated with some tumor characteristics, but they were also independent prognostic factors for breast cancer survival time.

CONCLUSIONS

In our study, an analysis of patients diagnosed with invasive breast cancer treated at *Hospital Santa Casa* in Belo Horizonte, Minas Gerais, Brazil, in 2011, revealed a significant association between the presence of TILs with invasive carcinomas of no special type and a younger age of patients. TILs were not significantly associated with high histological grade, estrogen receptor and progesterone receptor status, HER2 expression status, disease stage, tumor size, or axillary lymph node status. Some factors had a greater impact than others on survival in the multivariate analysis, such as tumor size, which had a greater impact than the axillary status, and T3 tumors had a worse outcome when compared to other tumor sizes. The presence of TILs was associated with longer survival time in the multivariate analysis, which confirms that TILs are a prognostic factor in breast cancer.

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AUTHORS' CONTRIBUTIONS

FMAF: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Resoucers,

Validation, Visualization, Writing – original draft, Writing – review & editing. CBN: Conceptualization, Data curation, Investigation, Methodology, Project administration, Supervision, Validation, Visualization, Writing – review & editing. FCLS: Conceptualization, Data curation, Formal analysis, Methodology, Supervision, Validation, Visualization, Writing – review & editing. MAB: Conceptualization, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – review & editing. DB: Conceptualization, Data curation, Formal analysis, Methodology, Validation, Visualization, Writing – review & editing.

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