

# The Prognostic Role of Tumor-Infiltrating Lymphocytes CD8 and Foxp3 and their Impact on Recurrence in Breast Cancer Patients

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## Abstract

**Background:** The presence of tumor-infiltrating lymphocytes (TIL) within tumor epithelium or stroma of breast cancer is a surrogate of an immune response to cancer development, but their significance remains controversial. We conducted this study to evaluate the correlation of CD8+ (cytotoxic T) lymphocyte infiltration and Foxp3+ Tregs and tumor characteristics and their impact on recurrence in patients with invasive breast cancers.

**Patients and methods:** CD8+ T cells and Foxp3+ Tregs were detected by immunohistochemistry using the paraffin-embedded tumor tissues from 68 patients with stage (I to III) breast cancer. Clinicopathological data including patient's age, tumor size and grade, stage, lymph node metastasis, estrogen and progesterone receptor status, Ki-67, and human epidermal growth factor receptor-2/neu, and recurrence were reviewed.

**Results:** The decreased mean number of CD8+ T cells was significantly associated with tumors with lymph node metastasis ( $P=0.02$ ), and immune-positivity of Ki-67 ( $P=0.00$ ). The increased number of Foxp3+ Tregs was significantly correlated with tumors with lymph node metastasis ( $P=0.01$ ) higher stage (stage III,  $P=0.03$ ), and immune-positivity for Ki-67 ( $P=0.02$ ).

Further analysis of the correlation using CD8+ T-cell/Foxp3+ Treg ratio showed significant correlation with tumors with lymph node metastasis ( $P=0.01$ ), and immune-positivity of Ki-67 ( $P=0.00$ ). Also, there were significant correlations between the increased Foxp3+Treg /CD4+ T-cell ratio and lymph node metastasis ( $P=0.00$ ), and immune-positivity of Ki-67 ( $P=0.02$ ).

**Conclusions:** Data showed that lymph node metastases, tumor stage, immunopositivity of Ki67, and non-triple-negative tumors were associated with high regulatory T-cell infiltration. The prognostic role of immunologic balance as a marker for recurrence must be evaluated more clearly in a larger study.

**Keywords:** Early breast cancer; Foxp3+ Treg; CD8+ T cell

## Introduction

The presence of tumor-infiltrating lymphocytes (TILs) either within the tumor stroma or tumor epithelium reflects the immune response of the host against the tumor, defined as cancer "immunoediting" [1]. Large tumors are in the "escape" phase during which immune cells are not able to stop tumor growth but their presence at least denotes stand-by immunocompetency [1-3] which can be reactivated by treatment.

Many TILs have been recognized. Of those, fork head box P3-positive (Foxp3+) regulatory T cells (Tregs), CD8+ T cells, and CD4+ T cells are known to be the main keys for immune surveillance and tolerance, respectively [4]. CD8+ T cells are mediators of antitumor immunity and can lyse tumor cells directly [5]. The clinical importance of CD8+ T cells has been suggested by many recent studies that reported a survival benefit in correlation with an increase in CD8+ T cells in large cohorts of various human cancer patients [6-12].

CD4+ T cells have an important role in antitumor immunity that was implicated by their helper or memory cell functions. CD4+ T cells may have several effector functions, such as priming tumor-specific cytotoxic T cells or macrophages that are involved in clearance of tumor cells [5]. In contrast, Tregs are known to have a very important role in escape of antitumor T-cell response in cancer cells [13], due to their ability to potently suppress immune reaction against tumors *in vivo* [14,15]. Tregs are a subset of T lymphocytes, constituting 5%-10% of CD4+T cells. Tregs regulate the immune response by suppressing the cytokine production and proliferation of effector T cells [16,17].

Tregs constitutively express CD25 (IL-2Ra-chain) on their surface

[18,19]. More recent studies have shown that the transcription factor Foxp3 is a key intracellular marker that has additional crucial functional and developmental importance for CD4+CD25+ Tregs [20].

Many studies have reported the presence of higher numbers of Tregs in the peripheral blood of patients with breast, gastric, esophageal, and liver cancer, compared with healthy controls [21-24]. Higher numbers of Tregs were associated with poor clinical outcome in many studies [21,25,26]. An increased Tregs population both in tumor tissues and peripheral blood was also reported in breast cancer, and a recent study demonstrated a significant infiltration of Foxp3+ Tregs intratumoral in high-risk breast cancer patients [21,27].

Different techniques are used for assessment of TILs. These techniques include simple counting of mononuclear cell infiltrate on routine hematoxylin and eosin (H&E) stained slides [28-30]; typing of an immune cell with immunohistochemistry [30-32]; immune-related gene expression [33-35] and weighing of the digital immune cell [36].

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Recent recommendations for the morphological assessment of TILs on H&E sections have been published to bring TILs closer to clinical application [30]. The aim of this study is to evaluate the correlation of CD8+ (cytotoxic T) lymphocyte infiltration and Foxp3+ Tregs and tumor characteristics and their impact on recurrence in patients with invasive breast cancers.

## Patients and Methods

### Patient selection

The present study included 68 patients who had been diagnosed with invasive ductal carcinoma, and underwent surgical excision followed by appropriate adjuvant treatment as indicated at Tanta University Hospital at the period from January 2010 to December 2011.

All patients provided an informed consent before surgery or core biopsy. Patients who received neoadjuvant chemotherapy or hormonal therapy were excluded.

All H&E stained slides for each patient were retrospectively reviewed, and we selected the most representative tumor block. All specimens were fixed in 10% formalin solution and embedded in paraffin for routine histopathologic analysis. The following Clinicopathological parameters were assessed for each patient, age, lymph node metastasis, tumor size, histologic grade, {was assessed using the Nottingham grading system [37]}, nuclear grade, hormonal receptor status including estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2) status, Ki-67, tumor recurrence, distant metastasis, and survival. All tumors stages were classified according to the American Joint Committee on Cancer (AJCC)-TNM (tumor, node, metastases) classification (seventh edition) [38].

The median follow-up period after surgery was 24 months. This protocol was approved by our ethical committee at Tanta University Hospital.

### Immunohistochemistry

For immunohistochemical analysis, breast carcinomas sections with a serial 4-mm were treated on electrostatic slides. Xylene first used for deparaffinization at formalin-fixed and paraffin-embedded tissues then treated by ethanol for rehydration. After quenching of endogenous peroxidase activity with 3% hydrogen peroxide for 30 min, the slides were subjected to EDTA antigenic retrieval buffer (pH 8.0) then used a microwave oven for adequate heating.

Bovine serum albumin/1x tris-buffered saline (TBS) was used at a concentration of 0.3% for incubation of the given slides for 20 minutes to minimize nonspecific and unwanted background staining. At normal room temperature, a primary antibody was added for only 30 minutes. After a series of TBS rinse, the bound antibody was detected by using a polymer secondary antibody from the DAKO EnVision+system (DAKO, Carpinteria, CA).

The slides were rinsed with TBS series and visualized with a 10 minute incubation of liquid D, 3,3'-diaminobenzidine in buffered substrate (DAKO) for 10 minutes followed by hematoxyline used for counterstaining.

Staining of lymphocytes was considered as internal positive controls, with given sections. Tonsils can be used as external controls. Negative controls were done by the omission of the antibodies.

### Assessment of immunohistochemical staining

Immunohistochemical staining for CD4/CD8 was considered positive when lymphocytes showed evident membranous immunoreactions. Immunohistochemical staining for Foxp3 was considered positive when lymphocytes showed evident nuclear immunostaining.

The average scoring of the number of Foxp3+lymphocytes, CD8+ and CD4+ T positive cells present within the malignant epithelium and just adjacent stroma (within the same high-powered field; HPF) was counted manually in 3 HPFs (x400) then the average of each marker-positive cells was calculated.

Expression patterns of Ki-67 were determined in a semi quantitative manner by light microscopy. Cases with >14% of ki-67-positive cancer cells were considered as those with higher proliferative activity (categorized as ki-67-positive cancers).

### Statistical analysis

The correlations between subsets of TILs (CD8+ T cells, Foxp3+ Tregs, CD8+ T-cell/Foxp3+ Treg ratio, and CD4+ T-cell/Foxp3+ Treg ratio) and clinicopathological characteristics listed before were analyzed by Spearman test. The statistical significance of the difference between the 2 groups was determined using the Mann-Whitney test. Simple logistic regression analysis was used for comparisons of independent variables with the dependent variable. Disease-free survival was measured from the date of diagnosis to the time of recurrence.

The Kaplan-Meier method was used for calculation of Univariate analysis. A P value of less than 0.05 was considered significant. The log-rank test was used for confirmation of significance.

Cox proportional hazards regression model was used for calculation of multivariate analysis.

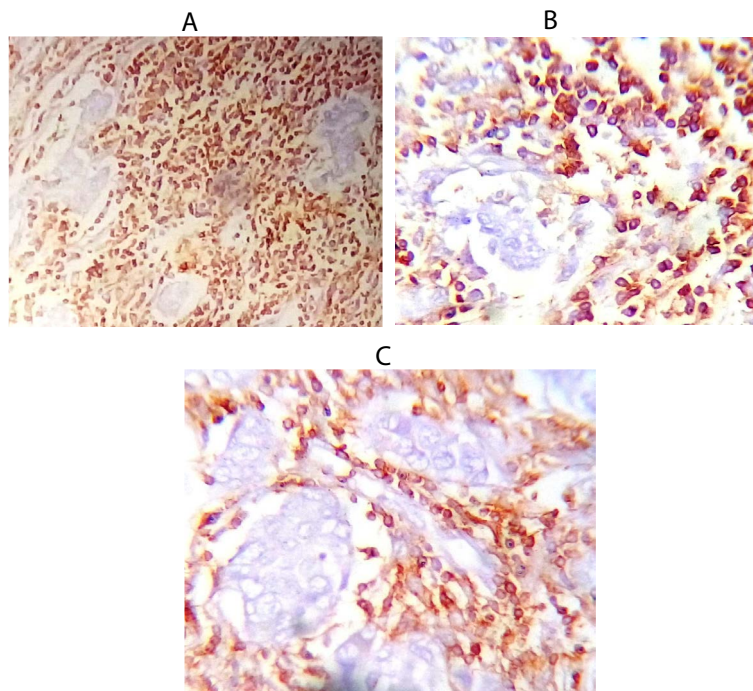
## Results

### Study population

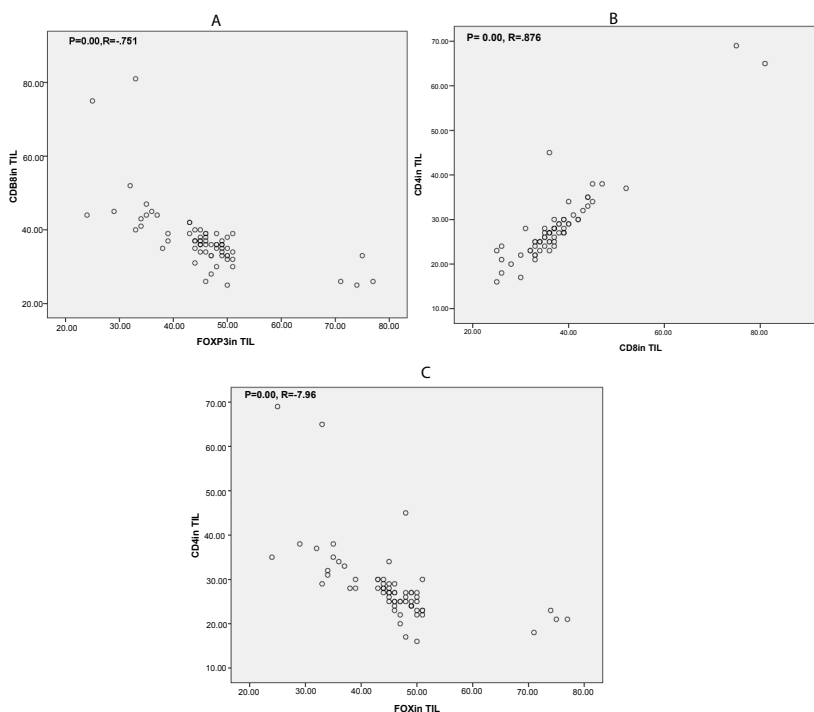
The clinicopathological characteristics of our patients are demonstrated in Table 1. The median follow-up duration of the study population was 24 months (range; 6-42). The median age of the patients was 54 years (range, 28-70), and 41.7% of the patients were postmenopausal. All of the patients had operable early stage breast cancer; 53 patients (78%) were clinical stage I or II and 15 patients (22%) were stage III. Forty-five patients (66.1%) had estrogen receptor-positive cancers, 15 patients (22%) had HER-2 amplified, and 24 patients (35.2%) had triple-negative cancers. All of the patients are still alive, and 7 patients (10.3%) had recurrent disease.

### Correlation of TIL subtypes with clinicopathological features

In this study, we observed that TILs were frequently found in both the tumor epithelium and stroma in the form of lymphoid aggregates. The representative pictures of lymphoid cells are shown in Figure 1. The correlations among the subtypes (CD4+, CD8+ T cell, and Foxp3+ Tregs) of tumor infiltrating lymphocytes were analyzed. There was a significant correlation between the expressions of each pair subtype by linear correlation analysis (Figure 2). The relationships between the mean numbers of each subtype of TILs (CD8+ T cells, Foxp3+ Tregs) per HPF with clinicopathological features of our study are shown in Table 1. Further analysis of ratio of the subtypes of tumor infiltrating lymphocytes (CD8+ T-cell/Foxp3+ Treg ratio and CD4+ T-cell/Foxp3+ Treg ratio) were also performed (Table 2).



**Figure 1:** (1A) Fox: Strong nuclear immunostaining of Fox with negative immunoreaction of malignant ductal cells inbetween (x200). (1B) CD8: Strong positive membranous immunoreaction for CD8, with negative immunohistochemistry staining of malignant ductal cells in between (x400). (1C) Strong positive membranous immunoreaction for CD4, with negative immunohistochemistry staining of malignant ductal cells in between (x400).



**Figure 2:** (A,B,C) Correlation between CD 4+, CD8+ T lymphocytes and FOXP3+ Tregs.

The decreased mean number of CD8+ T cells was significantly associated with tumors with lymph node metastasis ( $P=0.02$ ), and immune-positivity of Ki-67 ( $P=0.00$ ). The increased number of Foxp3+ Tregs was significantly correlated with tumors with lymph

node metastasis ( $P=0.01$ ) higher stage (stage III,  $P=0.03$ ), and immune-positivity for Ki-67 ( $P=0.02$ ).

Further analysis of the correlation using CD8+ T-cell/Foxp3+ Treg ratio showed significant correlation with tumors with lymph node

metastasis(P=0.01), and Ki-67 (P=0.00). Also, there were a significant correlations between the increased Foxp3+Treg /CD4+ T-cell ratio and lymph node metastasis (P=0.00), and Ki-67 (P=0.02).

### Prognostic significance of TIL subtypes in breast cancer

To investigate the prognostic impact of tumor infiltrating lymphocytes on disease-free survival (DFS), 68 patients who were diagnosed with early breast cancer were analyzed (Figure 3).

Although CD8+T cells, the number of Foxp3+ Tregs/HPF, CD8+ T-cell/Foxp3+ Treg ratio, and CD4+ T-cell/Foxp3+ Treg ratio were applied to various dichotomous cut-off points, none of them showed a significant correlation with overall DFS.

Patients with higher CD8+T-cell/Foxp3+ Treg ratio showed better disease free survival with borderline significance (P=0.06).

We also investigated the prognostic value of TIL subtypes. Univariate analysis indicated that DFS was significantly different according to age (P=0.024), LN (P=0.012), and KI 67 (P=0.012) (Table 2). CD8+ T cells, Foxp3+ Tregs, CD8+T-cell/Foxp3+ Treg ratio, and CD4+ T-cell/Foxp3+ Treg ratio had no significant impact on DFS. However, age, LN, and Ki-67 were no longer significant in multivariate analyses.

### Discussion

The immune system use complicated balance between different positive and negative signals to protect the body from foreign agents

Characteristics	No.cases	CD8+	Foxp3+	CD8+/Foxp3+	Foxp3+/CD4+
<b>Age</b>					
≤50	23	39.34	0.32	46.43	0.95
>50	45	36.62	0.43	45.17	0.84
<b>Tumor size</b>					
≤5	39	38.69	0.33	44.33	0.16
>5	29	36	0.16	47.31	0.78
<b>Grade</b>					
1-2	55	38.18	0.08	45.01	0.9
3	13	34.84	0.52	48.07	0.8
<b>LN</b>					
0-1	51	39.33	0.02	43.41	0.96
02-Mar	17	32.17	0.01	52.17	0.63
<b>Stage</b>					
I/II	53	37.28	0.18	45.92	0.86
III	15	38.46	0.03	44.46	0.95
<b>ER</b>					
Neg	23	36.92	0.72	45.04	0.86
pos	45	37.91	0.75	45.88	0.89
<b>PR</b>					
Neg	35	38.62	0.87	45.57	0.93
pos	33	36.39	0.76	46.63	0.82
<b>Her2</b>					
Neg	53	35.88	0.08	45.84	0.8
pos	15	43.4	0.07	44.73	1.16
<b>Ki</b>					
Neg	60	40.3	0	43.51	1.01
pos	8	33.82	0.02	48.41	0.7
<b>TN</b>					
Yes	21	41	0.17	41.57	1.07
no	47	36	0.02	47.4	0.8

Table 1: Correlation between clinicopathological characteristics and subtype of TILs.

Characteristics	Univariate Analysis		Multivariate Analysis	
	P	HR (95% CI)	P	HR (95% CI)
<b>Age</b>				
≤50	0.024	2.46	0.311	0.431-14.098
>50				
<b>Tumor size</b>				
≤5	0.489			
>5				
<b>Grade</b>				
1-2	0.077	3.457	0.161	0.611-19.556
3				
<b>LN</b>				
0-1	0.012	2.105	0.353	0.438-10.128
2-3				
<b>Stage</b>				
I/II	0.083	0.000	0.974	0.000-5.691
III				
<b>ER</b>				
Neg	0.21			
pos				
<b>PR</b>				
Neg	0.304			
pos				
<b>Her2</b>				
Neg	0.245			
pos				
<b>Ki</b>				
Ki	0.012	2.18	0.502	(0.224-21.252)
Neg				
<b>Pos</b>				
Pos	0.172			
TN				
<b>CD8+</b>				
≤37	0.157			
>37				
<b>Foxp3+</b>				
≤45	0.129			
>45				
<b>CD8+/Foxp3+</b>				
CD8+/Foxp3+	0.065	0.000	0.976	0
≤0.88				
<b>Foxp3+/CD4+</b>				
≤1.7	0.577			
>1.7				

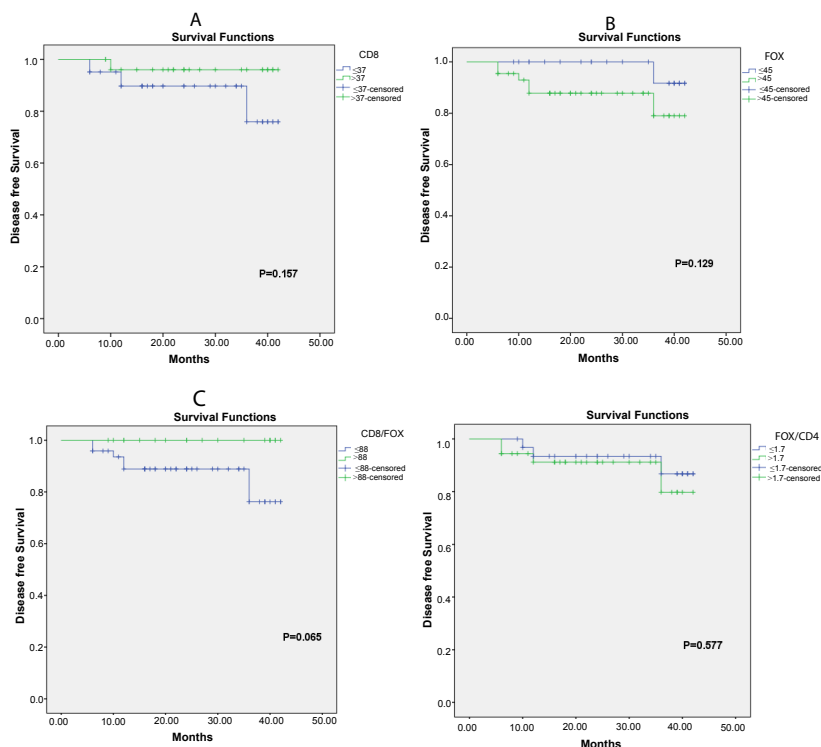
Table 2: Prognostic factors for disease-free survival in univariate and multivariate analysis.

[40]. Tumor growth and spread depends mainly on the interaction between the host immune system and malignant tumors.

The immune response of the host against tumor has been shown to change the response to chemotherapy. Denkert et al. reported that tumor infiltration by a high number of lymphocytes at diagnosis is associated with a higher likelihood of achievement of complete pathological response after neoadjuvant chemotherapy.

Besides clinical and treatment parameters, the host immune response affects the prognosis of cancer patients after standard treatment. TILs with their various types represent the reaction of the host to tumor antigens. Among subtypes of TILs, CD8+ T cells and Foxp3+ Tregs are the main keys for immune tolerance and surveillance, respectively [39]. Numerous recent studies have shown the importance of the balance between immune surveillance and immune evasion in tumor microenvironment [40-42].





**Figure 3:** A Kaplan-Meier curve for disease-free survival of all patients stratified by low or high numbers of CD8+ T lymphocytes (A) Foxp3+ Tregs (B) CD8+ T lymphocytes/FOXP3+ Tregs ratio (C) and FOXP3+ Tregs /CD4+ T lymphocytes ratio (D) P values <0.05 were considered significant.

Many randomized adjuvant clinical trials had evaluated the prognostic role of tumor-infiltrating lymphocytes (TIL) in breast cancer. In summary, TIL at baseline are associated with high-grade, high-proliferative, ER-negative tumors and represent a strong prognostic factor for some molecular subtypes, mainly for triple-negative breast cancer (TNBC) [43]. In all these trials, however, patients were treated with adjuvant chemotherapy; so, the prognostic role of TIL in chemotherapy-naïve patients is unknown. This information is important before defining the role of TIL in clinical practice. Moreover, few studies have studied the role of tumor-infiltrating lymphocytes in predicting sensitivity to specific treatments.

In our study, we investigated the infiltration of Foxp3 Treg, CD4, and CD8 T cells separately in the tumor epithelium and stroma, and assessed its association with clinical and pathological parameters, biologic subtypes, and patients' outcome. Immunohistochemical analyses were performed using full tissue sections instead of tissue microarrays to reduce selection bias. Each type of lymphocyte was counted manually on the immunostained slides. In our study, the decreased number of CD8+ T cells was significantly associated with poor prognostic factors including lymph node metastasis (P=0.02), and immunopositivity of Ki-67 (P=0.00). The increased number of Foxp3+ Tregs was significantly correlated with aggressive clinical characteristics as lymph node metastasis (P=0.01), higher stage (stage III, P=0.03), and Ki-67 (P=0.02).

In the further analysis, there were significant correlations between the increased Foxp3+ Treg/CD4+ T-cell ratio and lymph node metastasis (P=0.01), and immunopositivity of Ki-67 (P=0.02). The relation between tumor-infiltrating lymphocytes and aggressive biology of tumors shown here is in good correlation with the results from other studies, although there are still some contradictory data [44,45].

Breast cancer is a very heterogeneous disease, even after the recent molecular classification into 4 main subtypes according to the hormone receptor status, HER-2 expression, and Ki-67 [46-49]. Due to strong heterogeneity within the subgroups, this new classification, although it is based on biological and clinical behavior, is still unsatisfactory. TNBC has been known to be more heterogeneous than other subclasses and has poor prognosis compared with any other molecular subtype of tumors [50,51].

Although most patients with TNBC show aggressive clinical behavior and shorter survival, some TNBC patients have better clinical outcome. Recently, an immune response gene expression cluster (IR+) was used to identify a subgroup of ER-negative disease with a good prognosis [52]. Teschendorff et al. reported that the differences in clinical outcome of patients with ER-negative tumors are mainly related to differentially expressed genes in the complement and immune response pathways and that its association with prognosis may be independent of lymphocytic infiltration and lymph node status. Thus, the quality (IR+) of immune response might be more important than the quantity (lymphocytic infiltration) in the prognosis of ER-negative patients [52].

The results of our study showed that TNBCs may have favorable immune cell profiles than non-TNBCs: lower Foxp3+ Treg (P=0.02) in TNBCs. However, our results are inconsistent with data from the previous studies in western countries, which reported an association of a significant number of Foxp3+ Tregs infiltration with ER-negative tumors [21,53]. These apparent discrepancies may be due to the different technical methods used for assessment of TIL; Bates et al used tissue microarray for TILs examination that can limit systemic examination of TILs while Bohling counted Foxp3+ cells manually instead of the digital image analyzer. Another factor should be

considered that is ethnic differences between our study and others. Thus, our observations should be validated in a large cohort of Egyptian patients and in different ethnic group.

Although the prognostic significance of immune cell infiltrations including Foxp3+ Tregs were demonstrated in previous studies, [21] no immune cell subtypes or their ratio has been identified as an independent prognostic factor in our study. It might be partially explained by relatively short median follow-up time (24 months); thus, the number of events is still small to give a significant difference according to potential prognostic factors.

## Conclusion

In conclusion, we demonstrated that higher number of CD8+ T cells and lower number of Foxp3+ Tregs and Foxp3+Treg /CD4+ T-cell ratio were associated with favorable clinic-pathologic features, whereas higher numbers of Foxp3+ Tregs were associated with aggressive tumor behavior. In this study, patients with TNBCs had a lower number of Foxp3+ Treg. Although prognostic significance according to the subtypes and balance of tumor infiltrating lymphocytes remains unclear, immunologic microenvironment according to biological subtypes of breast cancer should be more clearly characterized in future studies.

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