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Tumor-infiltrating Lymphocytes are Independent Favorable Prognostic Indicator in 17-year Disease-Free Survival in Lymph Node-Negative Triple-Negative Breast Cancer Patient

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Authors' contributions

This work was carried out in collaboration between all authors. Author ALAE designed the study, wrote the protocol, and managed the literature searches. Authors ALAE and MCS performed the statistical analysis and writing of the first draft of the manuscript. Authors MTA and MGB performed the immunohistochemical analyses. Authors LC, SK and EA were consultants. All authors read and approved the final manuscript.

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ABSTRACT

Aims: To estimate the prognostic value of tumor-infiltrating lymphocytes, among other variables, in triple-negative breast cancer patients with a 17-year disease-free survival.
Study Design: A retrospective study of 79 patients was conducted to investigate treatment, and clinical, microscopic and immunohistochemical tumor characteristics.
Place and Duration of Study: Pathology Division, National Cancer Institute (INCA), Rio de Janeiro, RJ, Brazil, between January 1992 and December 1996.
Methodology: Histologically diagnosed 79 node-negative triple-negative breast cancer patients underwent partial or total mastectomy with axillary lymphadenectomy, with or without radiotherapy, chemotherapy and/or hormone therapy. Disease-free survival was estimate by the Kaplan-Meier method and log-rank test. Prognostic variables were obtained by Cox regression models.
Results: The 17-year disease-free survival was 50.6%. Disease-free survival was worse in patients aged 51-82 years, who underwent neoadjuvant chemotherapy and had skin compromise, geographic necrosis, grade 3 tumors, had no tumor-infiltrating lymphocytes, had vascular/lymphatic invasion, $CD44^{+}/CD24^{-/low}$ and elevated Ki-67. The risk of recurrence and/or metastasis, adjusted for the remaining variables of the final Cox model was 2.44 times higher for patients aged 51-82 years, 2.60 times higher for patients undergoing neoadjuvant chemotherapy, 3.97 times higher for grade 3 tumors and 0.34 times for patients with tumor-infiltrating lymphocytes.
Conclusion: The risk of recurrence and/or metastasis, adjusted for the remaining variables of the model, was about 2.5 times higher for older patients undergoing neoadjuvant chemotherapy. In grade 3 tumor patients, the risk increased almost fourfold. Patients with tumor-infiltrating lymphocytes had a 66% lower risk, i.e, tumor-infiltrating lymphocytes were shown to be a protective factor.

Keywords: Axillary lymph nodes; breast cancer; immunohistochemistry; prognosis; survival; triple-negative breast cancer; tumor-infiltrating lymphocytes.

1. INTRODUCTION

By definition, triple-negative (TN) breast cancer (BC) does not express estrogen receptor (ER), progesterone receptor (PR) or human epidermal growth receptor 2 (HER-2) on immunohistochemical study. It accounts for 7-20% of all BC and is subdivided into basal-like (BL) and non-basal-like tumors [1-3].

BLBC represents the majority of TNBC. These tumors are positive for one or more basal cell markers (cytokeratins: CK5/6, CK14, CK17), epidermal growth factor receptor (EGFR) [4-10], myoepithelial markers (vimentin, calponin, p63, etc), c-kit, P-cadherin, and laminin, among others [4-6].

TNBC and BLBC are more prevalent in younger women, particularly in females of African descent and Hispanics. Patients display a more aggressive clinical behavior, increased recurrence rates, and a poor prognosis [1,11,12]. Histologically, the majority of BL cancers are invasive ductal carcinoma (IDC) (or no-special type invasive carcinoma) and high histologic grade (HG) [3-6,8-10] tumors. Other tumor features are extensive geographical tumor necrosis, central fibrotic zones, finely delimited tumor margins and prominent lymphocytic infiltrate [13-15].

Non-basal-like TNBC are negative for both basal and myoepithelial markers [2,16] and patients may have more a favorable prognosis than those with BLBC [2,8].

The immune system is a promising target for the diagnosis and study of patient outcome related to BC. Evidence suggests that host immune response plays a critical role in tumor growth, progression and subsequent recurrence and/or metastasis [13,15,17,18]. Several studies have described dense T-lymphocyte tumor infiltration and are associated with a good prognosis [13-15,18-23]. Dense tumor-infiltrating lymphocytes (TIL) are more prominent in aggressive, highly proliferative, high-grade tumors and are linked to survival rates of BC patients, especially those with TNBC [13,15,19,22,23].

The aim of this study was to estimate the prognostic value of TIL, among other variables, for the 17-year disease-free survival (DFS) in a cohort of TN IDC patients, without axillary node metastases at the time of diagnosis.

2. MATERIALS AND METHODS

A retrospective observational hospital-based cohort study was conducted to evaluate the time between the first BC diagnosis and first relapse and/or distant metastasis. Histologically diagnosed node-negative BC patients underwent partial or total mastectomy with axillary lymphadenectomy, with or without radiotherapy, chemotherapy and/or hormone therapy at the National Cancer Institute (INCA, Rio de Janeiro, Brazil) from January 1992 to December 1996. Patient and treatment characteristics were obtained from medical charts. Histology slides (biopsies and/or surgical specimens) of tumors and axillary nodes, stained in hematoxylin and eosin (H&E), were revised by one of the authors (ALAE). Tumor reclassification followed World Health Organization criteria, 2012 [24]. Only node-negative patients categorized as IDC remained in the study [25,26]. Skin compromise, HG [27], vascular and/or lymphatic invasion, extensive geographical tumor necrosis (>30%) [28], central fibrotic zones (>30%) [28], and TIL [intratumoral, stromal, both locations; absent (<10%), scanty (10-49%), dense (≥50%) [29,30] were also evaluated. Immunohistochemical studies with monoclonal antibodies against ER, PR, HER-2 and Ki-67, among others, were conducted. The final cohort included 253 non-TN IDC and 95 TN IDC [26]. Immunohistochemical reactions with ER, PR and HER-2 were repeated for this study, since cut-off points for marker positivity have undergone changes over time. Paraffin sections from 79 specimens were stained. Sixteen cases were eliminated: eight cases tested positive for ER and/or PR, eight had insufficient material for analysis. All cases remained HER-2 negative. Immunohistochemical reactions with CK5, CK14, EGFR and calponin antibodies were also performed for the subclassification of TNBC in BLBC (CK5 and/or CK14 and/or EGFR were positive) and non-basal-like BC [6,10,16,31], and cancer stem cell markers CD24 and CD44 (immunophenotypes: $CD44^+/CD24^+$; $CD44^+/CD24^{-/low}$; $CD44^-/CD24^+$; $CD44^-/CD24^{-/low}$) [16,32,33] (Table 1). Visual evaluation was made by author ALAE at least twice. Results were semiquantitatively scored: positive versus negative reactions. Details on immunohistochemical methods employed were previously described [25,26]. All reactions included positive and negative controls. Previously known positive or negative IDC cases from this same cohort were used.

Table 1. Details of immunohistochemical study: Monoclonal antibody clones used, dilution, sources, positivity criteria and references

	Monoclonal antibody clones	Dilution	Source	Positivity	References
ER*	SP1	1:900	Spring	Nuclear staining ≥ 1%	[34]
PR#	PgR636	1:1200	Dako	Nuclear staining ≥ 1%	[34]
HER-2$	CB11	1:1600	Novocastra	**Negative 0**: no staining; **OR** membrane staining that is incomplete and is faint/barely perceptible and within <=10% of tumor cells. **Negative 1+**: incomplete membrane staining that is faint/barely perceptible within >10% of tumor cells. **No conclusive, equivocal, 2+**: circumferential membrane staining that is incomplete and/or weak/moderate and within >10% of tumor cells; **OR** complete and circumferential membrane staining that is intense and within <=10% of tumor cells. **Positive 3+**: circumferential membrane staining that is complete, intense, and within >10% of	[35]

				tumor cells.	
CK$^{\%}$5	D5/16B4	1:800	Cell Marque	Cytoplasm and/or membrane staining ≠ 0%	[6,10,16,31]
CK14	LL002	1:600	Cell Marque	Cytoplasm and/or membrane staining ≠ 0%	[6,10,16,31]
EGFR$^{\&}$	H11	1:100	Dako	Cytoplasm and/or membrane staining ≠ 0%	[6,10,16,31]
Calponin	Calp	1:6000	Cell Marque	Cytoplasm staining ≠ 0% on myoepithelial cells	[24]
CD24	SN3b	1:50	Neomarquers	Cytoplasm and cytoplasm membrane staining ≥ 34%	[16,32,33]
CD44	MRQ-13	1:200	Cell Marque	Cytoplasm and cytoplasm membrane staining ≥ 1	[16,32,33]
Ki-67	MIB-1	1:200	Dako	Nuclear staining > 25%	[36,37]

ER: estrogen receptor; PR#: progesterone receptor**;** HER-2$: human epidermal growth receptor 2; CK%: cytokeratin; EGFR&: epidermal growth factor receptor.*

Data analysis determined the distribution pattern of cohort characteristics. For continuous variables, measurements of the amplitude, mean, standard deviation (SD) and first, second and third quartiles were made. For categorical variables, absolute and relative frequencies were calculated. To evaluate the relation between different variables and outcomes (recurrence and/or metastasis), Fisher's exact test was used.

To estimate the probability of a 17-year DFS, the Kaplan Meier method was applied. The following criteria were used: initial event, primary breast cancer diagnosis; final event, recurrence and/or metastases; time of DFS, time between initial and final events or until loss to follow-up. Censoring occurred when patients were lost to follow-up or recurrence and/or metastases did not develop until 17 years after diagnosis. Variables with less than 10 cases per category were not included in survival analysis. To determine whether there were differences between the probabilities estimated, the log-rank test was used. Variables with *P-Values* <.20 in this test were included in Cox models.

3. RESULTS AND DISCUSSION

3.1 Results

In this cohort, the mean patient age was 52 years (SD±13.4 years) and 50.6% of the patients were younger than 50. The majority of patients were white (57.3%) with menarche occurring after 12 years of age (63.2%). The mean patient age was 13 years (SD±1.9 years) at menarche and 47 years at menopause (SD±5.7 years). More than 46% of patients were premenopausal at the time of diagnosis. Three patients were nulliparous and four women had never breastfed (Table 2).

All patients underwent breast surgery associated with axillary node resection, with or without radiotherapy and/or chemotherapy and/or hormone therapy, according to institutional treatment protocols. However, patients undergoing neoadjuvant chemotherapy, i.e., preoperative chemotherapy accounted for 26.6% of cases (Table 2).

The left breast was most commonly affected (58%). Mean tumor size was 4.7 cm (SD±2.3 cm); measuring 1.5-5.0 cm in 40.5%. Measurement was not assessed in 21 patients undergoing neoadjuvant chemotherapy. Most surgical specimens for analysis were obtained from radical surgeries (Table 2).

In more than 25% of surgical specimens there was skin compromise. The majority of IDC showed extensive geographical necrosis (53.2%) and/or central fibrosis (58.2%) (Table 2; Figs. 1A, 1B). Tumor margins were well-delimited in 27 (34.2%) tumors (Fig. 1A). TIL was present in 54.4% of IDC (scanty in 12 cases and dense in 31). It was intratumoral in four IDC (one scanty and three dense); stromal in 21 IDC (eight scanty and thirteen dense) and was found in both locations in 18 IDC (three scanty and fifteen dense) (Table 2; Fig. 1C).

The majority of TN IDC were composed of solid masses of neoplastic cells with rare or no tubule formation, exhibiting malignant cells with intense nuclear pleomorphism (63.3%) and high mitotic activity (54.4%) (Table 3; Figs. 1A, 1B, 1C). On histologic grading, only two IDC were grade 1, twenty-one IDC were grade 2 and fifty-six were grade 3 (Table 3). In 46.8% of IDC, there was vascular and/or lymphatic invasion. All lymph nodes were negative for metastases. Over 50%

of the patients had more than 19 lymph nodes examined (Table 3).

Regarding basal cell markers in immunohistochemical reactions, 51.9% of IDC were positive for CK5; 54.4% positive for CK14; 68.4% positive for EGFR and 43.0% positive for calponin. TN IDC with immunophenotype BL were classified in 81.0% of cases. Regarding cancer stem cell markers CD44 and CD24, IDC

Table 2. Distribution and probability of 17-year disease-free survival (DFS) of triple-negative breast cancer patients managed at HCI/INCA from 1992-1996, according to patient, treatment, macroscopic and microscopic tumor characteristics

Characteristics studied	Frequency			Probability of disease-free survival		
	Absolute	Relative	$P^{\$}$	DFS (17)	95%CI	$P^{\&}$
Total	**79**	**100.00**		**50.59**	**(34.55-64.61)**	
Age group at diagnosis						
28-50 years	40	50.63	.36	61.43	(40.53-76.88)	.06
51-82 years	39	49.37		32.93	(8.62-60.45)	
Skin colour[(4)]						
White	43	57.33	.35	58.40	(37.22-74.63)	.24
Non-white	32	42.67		38.41	(12.34-64.55)	
Age group at menarche[(3)]						
9-12 years	28	36.84	1.00	59.09	(37.01-75.69)	.96
13-18 years	48	63.16		50.71	(29.42-68.61)	
Menopause						
No	37	46.84	.49	45.91	(24.47-65.03)	.87
Yes	42	53.16		59.64	(41.12-74.04)	
Treatment received						
Surgery and RT and/or CT$^{\Phi}$	33	41.77	.33	51.63	(24.98-72.97)	.12
Surgery alone	25	31.65		60.81	(36.34-78.32)	
Neoadjuvant CT	21	26.58		42.40	(20.32-63.01)	
Laterality of tumor						
Right	33	41.77	.16	40.35	(18.43-61.44)	.10
Left	46	58.23		58.42	(36.03-75.35)	
Tumor size[(5)]						
1.5-5.0 cm	30	40.54	.09	59.00	(27.45-80.62)	.13
5.1-13.0 cm	23	31.08		52.20	(29.33-70.85)	
Not applied$^{@}$	21	28.38				
Type of surgical specimen						
Partial surgery	14	17.72	1.00	42.74	(9.17-73.89)	.53
Radical surgery	65	82.28		55.67	(41.54-67.67)	
Skin compromise						
No	57	72.15	.07	55.98	(35.48-72.27)	*.03*
Yes	22	27.85		37.01	(16.15-58.17)	
Extensive geographical necrosis						
No	37	46.84	.07	60.35	(33.00-79.46)	.08
Yes	42	53.16		42.24	(23.01-60.32)	
Central fibrosis						
No	33	41.77	.49	61.94	(41.62-76.97)	.43
Yes	46	58.23		41.56	(19.80-62.13)	
Tumor-infiltrating lymphocytes						
No	36	45.57	.16	39.43	(18.87-59.48)	*.05*
Yes	43	54.43		59.72	(36.00-77.09)	

Notes: (n) Corresponds to the number of cases without information on each variable. $ Corresponds to P-Value associated with Fisher's test. & Corresponds to P-Value associated with log-rank test. Φ RT = Radiotherapy; CT= Chemotherapy. @ Patients undergoing neoadjuvant chemotherapy

Table 3. Distribution and probability of 17-year disease-free survival (DFS) of triple-negative breast cancer patients managed at HCI/INCA from 1992-1996, according to tumor, microscopic and immunohistochemical characteristics

Characteristics studied	Frequency			Probability of disease-free survival		
	Absolute	Relative	$P^{\$}$	$DFS_{(17)}$	95%CI	$P^{\&}$
Nuclear pleomorphism						
Mild/moderate	29	36.71	.16	55.45	(24.15-78.3)	.22
Intense	50	63.29		47.96	(29.57-64.20)	
Number of Mitoses						
< 15 mitoses /10hpf$^{\Omega}$	36	45.57	*.01*	73.62	(53.80-85.96)	*.02*
≥15 mitoses 10/hpf	43	54.43		34.14	(15.18-54.20)	
Histological grade						
Grades 1 + 2	23	29.11	.08	59.40	(21.55-83.79)	.13
Grade 3	56	70.89		47.79	(30.48-63.21)	
Vascular/lymphatic invasion						
No	42	53.16	*.04*	68.12	(49.59-81.05)	*.04*
Yes	37	46.84		35.21	(15.37-55.89)	
Number of lymph nodes examined						
7-18 lymph nodes	38	48.10	*.04*	42.18	(23.20-60.04)	.09
19-39 lymph nodes	41	51.90		59.19	(31.26-78.94)	
CK5$^{\pi}$						
Negative	38	48.10	1.00	53.34	(30.46-71.72)	.78
Positive	41	51.90		47.53	(25.01-67.08)	
CK14$^{\pi}$						
Negative	36	45.57	1.00	51.57	(28.23-70.70)	.83
Positive	43	54.43		50.21	(27.92-68.93)	
EGFR						
Negative	25	31.65	*.03*	76.32	(51.94-89.45)	*.03*
Positive	54	68.35		37.30	(18.08-56.62)	
Calponin						
Negative	45	56.96	.24	40.88	(20.46-60.39)	.11
Positive	34	43.04		65.10	(44.61-79.59)	
Immunophenotype						
Hexanegative	15	18.99	.15	76.61	(43.33-91.86)	.12
Basal-like	64	81.01		44.82	(27.21-60.96)	
CD44						
Negative	40	50.63	.17	58.34	(34.43-76.16)	*.03*
Positive	39	49.37		44.50	(26.85-60.73)	
CD44/CD24 profile						
$CD44^{+}/CD24^{-/low}$	13	16.46	.07	33.33	(10.27-58.84)	*.03*
Other profiles	66	83.54		54.29	(35.81-69.54)	
Ki-67						
Low proliferative activity	23	29.11	.21	62.36	(31.32-82.52)	.12
High proliferative activity	56	70.89		45.87	(26.43-63.36)	

Notes: $ Corresponds to P-Value associated with Fisher's test. & Corresponds to P-Value associated with log-rank test. Ω hpf = high power field. π CK = cytokeratin

was positive for CD44 in 49.4% and positive for CD24 in 7.6% of cases. Concerning immunohistochemical profiles, 13 IDC (16.5%) showed $CD44^{+}/CD24^{-/low}$ and the remaining 66 tumors showed: $CD44^{+}/CD24^{+}$=26; $CD44^{-}/CD24^{+}$=21; and $CD44^{-}/CD24^{-/low}$=19. In relation to Ki-67, 70.9% of IDC had high proliferative activity (Table 3).

The 17-year DFS was 50.6% ($IC_{95\%}$=34.6-64.6%) (Table 2, Fig. 2). Twenty-two patients were alive until the end of follow-up. Twenty of these patients had no relapse and/or metastases; one patient had relapsed at the 202th month, and another had metastasis to distant lymph nodes at the 204th month; both received treatment. Twelve patients died without the disease. Twenty-eight

died with the disease. All relapsed and/or had metastases, dying from BC, 01-60 months after diagnosis (Fig. 2). Of the 49 patients who had no recurrence or metastasis, 12 died without disease, and 37 remained alive and disease-free. Of these 37 patients, twenty had complete follow-up and 17, incomplete follow-up. Seven patients had local recurrences, while 27 had metastases (18 to the lung, 12 to the central nervous system (CNS), eight to distant lymph nodes, five to the bones, two to the liver and one to the mediastinum).

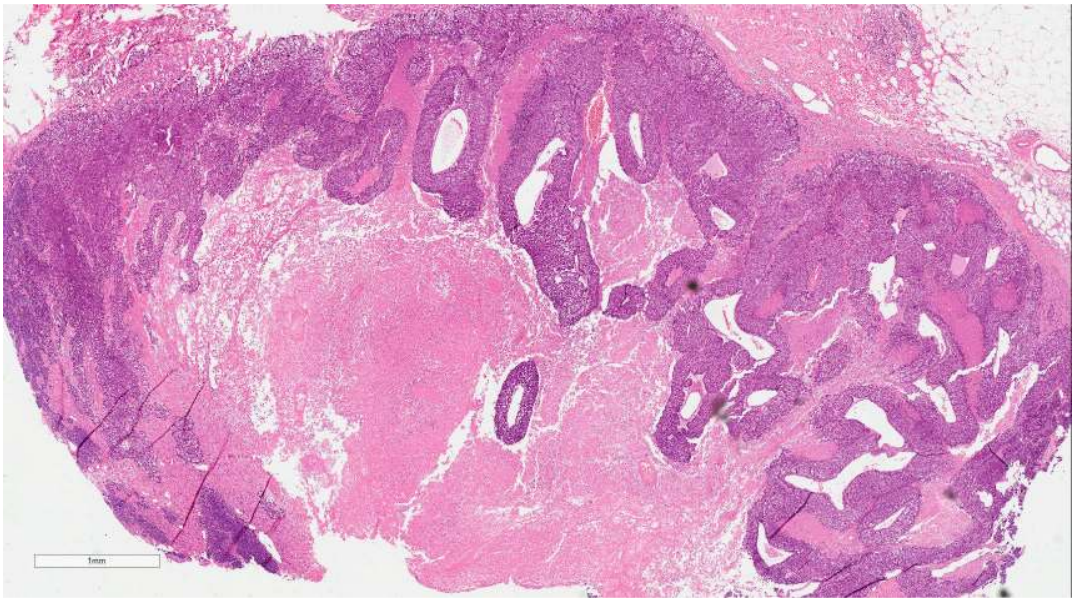

Fig. 1A. Solid masses of neoplastic cells with central extensive geographic necrosis. Well-delimited tumor margins. Hematoxylin and eosine (H&E) stained tumor sections (40x)

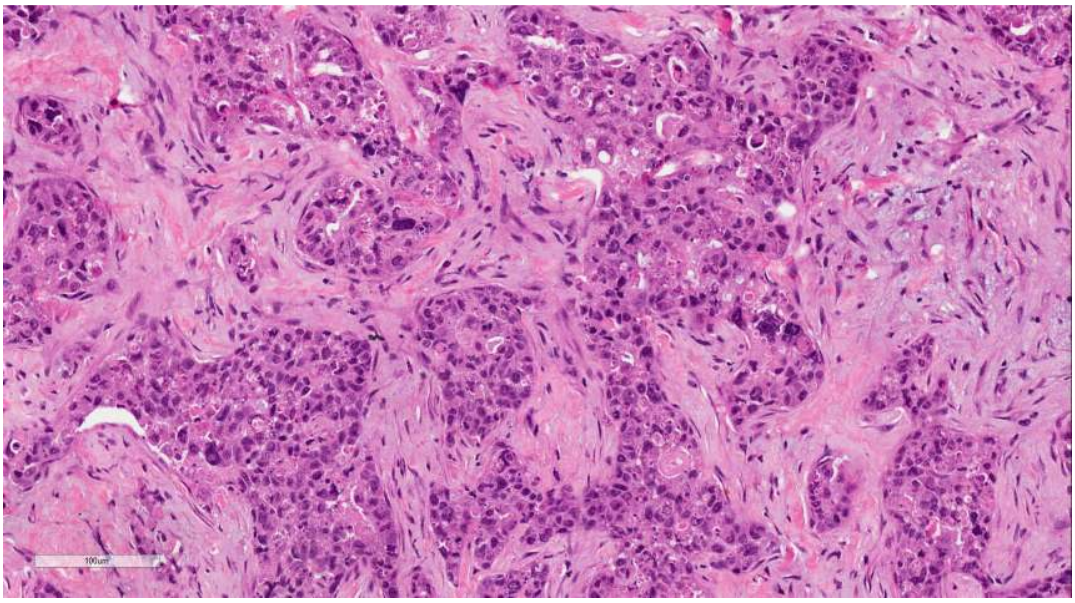

Fig. 1B. High-grade triple-negative invasive ductal carcinoma (IDC) showing cells with high-grade nuclei, mitotic activity, syncytial growth pattern, and extensive fibrosis. H&E stained tumor sections (400x)

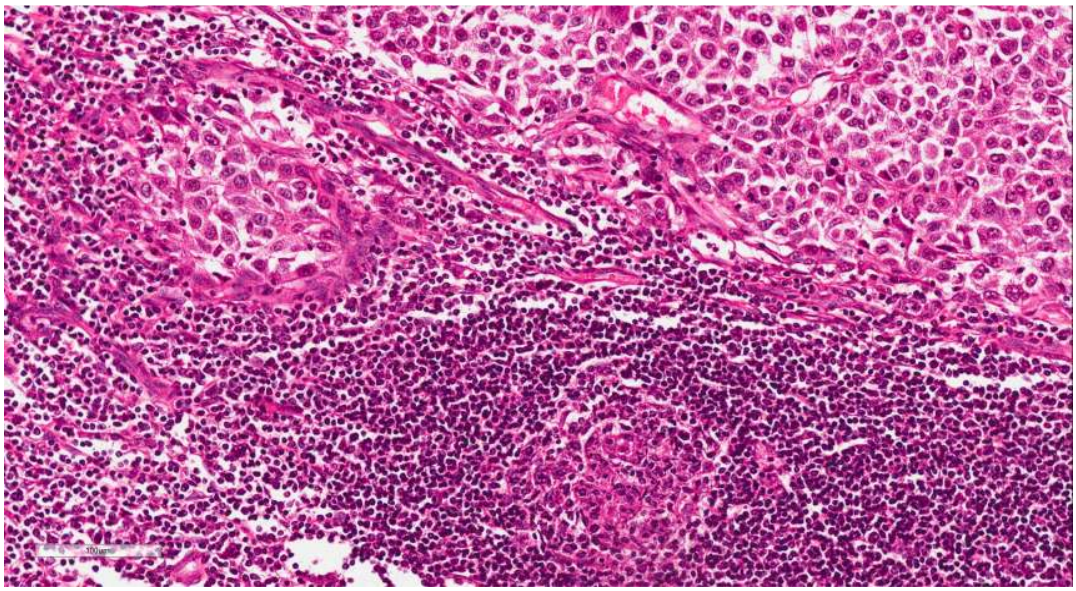

Fig. 1C. High-grade triple-negative IDC with a dense tumoral lymphocyte infiltrate. H&E stained tumor sections (400x)

The probability of survival was higher in cancer patients without skin compromise (*P*=.03), dense TIL (*P*=.05), up to 14 mitoses/10 hpf (*P*=.02), no vascular/lymphatic invasion (*P*=.04), and EGFR-negative (*P*=.03) and CD44-negative status (*P*=.03). Survival was also higher in patients with immunohistochemical profiles that differed from $CD44^{+}/CD24^{-/low}$ (*P*=.03) (Tables 2, 3).

Age group at diagnosis, treatment received, laterality, tumor size, skin compromise, extensive geographical necrosis, TIL, number of mitoses, HG, vascular/lymphatic invasion, number of nodes examined, EGFR, calponin, BL/non-BL immunophenotype, CD44, CD44/CD24 profile and Ki-67 were shown to be important prognostic factors on crude analysis using Cox models (*P*<0.20). Those variables were used to construct the final model for analysis of prognostic factors (Tables 2, 3).

Adjusted for the remaining conditions, the risk for recurrence and/or metastasis was 2.44 times higher in patients over 50 years of age (*P*=.03). The risk was 2.60 times higher in patients undergoing neoadjuvant chemotherapy than in those managed with surgery combined with radiotherapy and/or chemotherapy (*P*=.03). It was 3.97 times higher in grade 3 tumor patients than in grades 1 and 2 tumor patients (*P*<.01). Patients with TIL had an adjusted risk of recurrence and/or metastasis that was 66% lower than those without TIL (*P*<.01) (Table 4).

3.2 Discussion

In this study, the 17-year DFS for node-negative TNBC patients was 50.6%. Although the DFS was lower in BLBC patients, compared to those with non-basal-like tumors (44.8% vs. 76.6%), statistically significant differences were undetected. The small sample size and long-term survival study could possibly explain the lack of statistical significance. For the 17-year DFS in the whole TNBC group, age range from 51-82 years, treated with neoadjuvant chemotherapy, grade 3 tumors and absence of TIL were independent predictive factors for a worse prognosis.

According to some authors, TNBC is more aggressive at the onset of disease. Tumor recurrence and/or metastasis peaks in the first and third year after therapy. The majority of deaths occur in the first five years following treatment. Heterogenous occurrence of death among TNBC patients and those with other phenotypes decreases when follow-up is 10 years or longer [2-6,20,26,38,39]. Compared to non-TNBC patients, TNBC patients have a significantly shorter survival after the first metastatic event [1,3-5,38]. Eisenberg et al using the present study cohort, observed that 59% of deaths occurred in the first three years and 82% in the first five years following therapy in the TN IDC group. In non-TN IDC patients, 38% and 65% of deaths occurred in the first three and five years of follow-up, respectively [26].

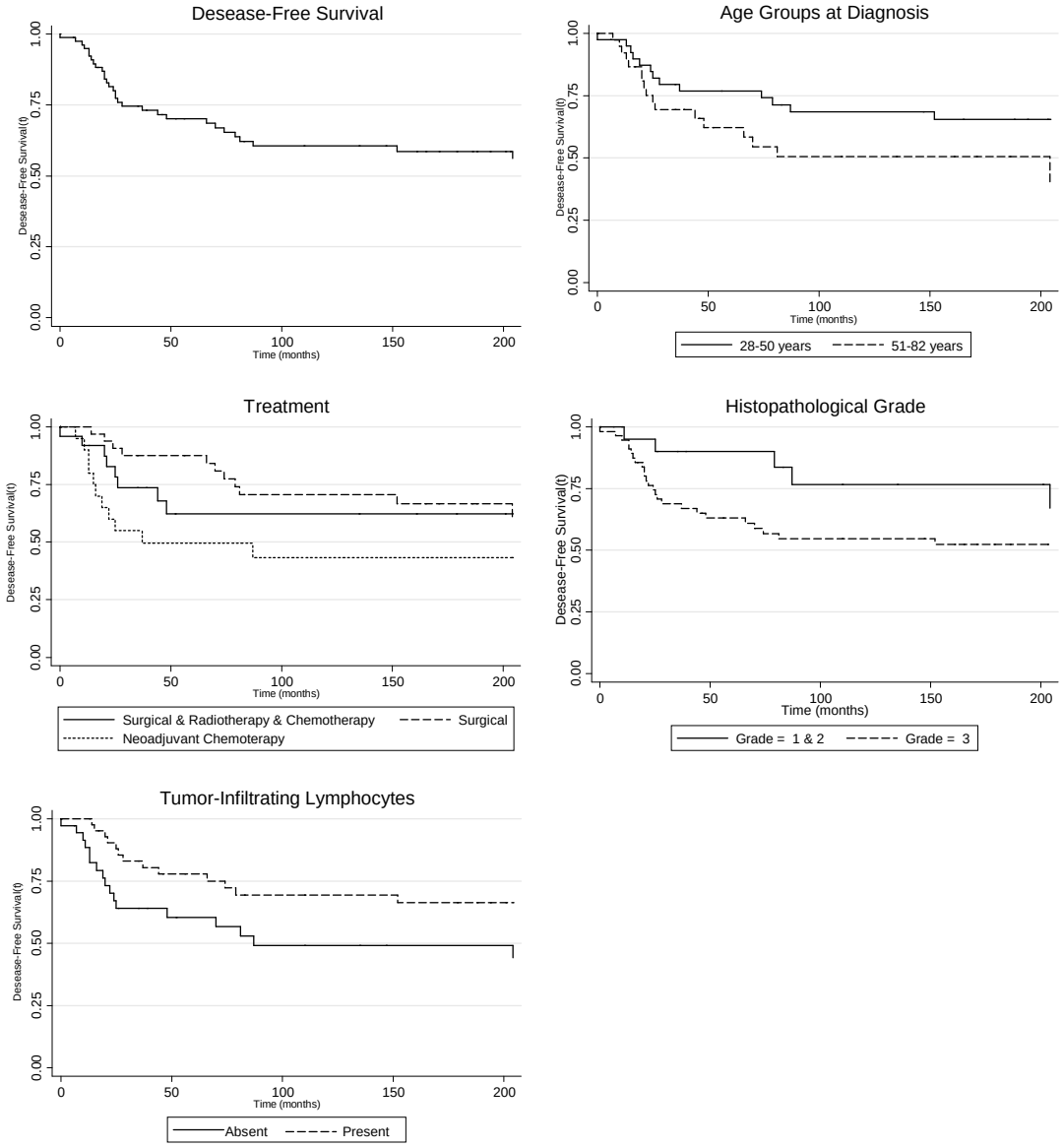

Fig. 2. 17-year disease-free survival curves in triple-negative breast cancer patients managed at HCI/INCA from 1992 to 1996, according to characteristics of multivariate Cox model

TNBC preferentially shows hematogenic dissemination, mainly to the viscera (liver and lungs) and CNS, producing axillary node and bone metastases less frequently than non-TN tumors [5,40]. In the present study, a similar situation occurred. Patients developed metastasis to the lungs (66.7%), CNS (44.4%), distant lymph nodes (29.6%), bones (18.5%), liver (7.4%) and mediastinum (3.7%).

In this cohort, there was a slight predominance of patients aged 28-50 years. The 17-year DFS was lower, while the risk of recurrence and/or metastasis was 1.84 times higher in patients aged 51-82 years. This age group remained an independent prognostic factor for worse DFS (HR=2.44), when compared to the 28 to 50-year age range (*P*=.03). Similar data were also observed in studies by Lehmann & Pietenpol and Adams et al. [11,13].

Concerning treatment received, the probability of a 17-year DFS was lower in patients receiving neoadjuvant chemotherapy, than in those

undergoing surgery combined with radiotherapy and/or chemotherapy. The risk of recurrence and/or metastasis was 2.37 times higher for the former group in comparison to the latter. In this study, treatment with neoadjuvant chemotherapy was an independent factor for worse prognosis (HR=2.60) remaining in the final Cox model (*P*=.03).

In this study, high-grade TNBC occurred in 70.9% of patients who had a lower DFS. Grade 3 tumor was an independent prognostic factor for a worse prognosis (HR=3.97) in the 17-year DFS (*P*<.01), when compared to grades 1 and 2 BC. Other studies in the literature also described a worse prognosis in grade 3 tumor patients [3-6,8-10].

In the present study, histological analysis of tumor showed intratumoral TIL, stromal TIL, and both locations. However, due to the small number achieved for analysis, only existing TIL were analyzed. TIL was present in 54.4% of IDC, and it was dense in 72.1% of these tumors. Garcia-Martinez et al. [41] found prognostic values similar to TIL results, with and without stratification into intratumoral and peritumoral or stromal TIL. The International TILs Working Group (2014) recommended that TILs should be reported only for the stromal compartment [23]. In this cohort, TIL stromal was present in 90.7% of IDC. Patients with TIL had the highest DFS in 17 years (*P*=.05) and a lower crude risk of recurrence and/or metastasis (*P*=.05). In the multivariate Cox model, a protective effect of 66% against the risk of recurrence and/or metastasis was seen, when controlled by other variables in the model (HR=0.34; *P*<.01). Results from the current study are similar to those observed in the literature researched, highlighting the predictive and prognostic impact of TIL on BC [13-15,22,29,42-44].

In a meta-analysis conducted by Ibrahim et al. [43] various studies suggested that host immune response plays a major role in tumor genesis, tumor development, disease progression and subsequently the occurrence of metastases. The intensity of immune response also influences cancer treatment effectiveness and benefits clinical outcome. Denkert et al. [42] reported that the immune system participates in tumor cell elimination and tumor growth control. Consequently, the presence of TIL is associated with improved patient outcome. According to Oh et al., immune cell infiltration is the major factor in the prevention of disease progression in rapidly proliferating tumors. A good correlation between HG and cell proliferation occurred in a concentration of high-grade tumors. Furthermore, those researchers believed that cell proliferation and immunity highly influence patient outcome. Both are defense mechanisms against rapidly progressive activity [45]. Swede et al. highlighted that several publications investigating the impact of TIL on clinical outcome in large cancer patient cohorts, reported that immune infiltration was the main prognostic factor in various types of cancer.

Table 4. Results of multivariate Cox model of triple-negative breast cancer patients managed at HCI/INCA from 1992 to 1996

Characteristics studied	HR	95%CI	$P^{\#}$
Age group at diagnosis			
28-50 years	1.00		
51-82 years	2.44	(1.10-5.41)	*.03*
Treatment received			
Surgery and RT and/or CT$^{\Phi}$	1.00		
Surgery alone	1.05	(0.40-2.71)	.93
Neoadjuvant chemotherapy	2.60	(1.10-6.13)	*.03*
Histological grade			
Grades 1 + 2	1.00		
Grade 3	3.97	(1.43-11.05)	<*.01*
Lymphocyte infiltrate			
No	1.00		
Yes	0.34	(0.16-0.73)	<*.01*

Notes: # Corresponds to p value associated with HR (Hazard Ratio) estimate.
Φ RT = Radiotherapy; CT = Chemotherapy.

Lymphocytes, especially T cells, are indicators of good prognosis in BC [7]. Similarly, Ibrahim et al. [15] in another meta-analysis (including eight studies from 2007 to 2014, representing a cohort of 2987 TNBC patients) and other researchers found that TIL was a robust independent prognostic marker mainly in TNBC, BLBC and HER-2-negative tumors. Patients with increased TIL had a lower risk of recurrence, distant metastases or death. According to those authors, TIL could be a prognostic biomarker in TNBC [13-15,30].

Several studies in the literature have researched TIL using immunohistochemistry to assess the clinical importance of subtyping T-lymphocytes: CD3, CD45, CD8, and FOXP3-positive [15,17-19,43,46]. Those studies obtained results similar to previous findings observed without T-cell stratification. Salgado et al. [23] stated that although immunohistochemistry may improve accuracy, it is currently unclear whether any further value can be obtained from these markers. The TILs working group does not recommend immunohistochemistry for detection of specific subpopulations outside research settings, until further evidence is available.

In two meta-analyses, pooled analysis showed that dense TIL indicates high pathologic response rates to neoadjuvant chemotherapy and may lead to favorable outcomes. Authors concluded that TIL may be an independent and robust marker for the prediction of pathologic complete response (pCR) rate following neoadjuvant chemotherapy, mainly in TNBC [15,18]. The International TILs Working Group (2014) has recommended that TILs be standardized and assessed in breast cancer [23]. However, the 14th St. Gallen International Breast Cancer Conference (2015), did not accept the presence of TILs as either a prognostic or predictive marker [47]. In the current cohort, the accurate assessment of pCR or lack of response to chemotherapy in 21 patients undergoing neoadjuvant chemotherapy was unfeasible. Sufficiently rigid criteria were not found in the histopathological case review for the evaluation and quantification of response.

In this study, DFS was shorter for patients with CD44-positive (*P*=.03) tumors. A $CD44^{+}/CD24^{-/low}$ profile was present in 16.5% of TNBC, and DFS was shorter in those patients (33.3%) compared to patient survival in other IHC profiles (54.3%) (*P*=.03). However, this immunohistochemical profile did not achieve statistical significance on multivariate Cox analysis. Expression of cancer stem cell markers (CD44 and CD24) has been reported in TNBC and BLBC [32,33,48,49]. Regarding survival and expression of cancer stem cells in BC, conflicting results were shown in the few studies available using immunohistochemistry. $CD44^{+}/CD24^{-low}$ phenotype was associated with a worse prognosis in TNBC in a study by Idowu et al. [49]. Ahmed et al. [48] observed that $CD44^{-}/CD24^{+}$ phenotype was solely associated with a worse prognosis. $CD44^{+}/CD44^{-/low}$ phenotype that was not associated with TNBC had a better prognosis. Those authors, however, found no difference in CD44 and CD24 expression between basal-like and non-basal-like TNBC. In a study by Ricardo et al. [33] CD44/CD24 phenotypes showed no differences compared with BC subtypes. Multivariate analysis conducted by Giatromanolaki et al. [32] indicated that $CD44^{-}/CD24^{-}$ phenotype was an independent variable of worse prognosis in a non-selected BC series. These results were also confirmed in TNBC.

High Ki-67 (>25%) was present in 70.9% of TNBC in this study and DFS was shorter in this group. Ki-67 did not achieve statistical significance on multivariate Cox analysis. These results were consistent with other findings in the literature [2,3,50].

Uchoa reported that despite its imperfection, histopathological testing is useful for predicting patient outcome and tumor response to clinical intervention. More recently, immunohistochemical technique has revolutionized cancer diagnosis. It is currently a fundamental tool in oncologic pathology. Histopathological and immunohistochemical aspects are highly accessible and mirror the expression of diverse genes [16].

This study had some limitations. It was a retrospective study with a small sample size of 79 patients. Dual observation was not performed by two pathologists. To minimize limitations, the same pathologist who was always blinded to clinical data and/or follow-up, read each slide at least twice with the naked eye. Discordances between both readings were reviewed with other experienced pathologists. Immunohistochemistry was not performed to subtype TIL and a morphometric approach was applied. Chemotherapy regimens and pathological response rates to chemotherapy were not assessed.

Nevertheless, this investigation had some positive aspects. A homogeneous sample of TN IDC patients was used. Tumor histology was reassessed at different times. Triple-negative status was confirmed by recent standardized criteria. All samples were surgical biopsies and/or surgical specimens. Core needle biopsies or tissue microarrays were not used to prevent sampling due to BC heterogeneity. There was a prolonged clinical follow-up period. This was probably the first Brazilian study to investigate long-term survival from TN IDC. It appears to be the second Brazilian publication on cancer stem cell markers (CD24 and CD44) in TNBC. All patients were diagnosed, treated and followed in a national referral oncology center. This ensured therapy homogeneity following diagnosis prior to BC classification based on immunohistochemical phenotype. Results were similar to many other findings described in the literature. Reproducibility suggests that there was no distortion in the estimates found.

4. CONCLUSION

The 17-year DFS in a hospital-based cohort composed of 79 women with TN IDC without axillary node metastases at the time of diagnosis was 50.6%. Adjusting for the remaining variables of the model, the risk of relapse and/or distant metastasis was 2.44 times higher for patients aged 51-82 years than for patients aged 28-50 years (P=.03). The risk was 2.60 times higher for patients receiving neoadjuvant chemotherapy than for those undergoing surgery associated with radiotherapy and/or chemotherapy (P=.03), and 3.97 times higher for grade 3 tumor patients, compared to grades 1 and 2 tumor patients (P<.01). The risk of relapse and/or distant metastasis was 0.34 times lower for patients with TIL, than for those without any TIL (P<.01).

In this study, TIL was evaluated as a biomarker of good prognosis for DFS and it was confirmed that TIL is a protective factor against tumor recurrence and/or distant metastasis. The risk of relapse and/or metastasis in these patients decreased by 66%.

CONSENT AND ETHICAL APPROVAL

Free informed consent was obtained from live patients.

The Research Ethics Committee of INCA approved this project (CAAE: 04360612.2.0000.5274) on 7/24/2012.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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