European Review for Medical and Pharmacological Sciences 2022; 26: 4207-4219

# The impact of tumor-infiltrating lymphocytes on tumor features and pathological characteristics in breast cancer patients: the role of cytotoxic T lymphocytes and regulatory T cells

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**Abstract. – OBJECTIVE: Though tumor-infiltrating lymphocytes (TILs) have a predictive impact in cancer patients, their association with presentation and prognosis in breast cancer is less consistent. This study aimed to assess the level of infiltrating cytotoxic T lymphocytes (CTLs) and regulatory T lymphocytes (Tregs) and their association with the clinicopathological features of breast cancer.**

**PATIENTS AND METHODS: Tissue samples from female patients (n=153) diagnosed with primary invasive breast cancer were stained with CD8 (a CTL marker) and Foxp3 (a Treg marker) using immunohistochemistry.**

**RESULTS: CTLs were distributed between tumor bed and stroma whereas Treg cells were mainly located in the stroma. The level of intratumoral CTLs correlated positively with Tregs in both tumor and stroma (rho=0.312, $p<0.001$ and rho=0.176, $p=0.031$; respectively). Stromal CTLs correlated positively with stromal Tregs (rho=0.319, $p=0.005$). Tumor size correlated inversely with the number of Treg cells in the tumor bed (rho= – 0.179, $p=0.028$). Tregs were associated with lymphovascular invasion status in the tumor bed ($p=0.042$). The ratio of intratumoral CTLs to Tregs was associated with estrogen receptor positivity and luminal subtype ($p=0.029$ and $p=0.045$, respectively). The median number of CTLs was significantly lower in patients using aspirin or antihypertensive medications compared to nonusers ($p=0.024$ and $p=0.03$, respectively).**

**CONCLUSIONS: TILs were distributed differently in tumor tissues of breast cancer patients. CTLs infiltrates were found in both tumor bed and stroma while Tregs were dominant in the stroma. TILs were also distinctly associated with tumor features. The impact of TILs on prognosis and treatment outcomes in Jordanian breast cancer patients needs further investigation.**

*Key Words:*
Breast cancer, Cytotoxic T lymphocytes, CD8, Regulatory T cells, Foxp3.

## Introduction

The tumor microenvironment (TME) involves a diverse and heterogeneous population of cells. These cells include fibroblasts, blood or lymphatic vessels, cancer cells, and infiltrative immune cells[1,2]. Regularly, tumors grow and progress in this complicated network of cells and interact with its components to expand and spread. The interaction between cancer cells and immune cells is best described as immunoediting. Immunoediting involves three phases: elimination, equilibrium, and escape[3,4]. In the elimination phase, the immune system detects and eliminates transformed cells through a process known as immunosurveillance[4]. The equilibrium phase involves the suppression of tumor expansion by residual tumor cells. Ultimately, tumor cells that survive the previous phases progress and grow to form clinically detectable tumors secondary to a deficient immune response[3,4]. Several mechanisms have been identified to explain the ability of cancer cells to escape the immune system. Such mechanisms include the downregulation of surface antigens, the stimulation of cancer cell survival pathways, recruitment of suppressive immune cells, and upregulation of immune checkpoints[3,5].

Several infiltrating immune cells were identified in the TME and are known to exert different effects on tumor progression[2]. Immune cells with antitu-

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mor effects include the natural killer cells, dendritic cells, M1 macrophages, T helper 1 lymphocytes, and cytotoxic T lymphocytes (CTLs)[2]. Alternatively, the M2 macrophages, myeloid-derived suppressor cells, T helper 2 lymphocytes, and regulatory T lymphocytes (Tregs) are known for their immunosuppressive effects[6,7]. Tumor-infiltrating lymphocytes (TILs) are immune cells detected in different types of solid tumors such as colon, ovarian, renal, lung, melanoma, and breast[2]. The degree and type of TILs have shown a potential prognostic and predictive value in solid tumors[8,9].

Breast cancer is an immunogenic tumor[3,10]. The immunogenicity of breast cancer is highly variable among the different molecular subtypes. Triple-negative breast cancer (TNBC) is the most immunogenic one compared to the human epidermal growth factor receptor 2 (HER2)-overexpressing and luminal cancers[11]. The relationship between breast cancer and TILs is not well-established. One study[12] on breast cancer found that 75% of TILs are T cells while B cells constitute about 20%. In general, higher levels of TILs in TNBC patients were associated with a favorable prognosis and response to chemotherapy than HER2-positive patients[13]. Results from the Neoadjuvant Gepar Quinto Trial indicated that increased TILs improved pathological complete response rates after chemotherapy treatment[14]. Alternatively, Huszno et al[15] showed that higher levels of TILs were associated with hormone receptor-negative status and HER2 overexpression. Yet, TILs lacked a prognostic value and did not predict overall survival (OS)[15].

Among TILs, CTLs and Tregs are key elements for immune attack and tolerance, respectively[16]. CTLs are CD8-positive T cells of the adaptive immune system and are the most powerful effectors in the anticancer immune response[17]. Regularly, CTLs recognize cancer cells in an antigen-specific manner[18]. Activated CTLs secrete cytotoxic molecules to induce a direct cytotoxic activity that correlated with better survival in breast cancer patients[19]. Tregs are a distinct subpopulation of helper T cells also known as CD25/CD4 forkhead box p3 (Foxp3)-positive T cells[18]. Treg cells are essential for the maintenance of immune self-tolerance to avoid immune system overstimulation and autoimmunity[18]. Bates et al[20] showed that the number of Treg cells was significantly higher in breast cancer patients with invasive tumors compared to ductal carcinoma *in situ*. In addition, a high number of Tregs was associated with lymph node involvement, high-grade tumors, and reduced OS in breast cancer patients. Studies[21,22] demonstrated that a higher CTLs to Tregs cell ratio is associated with improved survival and clinical outcomes in breast cancer patients receiving neoadjuvant chemotherapy. Alternatively, high Foxp3 expression and an increased Tregs to CTLs cell ratio correlated with worse prognosis and reduced survival in breast cancer patients[23].

The association between infiltrating CTLs and Tregs with the clinicopathologic features in Jordanian breast cancer patients is lacking. Besides, the impact of TILs among the different molecular subtypes is not well-characterized. This study aimed to describe the levels and localization of tumor-infiltrating CTLs and Tregs and to assess their association with the clinicopathologic characteristics in breast cancer patients.

## Patients and Methods

### Breast Cancer Patients and Tumor Data

Between 2014 and 2020, the archives of King Abdullah University Hospital (KAUH) revealed a total of 153 patients who met the inclusion criteria for this study. Eligible patients were adult women with a histologically confirmed diagnosis of primary invasive carcinoma of the breast. Patients who received any type of neoadjuvant therapy were excluded. Demographic and relevant medical information was retrieved from the electronic database of KAUH. According to the World Health Organization definition of obesity, patients were classified based on body mass index (BMI) into underweight, normal, overweight, and obese[24].

Tumor data were retrieved from pathology reports issued by the Pathology Department at KAUH at diagnosis. Pathological data included the size of the tumor, histopathologic type, the status of ipsilateral axillary lymph nodes, lymphovascular invasion (LVI) status, the expression of hormone receptors, and HER2. The stage of breast cancer was determined according to the tumor-node-metastasis cancer staging system of the American Joint Committee on Cancer[25]. According to the Nottingham Combined Histologic Grade system, patients were classified into grade I (low grade), grade II (intermediate grade), and grade III (high grade) carcinomas[26]. For HER2 expression status, scores of 0 or +1 on immunohistochemistry (IHC) indicated negative expression while a score of +3 indicated HER2 overexpression. For equivocal HER2 IHC results (score of +2), fluorescence *in situ* hybridization was applied. The molecular subtype of breast cancer was determined according to the expression status

of estrogen receptor (ER), progesterone receptor (PR), and HER2. These included luminal A (ER+ and/or PR+, HER2–), luminal B (ER+ and/or PR+, HER2+), HER2-enriched (ER–, PR–, HER2+), and triple-negative (ER–, PR–, HER2–)[27].

The protocol and procedure of the study were approved by the Institutional Review Board (IRB) committee of the Jordan University of Science and Technology (JUST) and KAUH (Research Grant Number: 14/126/2019). Informed consent was waived by the IRB committee because of the retrospective observational design in this study that involved the use of archival tumor samples.

### Immunohistochemistry

Formalin-fixed, paraffin-embedded archived tumor tissues for eligible patients were obtained from the Pathology Department. IHC was performed on sections that were cut at a thickness of 3 µm. Tumor sections were heated using an oven for 1 hr at 62°C and were let to cool down at room temperature. The staining procedure was performed using the fully automated Ventana BenchMark ULTRA IHC/ISH staining system[28] followed by the standard IHC procedures of the Pathology laboratory. The primary antibodies for the detection of CD8 (Ab4055, Abcam, Cambridge, MA, USA) and Foxp3 (Ab20034, Abcam, Cambridge, MA, USA) were added at dilutions of 1:500 and 1:300, respectively. The incubation time for the primary antibodies was 20 mins and 40 mins for CD8 and Foxp3, respectively. Tonsil tissue sections were used as positive control slides. Negative control slides were run with the primary antibody replaced by a buffer. Representative images for CD8 and Foxp3 immunohistochemical staining are shown in Figure 1.

### Evaluation of Immunostaining

CD8 is mainly distributed on the cell membrane and in the cytoplasm. Using high-power microscopy, five fields of view were selected randomly from each tissue section. The number of CD8-positive lymphocytes was determined by counting cells with moderate-to-strong staining intensity per high-power field (HPF) at 400x magnification. The average of the five fields was taken as the number of CD8-positive cells/tumor section. The same procedure was applied for Foxp3, however, ten random HPFs were examined from each tissue section. The detection of CD8 and Foxp3 was performed for lymphocytes infiltrating into cancer cell nests and in the stromal region for each section by the same method[6,29]. CD8 and Foxp3 cell count was classified into *'low'* and *'high'* based on the median value. Immunostaining was evaluated by two pathologists (R.M. and S.A.) who were blind to both the demographic and clinicopathologic data of patients. Discrepancies were resolved by joint discussion. The evaluation of immunostaining was performed avoiding areas of folded tissue, suboptimal preservation, necrosis, and technical artifacts.

### Statistical Analysis

Data were analyzed using the IBM SPSS statistical package (IBM Corp. Version 26.0. Armonk, NY, USA). Continuous variables are presented as mean ± standard deviation (SD) or median and interquartile range (IQR). Categorical variables are presented as frequency and percentages (n, %). The Mann-Whitney U test and Kruskal-Wallis analysis of variance were applied to compare two and multiple independent groups, respectively. Pearson's chi-square test of independence was used to examine associations between categorical variables. Correlations between continuous variables were assessed using Spearman's correlation test. All $p$-values were two-sided and statistical significance was indicated at $p<0.05$.

Some categorical variables were dichotomized ahead of performing statistical analysis to avoid a small sample size. The tumor stage was divided into early (I/II) and advanced (III/IV), and grade was categorized into grade (I/II) and grade (III). The histopathologic types were divided into invasive ductal carcinoma (IDC) and others. The molecular subtypes were grouped into luminal and non-luminal. These categories were determined based on cut points previously published in the literature[30].

## Results

### Demographic and Tumor Characteristics of the Study Population

The mean age of patients was 54.2±12.8 years (range 29 to 84, median 52, IQR 45-65). The mean BMI was 30.5±5.9 kg/m$^2$, ranging from 16.8 to 46.9 (median 30.1, IQR 26.2-34) (Table I). Hypertension and diabetes were the most frequent comorbidities (41.4% and 25.7%, respectively). The mean tumor size was 4.1±2.4 cm (range 1 to 20, median 3.5, IQR 2.6-4.9). The average number of lymph nodes affected was 5.6±7.8, with a range of 0 to 38 (median 2, IQR 0-7). IDC was the most frequent histopathologic type (73.9%). Fifty-seven patients (37.5%) had grade III disease. ER and PR positivity was reported in 88.7% and 81.7% of patients, respectively. Most patients had

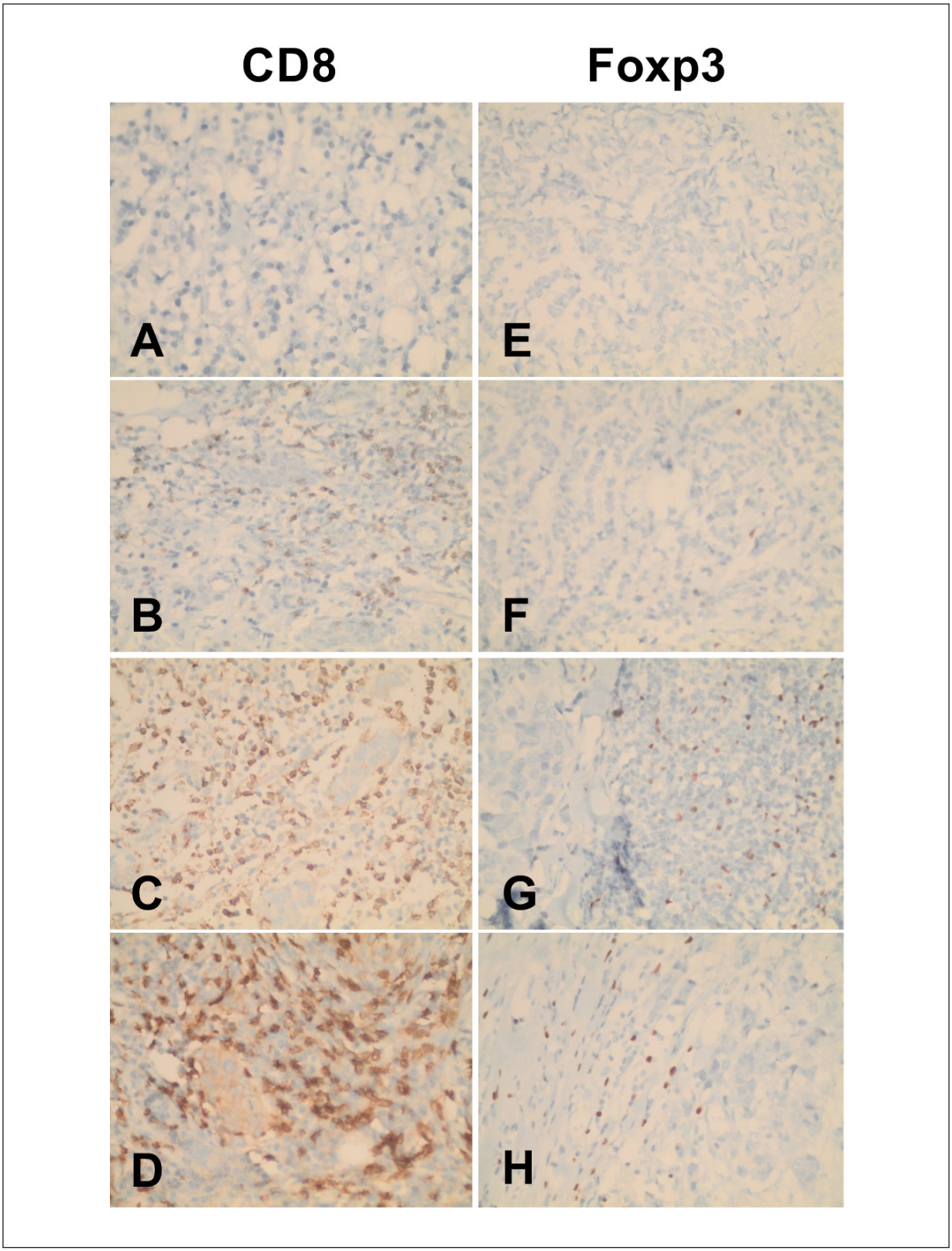


**Figure 1.** Immunohistochemistry staining for CD8 and Foxp3 in breast cancer tissues. Representative images for **(A)** negative; **(B)** weak; **(C)** moderate; and **(D)** strong staining for CD8. Representative images for **(E)** negative; **(F)** weak; **(G)** moderate; and **(H)** strong staining for Foxp3. [Magnification at x400]. CD8, the cluster of differentiation 8; Foxp3, forkhead box p3.

**Table I.** Breast cancer patients' demographics and tumor features‡.

| Characteristics | n (%) |
|---|---|
| BMI† | |
| Underweight | 3 (2.1) |
| Normal weight | 22 (15.1) |
| Overweight | 43 (29.5) |
| Obese | 78 (53.4) |
| Marital status | |
| Single | 9 (6.1) |
| Married | 135 (91.8) |
| Widowed/divorced | 3 (2.1) |
| Family history of breast cancer in first-degree relatives | |
| Present | 37 (25.2) |
| Absent | 110 (74.8) |
| Menopausal status | |
| Premenopausal | 64 (48.1) |
| Postmenopausal | 69 (51.9) |
| Comorbidities | |
| Hypertension | 63 (41.4) |
| Diabetes mellitus | 39 (25.7) |
| Ischemic heart disease | 13 (8.6) |
| Dyslipidemia | 11 (7.2) |
| Thyroid disorder | 10 (6.6) |
| Osteoporosis | 8 (5.3) |
| Respiratory disease | 6 (3.9) |
| Stroke | 5 (3.3) |
| Other | 23 (15.1) |
| Drug therapy | |
| Antihypertensives | 61 (40.4) |
| Antidiabetics | 39 (25.7) |
| Statins | 22 (14.6) |
| Aspirin | 18 (11.9) |
| Antisecretory | 18 (11.8) |
| Thyroxin | 9 (5.9) |
| Inhalers | 5 (3.3) |
| Other | 28 (18.5) |
| Tumor size | |
| T1 | 17 (11.1) |
| T2 | 97 (63.4) |
| T3 | 31 (20.3) |
| T4 | 8 (5.2) |

| Characteristics | n (%) |
|---|---|
| Lymph node status | |
| Negative | 43 (28.3) |
| Positive | 109 (71.7) |
| TNM stage | |
| I | 7 (4.6) |
| II | 66 (43.4) |
| III | 53 (34.9) |
| IV | 26 (17.1) |
| Grade | |
| Low (I) | 17 (11.2) |
| Intermediate (II) | 78 (51.3) |
| High (III) | 57 (37.5) |
| Histopathologic type | |
| IDC | 113 (73.9) |
| ILC | 10 (6.5) |
| Mixed | 20 (13.1) |
| Other | 10 (6.5) |
| ER | |
| Positive | 134 (88.7) |
| Negative | 17 (11.3) |
| PR | |
| Positive | 125 (81.7) |
| Negative | 28 (18.3) |
| HER2 | |
| Positive | 36 (25.5) |
| Negative | 105 (74.5) |
| LVI | |
| Identified | 74 (49.7) |
| Not identified | 75 (50.3) |
| Molecular subtype | |
| Luminal A | 98 (69.5) |
| Luminal B | 29 (20.6) |
| HER2-positive | 7 (5.0) |
| Triple-negative | 7 (5.0) |
| Surgery | |
| Mastectomy | 139 (90.8) |
| Wide local excision | 12 (7.8) |
| Breast conservation | 2 (1.3) |
| Chemotherapy | 106 (84.8) |

†BMI category was determined according to the World Health Organization system for classification of obesity into underweight (BMI<18.5 kg/m$^2$), normal (BMI 18.5-24.99 kg/m$^2$), overweight (BMI 25.0-29.99 kg/m$^2$), and obese (BMI≥30.0 kg/m$^2$). Other comorbidities include depression, rheumatic diseases, and osteoarthritis. Other therapies include antidepressants, steroids, and immunosuppressants. Other histopathologic types included medullary, metaplastic, mucinous, and neuroendocrine carcinoma.
‡The table has been adapted and modified with the publisher's permission from: Ayoub NM, Fares M, Marji R, Al Bashir SM, Yaghan RJ. Programmed Death-Ligand 1 Expression in Breast Cancer Patients: Clinicopathological Associations from a Single-Institution Study. Breast Cancer: Targets and Therapy 2021 Nov 13; 603-615. Originally published by and used with permission from Dove Medical Press Ltd.
BMI, body mass index; ER, estrogen receptor; HER2, human epidermal growth factor receptor 2; IDC, invasive ductal carcinoma; ILC, invasive lobular carcinoma; LVI, lymphovascular invasion; PR, progesterone receptor.

luminal tumors (90.1%). Other characteristics are shown in Table I.

### *The Number of CD8-Positive Cells, Foxp3-Positive Cells, and Their Ratio in Breast Cancer Tissues*

The median number of CD8-positive cells in the tumor bed was 3.4 cells/HPF (IQR 0.4-13.4). CD8-positive cell median number in the stroma was 3.2 cells/HPF (IQR 0.6-12.0). Seventy-seven patients (51.0%) had a low CD8-positive cell number, and 74 (49.0%) had a high CD8-positive cell number in the tumor bed. In the stroma, 78 (51.7%) and 73 patients (48.3%) had low and high CD8-positive cell counts, respectively. The median of Foxp3-positive cells in the

**Table II.** The correlations between CD8-positive, Foxp3-positive, and the ratio of CD8/Foxp3-positive cells in breast cancer tissues.

| Parameter | CD8-positive cells/Tumor | | CD8-positive cells/Stroma | | Foxp3-positive cells/Tumor | | Foxp3-positive cells/Stroma | | Ratio CD8:Foxp3-positive/Tumor | | Ratio CD8:Foxp3-positive/Stroma | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | rho | *p*-value | rho | *p*-value | rho | *p*-value | rho | *p*-value | rho | *p*-value | rho | *p*-value |
| CD8-positive cells/Tumor | - | - | 0.062 | 0.447 | 0.312 | <0.001* | 0.176 | 0.031* | 0.739 | <0.001* | -0.122 | 0.236 |
| CD8-positive cells/Stroma | 0.062 | 0.447 | - | - | -0.053 | 0.52 | 0.319 | <0.001* | -0.045 | 0.728 | 0.725 | <0.001* |
| Foxp3-positive cells/Tumor | 0.312 | <0.001* | -0.053 | 0.520 | - | - | 0.126 | 0.123 | -0.492 | <0.001* | -0.238 | 0.02* |
| Foxp3-positive cells/Stroma | 0.176 | 0.031* | 0.319 | <0.001* | 0.126 | 0.123 | - | - | 0.066 | 0.612 | -0.423 | <0.001* |
| Ratio CD8:Foxp3-positive cells/Tumor | 0.739 | <0.001* | -0.045 | 0.728 | -0.492 | <0.001* | 0.066 | 0.612 | - | - | 0.035 | 0.828 |
| Ratio CD8:Foxp3-positive cells/Stroma | -0.122 | 0.236 | 0.725 | <0.001* | -0.238 | 0.02* | -0.423 | <0.001* | 0.035 | 0.828 | - | - |

rho; Spearman's correlation coefficient. *Indicates statistical significance ($p<0.05$).

**Table III.** The association between the level of Foxp3-positive cells and clinicopathologic features of breast cancer.

| Characteristics | Foxp3-positive cells/Tumor | | | Foxp3-positive/Stroma | | |
|---|---|---|---|---|---|---|
| | Low (n=90) | High (n=62) | *p*-value | Low (n=80) | High (n=72) | *p*-value |
| Histopathologic type | | | 0.064 | | | 0.845 |
| IDC | 62 (68.9) | 51 (82.3) | | 60 (75.0) | 53 (73.6) | |
| Other | 28 (31.1) | 11 (17.7) | | 20 (25.0) | 19 (26.4) | |
| Stage | | | 0.502 | | | 0.826 |
| Early (I/II) | 41 (46.1) | 32 (51.6) | | 38 (47.5) | 35 (49.3) | |
| Advanced (III/IV) | 48 (53.9) | 30 (48.4) | | 42 (52.5) | 36 (50.7) | |
| Grade | | | 0.839 | | | 0.540 |
| Grade I/II | 56 (62.9) | 38 (61.3) | | 51 (64.6) | 43 (59.7) | |
| Grade III | 33 (37.1) | 24 (38.7) | | 28 (35.4) | 29 (40.3) | |
| ER | | | 0.989 | | | 0.589 |
| Positive | 78 (88.6) | 55 (88.7) | | 69 (87.3) | 64 (90.1) | |
| Negative | 10 (11.4) | 7 (11.3) | | 10 (12.7) | 7 (9.9) | |
| PR | | | 0.858 | | | 0.117 |
| Positive | 73 (81.1) | 51 (82.3) | | 69 (86.3) | 55 (76.4) | |
| Negative | 17 (18.9) | 11 (17.7) | | 11 (13.8) | 17 (23.6) | |
| HER2 | | | 0.640 | | | 0.108 |
| Positive | 20 (24.1) | 16 (27.6) | | 15 (20.0) | 21 (31.8) | |
| Negative | 63 (75.9) | 42 (72.4) | | 60 (80.0) | 45 (68.2) | |
| LVI | | | 0.042* | | | 0.327 |
| Identified | 49 (56.3) | 24 (39.3) | | 35 (45.5) | 38 (53.5) | |
| Not identified | 38 (43.7) | 37 (60.7) | | 42 (54.5) | 33 (46.5) | |
| Molecular subtype | | | 0.890 | | | 0.755 |
| Luminal | 75 (90.4) | 52 (89.7) | | 67 (89.3) | 60 (90.9) | |
| Non-luminal | 8 (9.6) | 6 (10.3) | | 8 (10.7) | 6 (9.1) | |

Data are presented as n (%). *Indicates statistical significance ($p<0.05$). Non-luminal tumors include HER2-enriched and triple-negative cancers. ER, estrogen receptor; HER2, human epidermal growth factor receptor 2; IDC, invasive ductal carcinoma; LVI, lymphovascular invasion; PR, progesterone receptor.

tumor was zero/HPF (IQR 0-0.5) and 0.4 cells/HPF (IQR 0-2.4) in the stroma. Ninety patients (59.2%) had a low and 62 (40.8%) had a high Foxp3-positive cell count in the tumor bed. In the stroma, 80 patients (52.6%) had a low, and 72 (47.4%) had a high Foxp3-positive cell count. The median ratio of CD8/Foxp3-positive cells was 11.2 (IQR 1.93-30.38) and 3.5 (IQR 0.6-10.6) in the tumor bed and stroma, respectively. As shown in Table II, CD8-positive cell number in the tumor bed positively correlated with Foxp3-positive cell number in both the tumor and stroma (rho=0.312, $p<0.001$ and rho=0.176, $p=0.031$; respectively). In addition, there was a positive and significant correlation between the numbers of stromal CD8-positive and Foxp3-positive cells (rho=0.319, $p=0.005$). The ratio of CD8/Foxp3-positive cells in the stroma was inversely correlated to the number of intratumoral Foxp3-positive cells (rho= – 0.238, $p=0.02$). Other correlations are shown in Table II.

### *The Association Between the Level of CD8-Positive Cells, Foxp3-Positive Cells, and Their Ratio With Tumor Characteristics in Breast Cancer Patients*

Age inversely correlated with the ratio of CD8/Foxp3-positive cells in the stroma (rho= – 0.316, $p=0.002$). Tumor size inversely correlated with Foxp3-positive cell count in the tumor bed (rho= – 0.179, $p=0.028$). No significant correlations were observed for the number of CD8-positive cells, Foxp3-positive cells, and their ratio with BMI or the number of involved lymph nodes.

The status of CD8-positive cells in the tumor bed or stroma was not associated with the tumor characteristics (**Supplementary Table I**). LVI was significantly associated with the level of intratumoral Foxp3-positive cells ($p=0.042$, Table III). The ratio of CD8/Foxp3-positive cells in the tumor bed was significantly associated with ER expression and molecular subtype ($p=0.029$ and $p=0.045$, respectively, Table IV). Patients with a high intratumoral ratio of CD8/Foxp3-positive

**Table IV.** The association between the ratio of CD8/Foxp3-positive cells and clinicopathologic features of breast cancer.

| Characteristics | Ratio CD8:Foxp3-positive cells/Tumor | | | Ratio CD8:Foxp3-positive cells/Stroma | | |
|---|---|---|---|---|---|---|
| | Low (n=124) | High (n=30) | *p*-value | Low (n=105) | High (n=49) | *p*-value |
| Histopathologic type | | | 0.393 | | | 0.015* |
| IDC | 89 (72.4) | 24 (80.0) | | 83 (79.8) | 30 (61.2) | |
| Other | 34 (27.6) | 6 (20.0) | | 21 (20.2) | 19 (38.8) | |
| Stage | | | 0.566 | | | 0.391 |
| Early (I/II) | 60 (49.2) | 13 (43.3) | | 47 (45.6) | 26 (53.1) | |
| Advanced (III/IV) | 62 (50.8) | 17 (56.7) | | 56 (54.4) | 23 (46.9) | |
| Grade | | | 0.344 | | | 0.622 |
| Grade I/II | 74 (60.7) | 21 (70.0) | | 63 (61.2) | 32 (65.3) | |
| Grade III | 48 (39.3) | 9 (30.0) | | 40 (38.8) | 17 (34.7) | |
| ER | | | 0.029* | | | 0.404 |
| Positive | 104 (86.0) | 30 (100.0) | | 89 (87.3) | 45 (91.8) | |
| Negative | 17 (14.0) | 0 (0.0) | | 13 (12.7) | 4 (8.2) | |
| PR | | | 0.066 | | | 0.362 |
| Positive | 97 (78.9) | 28 (93.3) | | 87 (83.7) | 38 (77.6) | |
| Negative | 26 (21.1) | 6 (6.7) | | 17 (16.3) | 11 (22.4) | |
| HER2 | | | 0.215 | | | 0.537 |
| Positive | 26 (23.2) | 10 (34.5) | | 26 (27.1) | 10 (22.2) | |
| Negative | 86 (76.8) | 19 (65.5) | | 70 (72.9) | 35 (77.8) | |
| LVI | | | 0.159 | | | 0.201 |
| Identified | 63 (52.5) | 11 (37.9) | | 46 (46.0) | 28 (57.1) | |
| Not identified | 57 (47.5) | 18 (62.1) | | 54 (54.0) | 21 (42.9) | |
| Molecular subtype | | | 0.045* | | | 0.375 |
| Luminal | 98 (87.5) | 29 (100.0) | | 85 (88.5) | 42 (93.3) | |
| Non-luminal | 14 (12.5) | 0 (0.0) | | 11 (11.5) | 3 (6.7) | |

Data are presented as n (%). *Indicates statistical significance ($p<0.05$)
Non-luminal tumors include HER2-enriched and triple-negative cancers.
ER, estrogen receptor; HER2, human epidermal growth factor receptor 2; IDC, invasive ductal carcinoma; LVI, lymphovascular invasion; PR, progesterone receptor.

cells were presented with ER-positivity and luminal tumors compared to those with a low ratio. The stromal ratio of CD8/Foxp3-positive cells was significantly associated with the tumor histopathologic type ($p$=0.015, Table IV).

### The Impact of Therapy on the Level of CD8-Positive Cells, Foxp3-Positive Cells, and Their Ratio in Breast Cancer Tissues

The effect of drug therapy on the levels of TILs and their ratio in breast cancer is shown in Table V. The median number of stromal CD8-positive cells was significantly lower in patients using aspirin and antihypertensive drugs compared to nonusers ($p$=0.024 and $p$=0.03, respectively). Further, the median number of Foxp3-positive cells in the tumor bed was significantly higher for patients using antidiabetic medications ($p$=0.029). The ratio of stromal CD8/Foxp3-positive cells was significantly lower in patients using aspirin and antihypertensive drugs compared to nonusers ($p$=0.045 and $p$=0.034, respectively) (Table V).

## Discussion

Immune cells are an important component of the TME[1]. TILs can be classified based on their position into intratumoral and stromal. Intratumoral TILs are lymphocytes located between tumor cells possessing cell-to-cell contact without intervening stroma. Stromal TILs, however, are scattered in the stroma between tumor cells without direct interaction with them[6]. In this study, we investigated the level of two main types of TILs: CTLs and Tregs. Antitumor effects of CTLs are mediated through direct cytolytic activity or by secreting cytokines such as interferon-γ and tumor necrosis factor-α[31,32]. Tregs play a key role in maintaining immune tolerance and preventing autoimmune reactions[33]. Tregs suppress antitumor immune response through cytolysis of CTLs and inhibition of dendritic cell maturation and function. Tregs also promote the overexpression of immune checkpoints and secretion of immunosuppressive cytokines such as interleukin-10 and transforming growth factor-β[33,34].

**Table V.** The level of CD8-positive cells, Foxp3-positive cells, and their ratio based on drug therapy in breast cancer patients.

| Parameter | Aspirin | | *p*-value | Antihypertensive drugs | | *p*-value | Antidiabetic drugs | | *p*-value |
|---|---|---|---|---|---|---|---|---|---|
| | Yes (n=18) | No (n=135) | | Yes (n=61) | No (n=92) | | Yes (n=39) | No (n=114) | |
| | Median (IQR) | Median (IQR) | | Median (IQR) | Median (IQR) | | Median (IQR) | Median (IQR) | |
| CD8-positive cells/Tumor | 3.2 (0.1-19.3) | 3.4 (0.45 -13.35) | 0.928 | 5.6 (0.45- 16.6) | 2.4 (0.4- 10.8) | 0.125 | 5.2 (0.4-14.2) | 3.2 (0.4-13.4) | 0.798 |
| CD8-positive cells/Stroma | 0.8 (0- 4.4) | 3.6 (1- 12.2) | 0.024* | 2.2 (0.05- 6.15) | 3.8 (0.9- 13.9) | 0.03* | 3.4 (1-7.6) | 3 (0.4-12.2) | 0.926 |
| Foxp3-positive cells/Tumor | 0.3 (0- 1.8) | 0 (0- 0.35) | 0.051 | 0 (0- 0.6) | 0 (0- 0.325) | 0.527 | 0.1 (0-1.1) | 0 (0-0.3) | 0.029* |
| Foxp3-positive cells/Stroma | 0.2 (0- 3.3) | 0.4 (0- 2.4) | 0.983 | 0.35 (0- 3.6) | 0.4 (0- 2.25) | 0.816 | 0.2 (0-3.2) | 0.4 (0-2.4) | 0.764 |
| Ratio CD8:Foxp3-positive cells/Tumor | 7.9 (0.74- 20.75) | 11 (2-31.5) | 0.35 | 16.8 (2.83-41.9) | 6 (1.12-27.33) | 0.28 | 7.4 (0.36-33.4) | 11.7 (3.15-31.13) | 0.581 |
| Ratio CD8:Foxp3-positive cells/Stroma | 0.24 (0-3.75) | 3.9 (0.99-12) | 0.045* | 2.3 (0.12-6.68) | 4.4 (1.33-12.29) | 0.034* | 3.04 (1.04-9.68) | 3.6 (0.51-11.67) | 0.986 |

*Indicates statistical significance ($p<0.05$)
Antihypertensive drugs included: Angiotensin-converting enzyme inhibitors, angiotensin receptor blockers, diuretics, calcium channel blockers, beta-adrenergic receptor blockers, alpha-adrenergic receptor blockers, and combinations of them.
Antidiabetic drugs included: Sulfonylureas, metformin, thiazolidinediones, insulin, and combinations of them.
IQR, interquartile range.

In this study, the localization of CTLs was comparable within the tumor bed and stroma. Tregs were dominant in the stroma. In agreement, Asano et al[35] found that infiltrating CTLs were detected in the tumor and stroma in 50.3% and 51.4% of breast cancer patients, respectively. In another study, however, greater infiltration of CTLs and Tregs was found in the stroma[21]. Contrary to our findings, Peng et al[23] showed a greater number of CTLs and Tregs in the stroma and tumor bed in breast cancer tissues, respectively. Our findings showed that the number of intratumoral CTLs positively correlated with the number of Tregs in both the tumor bed and the stroma of breast tumor tissues. This finding is not unlikely knowing that CTLs are responsible for tumor eradication while Tregs suppress immune system overactivation. Hence, the infiltration of CTLs to the tumor cells could be suggestive of immunogenicity of breast cancer and that Tregs are further recruited to the tumor bed to avoid the overactivation of CTLs[36]. CTLs and Tregs utilize similar methods for tumor infiltration[37,38]. Both cells express similar receptors that interact with endothelial selectins through the process of extravasation[39]. Additionally, CTLs and Tregs express chemokines responsible for the activation of adhesion molecules needed for their extravasation[40]. Thus, CTLs and Tregs could co-infiltrate into the tumor bed of breast cancer tissue. High endothelial venules which are blood vessels that normally exist in lymphoid organs, have been associated with the infiltration of TILs in breast cancer and could further explain the co-infiltration of CTLs and Tregs[41]. Earlier evidence[42-44] revealed an association between CTLs and Tregs and their concurrent infiltration in both ER-positive and ER-negative breast tumors. The ratio of CTL to Treg cell number is considered an indicator of cytotoxicity. In our study, the ratio of stromal CTLs to Tregs correlated inversely with the level of intratumoral Tregs. This finding can be explained by the low number of Treg cells infiltrating into the tumor bed compared to CTLs.

In our study, CTL infiltrates were not associated with tumor features. However, CTLs were associated with lymph node metastasis, grade, and stage of breast cancer in other studies[23,36]. Treg cells were associated with tumor size and LVI. Tsang et al[45] revealed a lack of association between the infiltration of CTLs and Tregs with the clinicopathologic characteristics of breast cancer patients. Existed evidence[21,46] showed that higher levels of CTL and Treg cells correlated with advanced tumor grade and hormone receptors. CTLs and Tregs were also associated with HER2 expression[21,46]. A higher Foxp3/CD8-positive cell ratio was associated with ER negativity[47]. In our study, the stromal CTL to Treg cell ratio was associated with invasive ductal histology of breast cancer. Alternatively, Glajcar et al[44] showed that the intraepithelial CTL to Treg cell ratio was associated with lobular histology and metastasis in patients with non-luminal tumors.

Our findings revealed a lack of association between CTLs and Tregs with the molecular subtype of breast cancer. However, the ratio of CTLs to Tregs in the tumor bed was associated with ER-positivity and the luminal subtype. Similarly, Kim et al[36] found no association between CD8- or Foxp3-positive cells with molecular subtypes despite a significant association between Treg cell levels and ER expression. In addition, Liu et al[21] showed a higher CTL to Treg cell ratio in luminal compared with non-luminal breast cancer. In contrast, Miyan et al[47] showed that the highest densities of CD8- and Foxp3-positive cells were detected in TNBC[47]. Alternatively, luminal A tumors were completely Foxp3-negative. Foxp3/CD8-positive cell ratio was highest in TNBC and lowest in luminal A patients according to the same study[47]. Overall, inconsistent findings have been observed in the literature regarding the expression of CTLs and Tregs and their relationship with clinicopathologic characteristics and outcomes in breast cancer. The variability can be explained, at least in part, by the populations studied, the methodologies used, and the immunoassay approach applied.

Our results demonstrate preliminary evidence for the effect of drug therapy on the level of TILs in the breast cancer microenvironment. The use of aspirin and antihypertensive medications reduced the levels of CTLs and the ratio of CTLs to Tregs in the stroma. Alternatively, antidiabetic treatment increased the number of intratumoral Tregs. Such results are not unlikely taking into consideration that many of these drugs have anti-inflammatory activity. Such effects could enhance the infiltration of Tregs into tumor tissue while reducing CTLs infiltration[48,49]. The antidiabetic drug metformin stimulated antitumor effects *via* increasing the number of infiltrating CD8-positive lymphocytes and suppressing their apoptosis *in vivo*[50]. Further, presurgical metformin has been shown to increase CTLs levels and decrease Tregs in head and neck squamous cell carcinoma in a recent study[51]. Because of the small number of patients in our study, the impact of drug therapy on TILs should be interpreted with caution. The impact of drug therapy on the antitumor immune response should be evaluated in larger populations of breast cancer patients.

TILs have emerged as promising targets for immunotherapy[52]. CTLs have been considered as the most effective immune cells to generate permanent antitumor activity in animal models[53]. The administration of dendritic cells and dendritic cell-induced antigen-specific CTLs enhanced the immune response and reduced the risk of relapse and metastasis in breast cancer patients[54]. The reactivation of CTLs from an exhausted state using immune checkpoint inhibitors is an effective immunotherapeutic strategy[17]. There is also mounting evidence[18,55] that depleting Tregs can restore antitumor immune response. Depletion of Tregs before surgery or radiation therapy enhanced antitumor immune activity and improved clinical outcomes in breast cancer patients[22]. Several approaches for Tregs depletion are under investigation. These include immune checkpoint inhibitors, low dose chemotherapy, and chemokine receptor blockade[34]. Nevertheless, systemic removal of Tregs may elicit detrimental autoimmunity[18,55]. Several strategies are under development to specifically target tumor-infiltrating Tregs without affecting tumor-reactive effector T cells[18,55].

The main limitations of this study were the retrospective nature and the mall sample size. Besides, the lack of survival data diminished the ability to evaluate the effect of TILs on patient survival as an outcome. However, the strengths of this study included the homogeneity of the population studied and using whole tumor sections for immunohistochemical staining.

## Conclusions

There is inconclusive evidence regarding the impact of TILs on clinicopathologic characteristics of breast cancer patients. In this sample of Jordanian patients, CTLs were localized in both tumor bed and stroma while Tregs were dominant in the stroma. While CTLs had no impact on clinicopathologic characteristics, both Tregs and the CTLs to Tregs cell ratio were associated with tumor features such as LVI, ER expression, and histopathologic type. The impact of TILs on prognosis and treatment outcomes in Jordanian breast cancer patients needs further investigation.

### Acknowledgments

Table I has been adapted and modified with the publisher's permission from 'Ayoub NM, Fares M, Marji R, Al Bashir SM, Yaghan RJ. Programmed Death-Ligand 1 Expression in Breast Cancer Patients: Clinicopathological Associations from a Single-Institution Study. Breast Cancer: Targets and Therapy 2021 Nov 13; 603-615'. Originally published by and used with permission from Dove Medical Press Ltd.

### Funding

This work was supported by a grant from the Deanship of Research at the Jordan University of Science and Technology (JUST) (Research Grant No. 20190529).

### Authors' Contributions

Mona Fares and Nehad M. Ayoub: conceived and designed the study, analyzed, and interpreted the results, and wrote the original manuscript. Raya Marji and Samir M. Al Bashir: performed immunohistochemical scoring. Osama M. Al-Shari: helped prepare and critically revise the manuscript. All authors read and approved the final manuscript.

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### Conflicts of Interest

The authors declare that they have no conflict of interest.

### Data Availability

The datasets generated and/or analyzed during the current study are not publicly available due to ethical reasons but are available from the corresponding author on reasonable request.

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