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

Ana Lucia Amaral Eisenberg¹, Leila Chimelli¹, Mirian Carvalho de Souza², Fernanda Maria Braga Marinho³, Maria Theresa Accioly¹, Marcelli Gatto de Brito¹, Sérgio Koifman⁴ and Eliana Abdelhay⁵

¹Pathology Division (DIPAT), Nacional Cancer Institute (INCA), Rua Cordeiro da Graça, 156, Santo Cristo, Rio de Janeiro, RJ, CEP: 20220-400, Brazil.
²Epidemiology Division, Nacional Cancer Institute (INCA), Rua Marquês de Pombal, 125, 7th floor, Centro, Rio de Janeiro, RJ, CEP: 20230-240, Brazil.
³Mastology, Hospital do Câncer III, Nacional Cancer Institute (INCA), Rua Visconde de Santa Isabel, 274, Vila Isabel, Rio de Janeiro, RJ, CEP: 20560-120, Brazil.
⁴Department of Epidemiology, National School of Public Health, Oswaldo Cruz Foundation (FIOCRUZ), Brazil.
⁵Stem Cell Laboratory, Bone Marrow Transplantation Unit, Nacional Cancer Institute (INCA), Praça Cruz Vermelha, 23, Centro, CEP: 20230-130, Rio de Janeiro, RJ, Brazil.

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.

Article Information

DOI: 10.9734/BJMMR/2016/25507

Editor(s):
(1) Syed Faisal Zaidi, Department of Basic Medical Sciences, College of Medicine, King Saud Bin Abdulaziz University-HS, National Guard Health Affairs, King Abdulaziz Medical City, Kingdom of Saudi Arabia.

Reviewers:
(1) Viktoria – Varvara Palla, G. Genimatas General Hospital, Athens, Greece.
(2) Anonymous, Unidad de Investigación Médica en Genética Humana, IMSS, Mexico.
(3) Amrani Mariam, University Mohamed V, Rabat, Morocco.
(4) Ketan Vagholkar, D.Y.Patil University School of Medicine, India.
Complete Peer review History: http://sciencedomain.org/review-history/14489

Received 7th March 2016
Accepted 18th April 2016
Accepted 6th May 2016

*Corresponding author: E-mail: alamaral@inca.gov.br;
*memoriam
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+/low; 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]        |
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(4)                       |           |            |          |       |
| 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(6)                    | 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(8)                                  |           |            |          |       |
| 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(8)                                 | 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. $ 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   | \(P^a\)   | DFS | 95%CI | \(P^b\) |
| **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\(^Ω\) | 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\(^π\)**          |           |           |           |     |       |         |
| Negative                | 38        | 48.10     | 1.00      | 53.34 | (30.46-71.72) | .78 |
| Positive                | 41        | 51.90     |           | 47.53 | (25.01-67.08) |     |
| **CK14\(^π\)**         |           |           |           |     |       |         |
| 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 202\(^{th}\) month, and another had metastasis to distant lymph nodes at the 204\(^{th}\) 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)
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].
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 | 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=0.03) tumors. A CD44+/CD24low 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=0.03). 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+/CD24low 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+/CD44low 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. Those 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.

REFERENCES

1. Akasbi Y, Bennis S, Abbass F, et al. Clinicopathological, therapeutic and prognostic features of the triple-negative tumors in moroccan breast cancer patients (experience of Hassan II university hospital in Fez). BMC Res Notes. 2011;4:500.

2. Minami CA, Chung DU, Chang HR. Management options in triple-negative breast cancer. Breast Cancer (Auckl). 2011;5:175-99.

3. Rakha EA, Chan S. Metastatic triple-negative breast cancer. Clin Oncol (R Coll Radiol). 2011;23(9):587-600.

4. Reis-Filho JS, Tutt AN. Triple negative tumours: A critical review. Histopathology. 2008;52(1):108-18.

5. Albergaria A, Ricardo S, Milanezi F, et al. Nottingham prognostic index in triple-negative breast cancer: A reliable prognostic tool? BMC Cancer. 2011;11:299.

6. Badve S, Dabbs DJ, Schnitt SJ, et al. Basal-like and triple-negative breast cancers: a critical review with an emphasis on the implications for pathologists and oncologists. Mod Pathol. 2011;24(2):157-67.

7. Swede H, Gregorio DI, Tannenbaum SH, et al. Prevalence and prognostic role of triple-negative breast cancer by race: A surveillance study. Clin Breast Cancer. 2011;11(5):332-41.

8. Verma S, Provencher L, Dent R. Emerging trends in the treatment of triple-negative breast cancer in Canada: A survey. Curr Oncol. 2011;18(4):180-90.

9. Sood N, Nigam JS. Correlation of CK5 and EGFR with clinicopathological profile of triple-negative breast cancer. Patholog Res Int; 2014. Article ID 141864.

10. Bose S. Triple-negative breast carcinoma: Morphologic and molecular subtypes. Adv Anat Pathol. 2015;22(5):306-13.

11. Lehmann BD, Pietenpol JA. Identification and use of biomarkers in treatment strategies for triple-negative breast cancer subtypes. J Pathol. 2014;232:142-150. Published online in Wiley Online Library.
12. Mayer IA, Abramson VG, Lehmann BD, Pietenpol JA. New strategies for triple-negative breast cancer: Deciphering the heterogeneity. Clin Cancer Res. 2014;20(4):782-90.

13. Adams S, Gray RJ, Demaria S, et al. Prognostic value of tumor-infiltrating lymphocytes in triple-negative breast cancers from two phase III randomized adjuvant breast cancer trials: ECOG 2197 and ECOG 1199. J Clin Oncol. 2014;32(27):2959-66.

14. Loi S, Michiels S, Salgado R, et al. Tumor infiltrating lymphocytes are prognostic in triple negative breast cancer and predictive for trastuzumab benefit in early breast cancer: results from the FinHER trial. Ann Oncol. 2014;25(8):1544-50.

15. Ibrahim EM, Al-Foheidi ME, Al-Mansour MM, Kazkaz GA. The prognostic value of tumor-infiltrating lymphocytes in triple-negative breast cancer: A meta-analysis. Breast Cancer Res Treat. 2014;148(3):467-76.

16. Uchôa DM. Expressão de marcadores de células-tronco tumorais em carcinomas mamários basais e pentanegativos. Estudo em uma série de tumores triplonegativos: Tese de Doutorado; Ciências Médicas; Universidade Federal do Rio Grande do Sul; 2012. Available:https://www.lume.ufrgs.br/bitstream/handle/10183/61723/000866360.pdf?sequence=1

17. Ali HR, Provenzano E, Dawson SJ, et al. Association between CD8+ T-cell infiltration and breast cancer survival in 12,439 patients. Ann Oncol. 2014;25(8):1536-43.

18. Mao Y, Qu Q, Zhang Y, Liu J, Chen X, Shen K. The value of tumor infiltrating lymphocytes (TILs) for predicting response to neoadjuvant chemotherapy in breast cancer: A systematic review and meta-analysis. PLoS One. 2014;9(12):1-21.

19. West NR, Kost SE, Martin SD, et al. Tumour-infiltrating FOXP3(+) lymphocytes are associated with cytotoxic immune responses and good clinical outcome in oestrogen receptor-negative breast cancer. Br J Cancer. 2013;108(1):155-62.

20. Dieci MV, Criscitiello C, Goubar A, et al. Prognostic value of tumor-infiltrating lymphocytes on residual disease after primary chemotherapy for triple-negative breast cancer: A retrospective multicenter study. Ann Oncol. 2014;25(3):611-8.

21. Dieci MV, Mathieu MC, Guarneri V, et al. Prognostic and predictive value of tumor-infiltrating lymphocytes in two phase III randomized adjuvant breast cancer trials. Ann Oncol. 2015;26(8):1698-704.

22. Ohtani H, Mori-Shiraishi K, Nakajima M, Ueki H. Defining lymphocyte-predominant breast cancer by the proportion of lymphocyte-rich stroma and its significance in routine histopathological diagnosis. Pathol Int. 2015;65(12):644-51.

23. Salgado R, Denkert C, Demaria S, et al. The evaluation of tumor-infiltrating lymphocytes (TILs) in breast cancer: Recommendations by an International TILs Working Group 2014. Ann Oncol. 2015;26(2):259-71.

24. Lakhani SR, Ellis IO, Schnitt SJ, Tan PH, van de Vijver MJ, eds. WHO classification of tumours of the breast. Lyon: IARC; 2012.

25. Eisenberg ALA, Koifman, S. Sobrevida de cinco anos em pacientes com carcinoma ductal infiltrante de mama com linfonodos axilares negativos. Revista Brasileira de Medicina. 2006;63(4):152-163. Available:http://www.moreirajr.com.br/revistas.asp?fase=r003&id_materia=3281

26. Eisenberg ALA, Pinto, IV, Koifman, S. Triple-negative breast cancer in Brazilian women without metastasis to axillary lymph node: Ten-year survival and prognostic factors. Br J Med Med Res. 2013;3(4):880-896.

27. Elston CW, Ellis IO. Pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: experience from a large study with long-term follow-up. Histopathology. 1991; 19(5):403-10.

28. Zhang Y, Ou Y, Yu D, et al. Clinicopathological study of centrally necrotizing carcinoma of the breast. BMC Cancer. 2015;15:282.

29. Pagès F, Galon J, Dieu-Nosjean MC, Tartour E, Sautes-Fridman C, Fridman WH. Immune infiltration in human tumors:
A prognostic factor that should not be ignored. Oncogene. 2010;29(8):1093-102.

30. Loi S, Sirtaine N, Piette F, et al. Prognostic and predictive value of tumor-infiltrating lymphocytes in a phase III randomized adjuvant breast cancer trial in node-positive breast cancer comparing the addition of docetaxel to doxorubicin with doxorubicin-based chemotherapy: BIG 02-98. J Clin Oncol. 2013;31(7):860-7.

31. Nielsen TO, Hsu FD, Jensen K, et al. Immunohistochemical and clinical characterization of the basal-like subtype of invasive breast carcinoma. Clin Cancer Res. 2004;10(16):5367-74.

32. Giatromanolaki A, Sivridis E, Fiska A, Koukourakis MI. The CD44+/CD24- phenotype relates to 'triple-negative' state and unfavorable prognosis in breast cancer patients. Med Oncol. 2011;28(3):745-52.

33. Ricardo S, Vieira AF, Gerhard R, et al. Breast cancer stem cell markers CD44, CD24 and ALDH1: Expression distribution within intrinsic molecular subtype. J Clin Pathol. 2011;64(11):937-46.

34. Hammond ME, Hayes DF, Dowsett M, et al. American society of clinical oncology/College of American Pathologists guideline recommendations for immunohistochemical testing of estrogen and progesterone receptors in breast cancer (unabridged version). Arch Pathol Lab Med. 2010;134(7):e48-72.

35. Wolff AC, Hammond ME, Hicks DG, et al. Recommendations for human epidermal growth factor receptor 2 testing in breast cancer: American society of clinical oncology/College of American pathologists clinical practice guideline update. J Clin Oncol. 2013;31(31):3997-4013.

36. Mello ES, Alves VAF. Determinação da fração de proliferação celular no carcinoma de mama pela marcação imunistoquímica do antígeno nuclear Ki-67: comparação do método quantitativo e semi-quantitativo. Jornal Brasileiro de Patologia. 1999;35:200-205.

37. Carvalho FM, Bacchi LM, Santos PP, Bacchi CE. Triple-negative breast carcinomas are a heterogeneous entity that differs between young and old patients. Clinics (Sao Paulo). 2010;65(10):1033-6.

38. Eiermann W, Bergh J, Cardoso F, et al. Triple negative breast cancer: proposals for a pragmatic definition and implications for patient management and trial design. Breast. 2012;21(1):20-6.

39. Penault-Llorca F, Viale G. Pathological and molecular diagnosis of triple-negative breast cancer: a clinical perspective. Ann Oncol. 2012;23(Suppl 6):v19-22.

40. Winczura P, Sosinska-Mielcarek K, Duchnowska R, et al. Immunohistochemical predictors of bone metastases in breast cancer patients. Pathol Oncol Res. 2015;21(4):1229-36.

41. Garcia-Martinez E, Gil GL, Benito AC, et al. Tumor-infiltrating immune cell profiles and their change after neoadjuvant chemotherapy predict response and prognosis of breast cancer. Breast Cancer Res. 2014;16(6):488.

42. Denkert C, Loibl S, Noske A, et al. Tumor-associated lymphocytes as an independent predictor of response to neoadjuvant chemotherapy in breast cancer. J Clin Oncol. 2010;28(1):105-13.

43. Ibrahim EM, Al-Foheidi M, Al-Mansour MM, Kazkaz GA, Yunus TE. The Prognostic and predicting roles of tumor-infiltrating lymphocytes in breast cancer: a meta-analysis. Open Breast Cancer J. 2014;6:11.

44. Dushyanthen S, Beavis PA, Savas P, et al. Relevance of tumor-infiltrating lymphocytes in breast cancer. BMC Med. 2015;13:202.

45. Oh E, Choi YL, Park T, Lee S, Nam SJ, Shin YK. A prognostic model for lymph node-negative breast cancer patients based on the integration of proliferation and immunity. Breast Cancer Res Treat. 2012;132(2):499-509.

46. Chen Z, Chen X, Zhou E, et al. Intratumoral CD8(+) cytotoxic lymphocyte is a favorable prognostic marker in node-negative breast cancer. PLoS One. 2014;9(4):e95475.

47. Coates AS, Winer EP, Goldhirsch A, et al. Tailoring therapies-improving the management of early breast cancer: St Gallen international expert consensus on the primary therapy of early breast cancer. Ann Oncol. 2015;26(8):1533-46.

48. Ahmed MA, Aleskandarany MA, Rakha EA, et al. A CD44(-)/CD24(+) phenotype is a poor prognostic marker in early invasive
breast cancer. Breast Cancer Res Treat. 2012;133(3):979-95.

49. Idowu MO, Kmieciak M, Dumur C, et al. CD44(+)/CD24(-/low) cancer stem/progenitor cells are more abundant in triple-negative invasive breast carcinoma phenotype and are associated with poor outcome. Hum Pathol. 2012;43(3):364-73.

50. Hernandez-Aya LF, Chavez-Macgregor M, Lei X, et al. Nodal status and clinical outcomes in a large cohort of patients with triple-negative breast cancer. J Clin Oncol. 2011;29(19):2628-34.

© 2016 Eisenberg et al.; This is an Open Access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/4.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Peer-review history:
The peer review history for this paper can be accessed here:
http://sciencedomain.org/review-history/14489