**Clinical Relevance of Determination of Tumour Infiltrating Lymphocytes in Breast Carcinoma**

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**ABSTRACT**

**Objective:** The main purpose of this research was to determine the prognostic relevance of tumour infiltrating lymphocytes (TILs), CD4+ and CD8+ lymphocytes in patients with breast cancer.

**Methods:** Tissue samples were obtained from 120 breast cancer patients with operable disease and ductal histology. The intensity of lymphocyte infiltrate (LI) was graded from 0 to 3 semi-quantitatively on H&E stained whole sections. CD4+ and CD8+ T lymphocytes were evaluated in the tumor samples using monoclonal antibodies.

**Results:** A positive correlation between the level of TILs, as well as CD4+ lymphocytes and the size of the tumor, the presence of metastases in the axillary lymph nodes, histological grade, stadium and relapse of the disease was determined. High LI and high expression of CD4+ lymphocytes was seen in triple negative and HER2 positive tumors. A positive correlation between the level of CD8+ lymphocytes and histological grade, and positive HER2 expression was also found. A inverse correlation between the level of TILs, CD4+ and CD8+ lymphocytes and the presence of estrogen and progesterone receptors as well as the patients' age was detected. The patients with the higher level of TILs and CD4+ lymphocytes had distinctly worse disease free survival (DFS).

**Conclusion:** This research may contribute to development of the breast carcinoma "immunologic grade" which would reflect the intensity of the immune response of each individual patient and which would impact the prognosis of the disease, together with the other standard prognostic parameters.

**Keywords:** Breast cancer, CD4+, CD8+ T lymphocytes, tumor infiltrating lymphocytes

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## INTRODUCTION

The main problem in treatment of breast cancer is how, upon clinical classification and morphological characteristics of the tumor, to anticipate its further behavior. For the purpose of application of the adequate further therapy of breast cancer and discovery of aggressive tumor types, and after the surgical treatment, there is a constant need for finding some new indicators which might help in identification of the patients with increased risk of developing of the disease relapse.

All immune cell types may be found in a tumour, including macrophages, dendritic cells, mast cells, natural killer (NK) cells, naive and memory lymphocytes, B cells and effector T cells (1). The composition of leukocytes infiltrating breast tumors was dissected in great detail by Gu-Trantien et al. (2). In the leukocytic infiltrate, T-lymphocytes constituted 75% of cells, the proportion of B-lymphocytes was below 20%, monocytes constituted fewer than 10% of cells, and natural killer T-cells made up fewer than 5% of all leukocytes.

Cancer cells constantly modulate the host antitumor immune response in a process called immunoediting. During this process, the balance between antitumor and tumor-promoting immunity can be tilted to protect against the neoplasia development or, on the contrary, to support tumor growth (3). Therefore, the immune system can release factors that promote neoplastic cells survivor, growth, and invasion (4). Thus, paradoxically, immune system acts as an extrinsic tumor suppressor but can also promote cancer initiation, promotion, and progression, so the question related to relevance of tumor infiltrating lymphocytes (TILs) in ductal breast cancer arises. Therefore, the main goals of this research were to determine the correlation of TILs, CD4+ lymphocytes (T helper cells) and CD8+ lymphocytes (cytotoxic T cells) with standard prognostic factors in breast cancer, and how the differences in the level of infiltration of the lymphocytes in tumor affect the disease-free survival (DFS) and cancer-specific overall survival (OS).

## SUBJECTS AND METHODS

This research included 120 patients with invasive ductal carcinoma, with a tumor localized only within the breast without inclusion of skin and the pectoral muscle, with the tumor size of up to 5 cm, without distant visceral and bone metastases, who were operated at the Oncology Institute of Vojvodina. The research neither included patients who received neoadjuvant chemotherapy, nor those with multifocal and multicentric tumors. The clinical and pathological parameters of all the cases, including histological type, grade, lymph node status, tumor size, ER, PgR and HER2/neu expressions, pathological stage and follow-up were obtained from the archive and medical records of the hospital.

The average age of the patients at the time of diagnosis was 56.18 years (SD = 10.51), ranged from 31 to 77. The median follow-up was 77 months (range 11 to 93). During the follow-up period, 31 patients relapsed and 14 patients died. The clinical and pathological characteristics of patients with breast cancer are summarized in Table 1. The use of the specimens and data for research purposes was approved by the Ethics Committee of Oncology Institute of Vojvodina. Formalin-fixed paraffin-embedded breast cancer tissue was cut (5 μm). Dewaxed sections were stained with Hematoxylin-Eosin (HE) and analyzed.

Immunohistochemical reaction were performed using monoclonal antibodies against CD4+ (FLEX Monoclonal Mo a Hu CD4, Clone 4B12, DAKO, RTU) and CD8+ (FLEX Monoclonal Mo a Hu CD8, Clone 144B, DAKO, RTU). The intensity of lymphocyte infiltrate (LI) was graded from 0 to 3 semi-quantitatively. Grade 0 corresponded to negligible number of tumor infiltrating lymphocytes, and grades 1–3 corresponded to increasing degree of LI. Grades 0–1 were considered low LI and grades 2–3 were considered high LI (5). The immunophenotyped cells (CD4+ and CD8+ lymphocytes) were also semi-quantitatively graded.

### Statistical analysis

In order to determine the correlation between the low and high degree of LI, as well as CD4+

and CD8+ lymphocytes in tumor with different clinicopathologic parameters, Spearman rank order correlation was performed. The specific OS and DFS were estimated by using the Kaplan-Meier method and comparison between study groups was performed with the log-rank test. For testing of effects of various prognostic indicators, as well as the degree of tumor infiltrating lymphocytes, CD4+ and CD8+ lymphocytes in tumor to a recurrent occurrence of the disease and the fatal outcome of the disease, the Cox regression analysis was applied. In all tests, the significance level was set at 0.05. The statistical analyses were performed using the Software SPSS 11.5 for Windows.

## RESULTS

Dense LI was observed in 17 (14%) breast cancers, moderate in 45 (38%), and poor in 52 (43%) breast cancers. LI was not detected in 6 (5%) tumors. In the greatest number of tumors with LI, the infiltrate was present in tumor margins (in 96% of tumors), and in 37% of tumors, the centrally localized infiltrate was also detected. High expression of CD4+ lymphocytes was determined in 9 (8%) breast cancers, moderate in 53 (44%), and poor in 52 (43%) breast cancers. Staining with the monoclonal antibody against CD4 exhibited no expression in 6 (5%) tumors. High expression of CD8+ lymphocytes was seen only in 1 (1%) breast cancers, moderate in 28 (23%), and poor in 79 (66%) breast cancers. CD8+ lymphocytes not detected in 12 (10%) tumors.

As it can be observed from Table 2, a high positive correlation between tumor infiltrating lymphocytes (TILs) and CD4+ expression was determined ($r = 0,833$; $p < 0,001$), while between the expression of CD8+ and TILs ($r = 0.507$; $p < 0.001$), as well as between CD4 + and CD8 + lymphocytes ($r = 0.468$; $p < 0.001$), obtained the moderate positive correlation. A positive correlation between the level of TILs, as well as CD4+lymphocytes and the size of the tumor, the presence of metastases in the axillary lymph nodes, histological

grade, stadium and the disease relapse was determined. High LI and high expression of CD4+ lymphocytes was seen in triple negative and HER2 positive tumors. A positive correlation between the level of CD8+ lymphocytes and histological grade, as well as positive HER2 expression was also determined. A negative correlation between the level of TILs, CD4+ and CD8+ lymphocytes and the presence of estrogen and progesterone receptors, as well as the patients' age, was found.

Prognostic value of the expression of TILs, CD4+ and CD8+ lymphocytes was analyzed. The patients with higher level of TILs and CD4+ lymphocytes had distinctly worse DFS (log-rank test: p=0.012 for TILs, Figure 1 and log-rank test: p=0.045 for CD4+, Figure 2). Also the patients with higher level of TILs and CD4+ lymphocytes had distinctly worse cancer-specific OS, but the differences were not statistically significant, probably because of the small group of patients with fatal outcome of the disease (log-rank test: p=0.288 for TILs and log-rank test: p=0.295 for CD4+ lymphocytes). Between CD8+ lymphocytes and DFS no correlation was observed. By implementation of the Cox regression analysis, TILs (nor CD4+ and CD8+ lymphocytes) were not observed as significant disease prognosis predictors.

## DISCUSSION

The most significant, independent prognostic factors in breast cancer are the size of the tumor and the number of positive axillary lymph nodes (6). A positive correlation between the level of TILs, CD4+ lymphocytes and the presence of positive axillary lymph nodes was determined. In a sample of 23 breast cancers, Macchetti et al. (7) had also proved a positive correlation between a high degree of infiltration of CD4+ lymphocytes and the presence of metastases in the axillary lymph nodes. In our research, a positive correlation between the degree of LI in tumor, as well as CD4+ lymphocytes and its size was found. Tsang et al. (5) and Loi et al. (8), had also determined a positive correlation between the intensity of LI and the size of the tumor. However, these researches, did not observe only the ductal breast

cancer, and it is well known that the degree of LI significantly varies in different histological types (5).

This research determined a positive correlation between the level of TILs, CD4+ and CD8+ lymphocytes and HER2/neu amplification, which is in accordance with the literature data (5,8-10). This correlation is explained by the fact that the signals within the framework of EGF family of receptors may lead to activation of NF-κb signaling pathway in breast carcinoma. Activation of this signaling pathway causes a lymphocyte activation and inflammation (11). Our paper determined a positive correlation between a triple negative tumor and TILs. The same results were obtained for CD4+ lymphocytes. The researches of Loi et al. (8), also confirmed that in triple negative tumor, a dense LI was expressed but the research did not observe only ductal breast cancers.

Upon previous studies as well as based upon our own research, it may be concluded that HER2 positive and triple negative tumors are more immunogenic than luminal carcinoma of A type, which is the result of greater presence of lymphocytes in microenvironment of these tumors. This might be used in identification of those patients who would benefit more from immunotherapy, considering that tumors with dense and poor lymphocytic infiltrate have different biological behavior. This research determined a positive correlation between degree of LI and histological grade. Furthermore, a greater degree of LI was also observed in triple negative and HER2 positive tumors, while it is known that these tumors are characterized by a high histological grade (14). This research determined a negative correlation between the patients' age and the level of LI.

Aging causes disorders in cellular signaling, production of cytokines (domination of pro-inflammatory cytokine profiles), the number of antigens recognized by T lymphocytes is reduced, activity of NKT and CTL cells is reduced, so this is also the way of explaining of the greater tumor incidence in the older age (15). This study, as well as most of the other studies, determined a negative correlation between hormone receptors and the degree of LI (8-10).

Hormone positive and negative breast cancers represent two separate groups of tumors which have different pathological and clinical behavior as well as different gene expression profiles, so, in accordance with this, they have different interactions with immune system (16). The presence of lower level of TILs in hormone positive tumors may be explained by the facts that ER+ carcinomas are occurring more often in older women, they are more often well differentiated, so they more rarely HER-2 positive (6). In our research, a lower degree of LI was observed in well differentiated tumors, without HER2/neu amplification and in older patients.

Our research showed that there is a positive correlation between the stage of the disease and the degree of LI as well as CD4+ lymphocytes. In available literature, only one study, performed on a sample of 48 patients (13) was found, which also obtained a positive correlation between the stage of the disease and the level of TILs. This correlation may be the consequence of the TILs ability to produce and release vascular endothelial growth factor and the fibroblast growth factor, which may induce lymphangiogenesis and angiogenesis, which then may lead to spread of malignant cells (17). TILs may release larger quantities of matrix metalloproteinase enzymes, as well as immune inhibitory cytokines like interleukine 4 (IL-4), IL-10 and transforming of the ß growth factor, which may all affect the disease progression (18). These cytokines were not detected in benign breast lesions, thus suggesting the significance of TILs in immunosuppression (19).

Traditionally, the CD8+ cytotoxic T lymphocytes are considered as a key component of anti-tumor immunity, and breast tumors with a higher level of infiltration of these cells have been associated with better prognosis (20). However, studies have shown that CD8+ cells often do not function fully in vivo conditions, if there is a lack of adequate CD4+ lymphocytes. It appears that CD4+ lymphocytes play a key role in activating and shaping the immune response (21). Differences in the results related to the CD8+ lymphocytes in the tumor can be explained by the heterogeneity of the population of cytotoxic lymphocytes.

Similar to CD4+ lymphocytes, CD8+ lymphocytes can be divided into various subgroups, based on the cytokines they express. Various subgroups of CD4+ and CD8+ lymphocytes have a different function and effects on the prognosis of the disease (22).

Clinical relevance of TILs has been observed in breast cancer, but the overall significance was still poorly defined. The possible reasons for this is that the results of previous studies are difficult to compare due to the differences in methodology (flow cytometry vs. immunohistochemistry; full section HE vs. tissue microarray-TMA), use of different methods in interpretation of immunophenotyping (quantitative vs. semi-quantitative) and the subsets of lymphocytes or breast cancers being analyzed.

## CONCLUSION

The results of this research indicate the correlation of TILs and CD4+ lymphocytes with numerous negative prognostic factors and shorter DFS, which all indicates that TILs, as well as CD4+ lymphocytes, are bad prognostic factors in patients with ductal breast cancer. This research may contribute to development of "immunological grade" of breast carcinoma which would reflect the intensity of the immune response of each individual patient and affect the disease prognosis together with the other standard prognostic parameters. Furthermore, understanding of the role of tumor infiltrating lymphocytes with regard to immune response in tumors is of a crucial significance for development of a new immunotherapeutic strategy in breast cancer treatment.

## AUTHORS' NOTE

I Kolarov-Bjelobrk conceived paper, oversaw data collection, conducted data analysis, wrote manuscript and approved final version. T Ivkovic-Kapicl participated in study design, data analysis and interpretation, critically revised manuscript and approved final version. D

Jovanovic participated in study design, data analysis, and interpretation of data and revision of manuscript and approved final version. J Trifunovic participated in study design, interpretation of data and revision of manuscript and approved final version. J Radic participated in study design and interpretation of data; critically revised manuscript and approved final versio. V Vidovic participated in study design and interpretation of data, critically revised manuscript and approved final version. B Vranjkovic participated in study design and interpretation of data, critically revised manuscript and approved final version. I Djan participated in study design and interpretation of data, critically revised manuscript and approved final version. L Popovic participated in study design and interpretation of data, critically revised manuscript and approved final version. The authors declare thatthey have no conflicts of interest.

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Table 1: Clinical and pathological characteristics of patients with breast cancer

| **Parameter** | **%** | **n (120)** |
|---|---|---|
| pT | | |
| ≤ 2cm | 49.2 | 59 |
| > 2cm | 50.8 | 61 |
| lgl | | |
| positive | 44.2 | 53 |
| negative | 55.8 | 67 |
| grade | | |
| G1 | 11.7 | 14 |
| G2 | 42.5 | 51 |
| G3 | 45.8 | 55 |

| | | |
|---|---|---|
| ER | | |
| negative | 27.5 | 33 |
| positive | 72.5 | 87 |
| PR | | |
| negative | 35.8 | 43 |
| positive | 64.2 | 77 |
| HER2 | | |
| negative | 81.7 | 98 |
| positive | 18.3 | 22 |
| triple- | | |
| yes | 14.2 | 17 |
| no | 85.8 | 103 |
| age | | |
| ≤ 50 | 31.7 | 38 |
| > 50 | 68.3 | 82 |
| stage | | |
| I | 35.8 | 43 |
| II | 50.8 | 61 |
| III | 13.3 | 16 |

Table 2: Correlation between TILs, CD4+, CD8+ lymphocytes and clinicopathological parameters

| | | **CD4+** | **CD8+** | **TILs** |
|---|---|---|---|---|
| CD4+ | r | 1,000 | **0,468** | **0,833** |
| | p | . | <0.0001 | <0.0001 |
| CD8+ | r | **0,468** | 1,000 | **0,507** |
| | p | <0.0001 | . | <0.0001 |
| TILs | r | **0,833** | **0,507** | 1,000 |
| | p | <0.0001 | <0.0001 | . |
| pN | r | **0,256** | 0,125 | **0,289** |
| | p | 0,005 | 0,173 | 0,001 |
| pT | r | **0,246** | 0,096 | **0,201** |
| | p | 0,007 | 0,297 | 0,028 |
| G | r | **0,398** | **0,235** | **0,398** |
| | p | <0.0001 | 0,010 | <0.0001 |
| ER | r | **-0,260** | **-0,306** | **-0,260** |
| | p | 0,004 | 0,001 | 0,004 |
| PR | r | **-0,305** | **-0,309** | **-0,305** |
| | p | 0,001 | 0,001 | 0,001 |
| HER2 | r | **0,243** | **0,236** | **0,329** |
| | p | 0,008 | 0,010 | <0.0001 |
| Triple negative | r | **0,202** | 0,161 | **0,202** |
| | p | 0,027 | 0,078 | 0,027 |
| Age | r | **-0,243** | **-0,299** | **-0,357** |
| | p | 0,007 | 0,001 | <0.0001 |
| Stadium of the disease | r | **0,311** | 0,072 | **0,276** |
| | p | 0,001 | 0,435 | 0,002 |
| Relapse | r | **0,266** | 0,156 | **0,304** |
| | p | 0,003 | 0,089 | 0,001 |
| Death | r | -0,092 | -0,098 | -0,092 |
| | p | 0,319 | 0,287 | 0,319 |

*p < 0.05

TILs: Tumor infiltrating lymphocytes, p: pathological, T: tumor size, N: node status, G: histological grade, ER: estrogen receptor, PR: progesterone receptor, r: Spearman's rank correlation coefficient

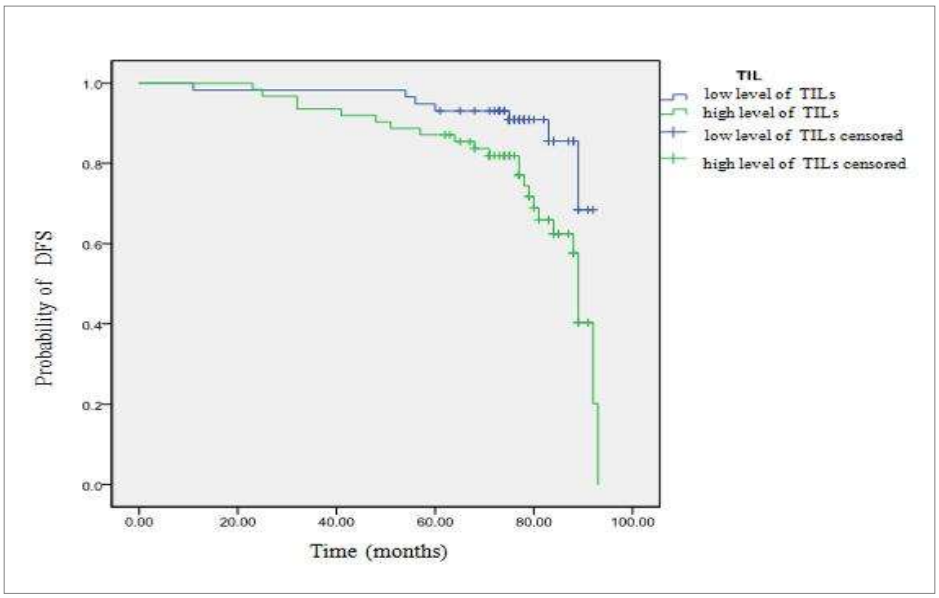


Fig. 1: Disease free survival in relation to expressions of TILs.

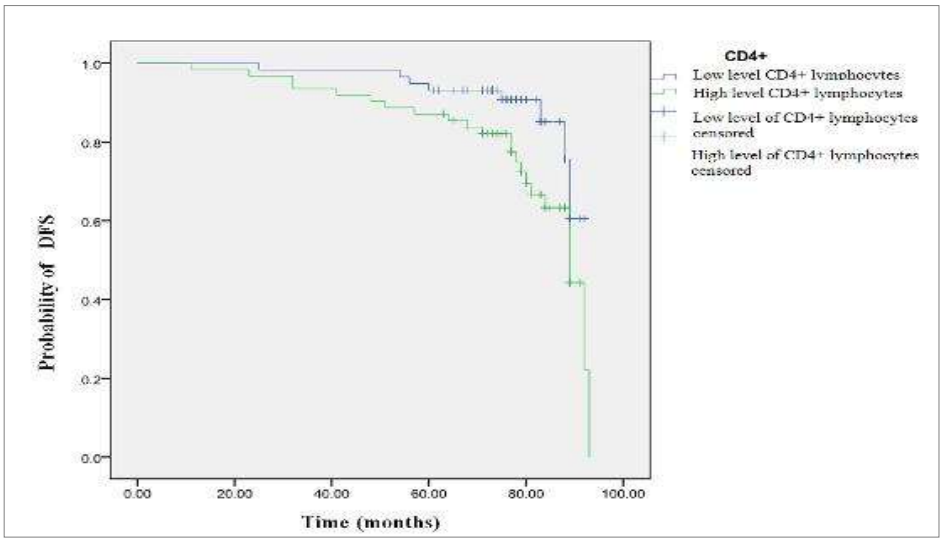


Fig. 2: Disease free survival in relation to expressions of CD4+ lymphocytes.