The Egyptian Journal of Hospital Medicine (April 2022) Vol. 87, Page 1216-1225

# Expression of Tumor infiltrating Lymphocytes (Tils), Forkhead Box P3 (FOXP3) and Cyclooxygenase-2 (COX2) in Breast Cancer Patient's Tumor Tissue, A Single Institute Study

**Hagar. A. Alagizy*[1], Hayam Abdel-Samie Aiad[2], Moshira Mohamed Abdel-Wahed[2], Mahmoud Abdelsattar Elshenawy[1], Heba Gaber Abou-Sheishai[2] , Hala Said El-Rebey[2]**

Departments of [1]Clinical Oncology & Nuclear Medicine and [2]Pathology,
Faculty of Medicine, Menoufia University, Shebin El-Kom, Egypt

***Corresponding author:** Hagar Alagizy**, Mobile: (**+20)01017843828, **E-mail:** hagar76@hotmail.com

## ABSTRACT

**Background:** Breast cancer (BC) is the most common cancer worldwide and is the main cause of cancer related death in women. Forkhead boxp3 (FOXP3) is a transcription factor that plays an important role in the development and function of immune regulatory T cells (Tregs). A strong relation between the COX-2/PGE2 pathway and FOXP3-positive Tregs has been proposed.

**Objective**: We implemented this study to evaluate the prognostic significance of FOXP3 and COX-2 expression and relation with overall survival in BC patients.

**Patients and Methods:** This study was conducted on archival cases of 66 BC patients. Tumor infiltrating lymphocytes was evaluated in H&E slides whether peri-tumoral or intra-tumoral and were classified as absent, low, moderate, and high infiltration. Sections from each case were stained for FOXP3 and Cyclo-oxygenase 2(COX2). Expression of FOXP3 was assessed in malignant epithelial tissues and in tumor infiltrating lymphocytes (intra-tumoral and peri-tumoral). Expression of COX-2 was assigned in tumor epithelial cells and correlated with overall survival.

**Results:** Higher FOXP3 H. scores in epithelial cells were associated with younger age, low grade tumors, non-triple negative group. Higher FOXP3 H. scores in peri-tumoral lymphocytes were significantly associated with young age. Higher H. scores of COX2 expression was associated with PR negative cases. OS was improved in patients with high infiltration by peri-tumoral infiltrating lymphocytes and high FOXP3 positive intra-tumoral lymphocytes scores.

**Conclusions:** Dense TILs as well as high FOXP3 expression are considered as good prognostic factors. On the other hand, COX2 might be considered to have poor prognostic role.

**Keywords:** Tumor Infiltrating Lymphocytes, FOXP3, COX2, Immunohistochemical Expression, Breast Cancer.

## INTRODUCTION

Breast cancer (BC) is the most diagnosed cancer worldwide and is the main cause of death due to cancer in women in the less developed countries of the world [(1)].

In Egypt, breast cancer ranks as the first malignancy affecting females, contributing 30% of all female cancers [(2)].

Additionally, it accounts for 15.4% of cancers in both sexes [(3)].

Forkhead boxp3 (FOXP3) is a transcription factor that plays an important role in the development and function of immune regulatory T cells (Tregs) [(4)]. Increased Tregs activity is a supposed crucial mechanism of immune evasion by tumors and a wealthy milieu of molecules capable of increasing the number of FOXP3+ Tregs has been identified in the tumor microenvironment. This increase in FOXP3+ Tregs by tumor cells appears to be a powerful hindrance to attempts at cancer immunotherapy [(5)].

The prognostic significance of FOXP3+ tumor-infiltrating lymphocytes (TILs) in BC has been a site of debate. Some studies reported that FOXP3+ T-cell infiltration is associated with poor clinical outcomes [(6)], whereas others found no significant prognostic role for FOXP3+ infiltration in BC [(7)].

Cyclooxygenase-2 (COX-2) is one of the two isoforms of COX which is usually unexpressed in most normal tissues but induced rapidly by mitogenic and inflammatory stimuli. Furthermore, COX-2 is broadly expressed and strongly linked to a bad prognosis in a range of malignant tumors. Therefore, COX-2 may have a strong prognostic role in cancer patients [(8)].

An intimate relationship between the COX-2/PGE2 pathway and FOXP3-positive Tregs has been proposed [(9)].

Furthermore, reduction of FOXP3 protein levels was obtained using both non-selective COX and COX-2 selective inhibitors in a lot of tumors [(10)].

We implemented this study to evaluate the prognostic significance of FOXP3 and COX-2expression in breast cancer patients and correlate their expression with the clinicopathological data.

## PATIENTS AND METHODS:

This retrospective study was conducted on archival cases of 66 Egyptian BC patients. Cases were diagnosed in the Pathology Department, Faculty of Medicine, Menoufia University during the period between January 2010 and March 2015.

Eligible patients were adult females with a known diagnosis of breast invasive ductal carcinoma, of no special type (IDC, no special type) with available paraffin-embedded blocks of mastectomy specimens for serial cutting and examination, available clinical, survival, and follow up data and available estrogen receptor (ER), progesterone receptor (PR) and human



Received: 8/10/2021
Accepted: 6/12/2021

epidermal growth factor receptor 2 (HER2)/neu immunohistochemical (IHC) stained slides. BC cases subjected to neoadjuvant therapy were excluded from the study. This study and our access to the de-identified data were approved by the Clinical Research Ethics Committee of the Faculty of Medicine, Menoufia University Egypt.

Clinical data were collected from the patients' medical records and included Age; < 50 years and ≥ 50 years [(11)] as well as Menopausal state.

**Histopathological assessment:**

Re-evaluation of hematoxylin and eosin (H&E) stained sections to confirm a diagnosis and to determine the following parameters; Tumor grade [(12)], TNM staging according to AJCC 8th edition [(13)], Lymphovascular invasion (LVI) (present or absent), and Tumor-infiltrating lymphocytes in H&E (Based on low-magnification (× 100), average TIL densities were calculated by expressing stromal areas occupied by lymphocytes as percentages of total intra-tumoral (intra-tumoral TILs) or peri-tumoral (peri-tumoral TILs) stromal areas. The findings were categorized according to three possible cut-off points for TIL proportions as absent 0-10%, low 30-50%, moderate 30-50% and high >50%) [(14)].

**ER, PR and, HER2/neu IHC staining:**

Re-evaluation of the IHC stained slides. ER& PR-stained slides; positive if ≥1% of tumor cells showed nuclear staining [(15)]. HER2/neu stained slides; evaluated according to ASCO/CAP guidelines where positive cases (3+) showed complete membrane staining in >10% of tumor cells [(16)].

Molecular classification: Cases were classified according to IHC staining of ER, PR and, HER2/neu as follows: (1) Luminal group (2) HER2/neu positive group (3) triple negative.

Immunohistochemistry: Sections of 4 μm thickness, from each case, were stained for FOXP3 and COX-2. A Negative control slide was included in each run by omitting the primary antibody. Positive control for each run was also included; normal tonsillar tissue for FOXP3 and colorectal carcinoma for COX-2. The method used for immunostaining was a streptavidin-biotin amplified system. The primary antibodies used are (1) Rabbit monoclonal antibody against FOXP3 (Master diagnostica, Granada, Spain), 7 ml ready to use. (2) Rabbit monoclonal antibody against COX-2 (Thermo scientific, Fremont, CA, USA cat. #RM-9121-S0), 0.1 ml concentrated with a dilution of 1:100.

Expression of FOXP3 was assessed in; malignant epithelial tissues and tumor-infiltrating lymphocytes (intra-tumoral and peri-tumoral). The staining pattern was cytoplasmic in tumor cells and nuclear in tumor-infiltrating lymphocytes. FOXP3 expression was assigned in tumor epithelial cells as positive if > 25% of tumors were stained by FOXP3. FOXP3 expression was assigned in tumor-infiltrating lymphocytes as; 0:no positive cells, 1+:1%-25% positive cells, 2+:26%-50% positive cells and 3+:51%-100% positive cells. Scores of 0 and 1+ were defined as negative and scores of 2+ and 3+ as positive [(17)].

**Expression of COX-2 was assigned in;** tumor epithelial cells only where it was considered positive if ≥ 10% of the tumor showed cytoplasmic staining of COX-2 [(18)].
The staining intensity of both markers was assigned subjectively as mild, moderate, and strong then H.score was calculated to all studied cases according to the following equation: H.score = 1 x % of mildly stained cells + 2 x % of moderately stained cells + 3 x % of strongly stained cells .

**Survival data analysis**:

Survival data were collected from the archives of the Clinical Oncology Department, Menoufia University. The overall survival (OS) for the studied cases was calculated from the date of primary breast cancer diagnosis to the date of death or last follow-up.

**Ethics approval and consent to participate:**

**This study and our access to the de-identified data and the use of archival paraffin blocks were approved by the Clinical Research Ethics Committee of the Faculty of Medicine, Menoufia University Egypt.**

***Statistical analysis***

Data were collected, tabulated, and statistically analyzed using the Statistical Package for Social Science (SPSS) version 22.0 program: (Inc., Chicago, IL, USA).Contingency tables were analyzed with descriptive statistics (Arithmetic mean, Standard deviation (SD), Percentage (%), Median and Range) and analytic statistics(Chi- square ($X^2$) test, Mann-Whitney (Z) test, Kruskal-Wallis (K) test, Pearson correlation (r), Kaplan Meier survival curves, Multivariate cox regression analysis, and Student t test). The probability values <0.05 were regarded as statistically significant.

**RESULTS**

Patient's age ranged from 24 years to 84 years with a ±SD of 48.30 ± 10.15. Thirty-one cases (47 %) were postmenopausal. Most of the cases (51 cases, 77.3%) were grade 2. Also, in most cases (41 cases, 62.1%) were the T2 stage. Data about nodal status was available for 65 cases only where N0 was detected in 10 cases (15.2 %), N1 in 17 cases (25.8%), N2 in 26 cases (39.4%), and N3 in 12 cases (18.2%). Only 17 cases (25.8%) showed LVI. Seventeen cases (25.8%) were ER-positive, 12 cases (18.2%) were PR positive, and 17 cases (25.8%) were HER2/neu positive. Thirty-seven cases were triple-negative (56.1%), 12 cases were HER2/neu positive (18.2%) and 17 cases were luminal (25.7%).

**Tumor-infiltrating lymphocytes profile:**

In routine H&E staining, 2 cases showed absence of intra-tumoral lymphocytes infiltration (3%), 18 cases showed low infiltration (27.3%), 16 cases were moderately infiltrated (24.2 %) and 30 cases were highly infiltrated (45.5%) (Figure 1: A, B, C). In respect to peri-tumoral infiltrating lymphocytes, eight cases showed absent infiltration (12.1%), 15 cases showed low infiltration (22.7%), 25 cases were moderately infiltrated (37.9%) and 18 cases were highly infiltrated (27.3%) (Figure 1: D, E, F).

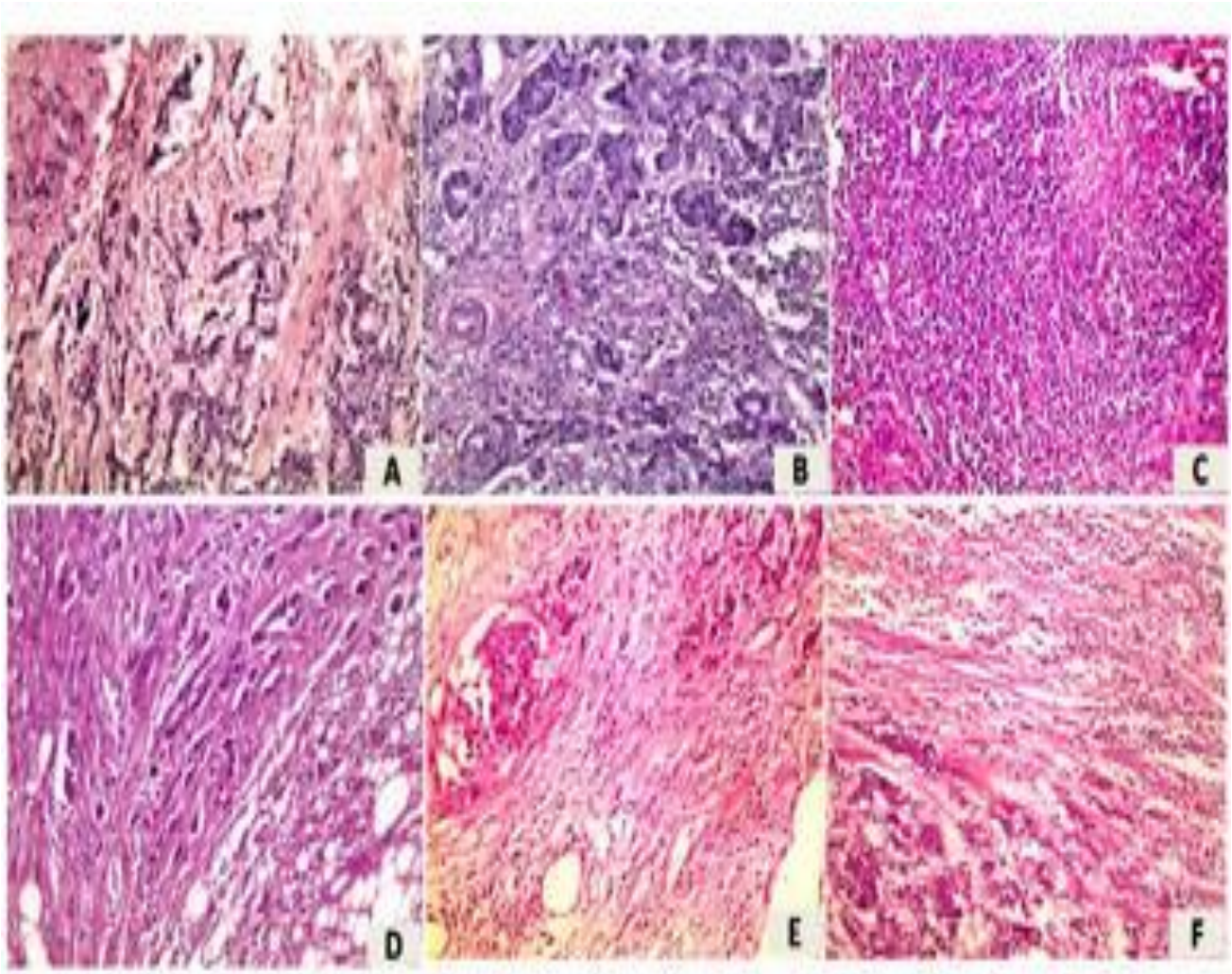


**Figure (1):** IDCs showing; A: mild intra-tumoral lymphocytic infiltration, B: moderate infiltration by intra-tumoral lymphocytes, C: high infiltration by intra-tumoral lymphocytes, D: mild infiltration by peri-tumoral lymphocytes, E: moderate infiltration by peri-tumoral lymphocytes and F: high infiltration by peri-tumoral lymphocytes. (H&Ex100 for A, B, D, E and F; H&Ex200 for C).

No significant relationship was found between intra-tumoral or peri-tumoral infiltrating lymphocytes regarding the clinicopathological parameters of the studied BC cases.

**Immunohistochemical profile of FOXP3:**

***1) IHC expression of FOXP3 in tumor cells of BC cases:*** Sixty-three (95.5%) cases showed positive expression of FOXP3 (Figure 2: A, B, C).

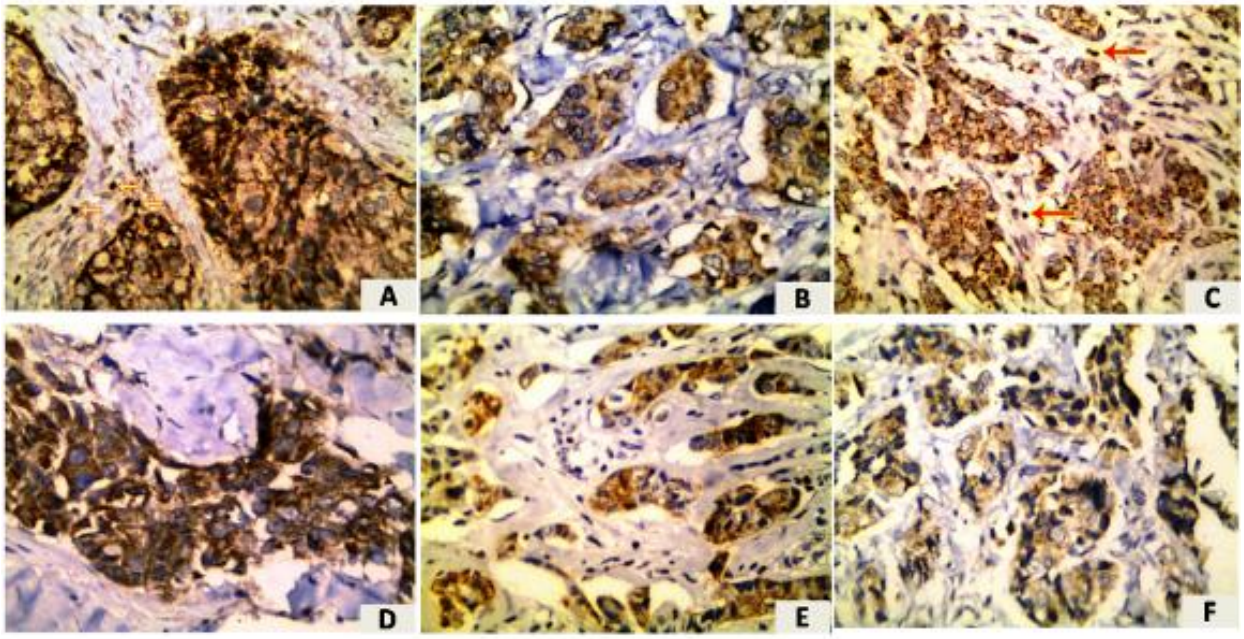


**Figure (2):** IDCs showing; A: strong cytoplasmic FOXP3 expression in tumor cells and few surrounding intra-tumoral lymphocytes showed strong nuclear staining (red arrows), B: moderate cytoplasmic FOXP3 expression in tumor cells, C: mild cytoplasmic FOXP3 expression in tumor cells and few intra-tumoral lymphocytes showing positive FOXP3 expression (arrows), D: strong cytoplasmic COX-2 expression in tumor cells, E: moderate COX-2 expression in tumor cells and F: mild cytoplasmic COX-2 expression in tumor cells. (IHC X400 for all).

The FOXP3 H. score ranged from 0 to 255 with ± SD of 118.18 ± 60.62 and a median of 115.

***2) IHC expression of FOXP3 in intra-tumoral infiltrating lymphocytes:*** Five (7.8%) cases were of score 0, 18 (28.1%) cases were of score 1+, 19 (29.7%) cases were of score 2+ and 22 (34.4%) cases were of score 3+ (Figure 3: A, B, C).

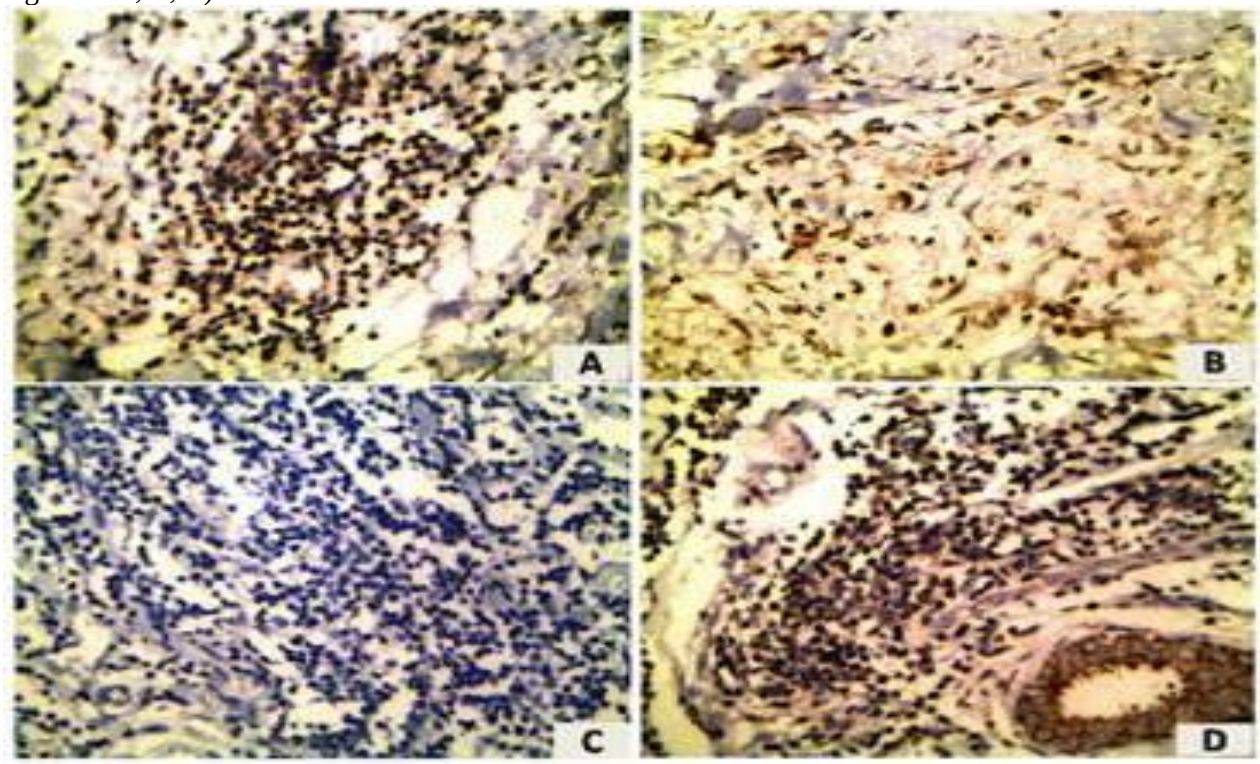


**Figure (3):** IDCs demonstrating; A: High intra-tumoral lymphocytic infiltration with focal strong nuclear FOXP3 expression, B: Mild intra-tumoral lymphocytic infiltration with scattered cells showed nuclear FOXP3 expression, C: Intra-tumoral infiltrating lymphocytes showed negative immunoreactivity for FOXP3 and D: High Peri-tumoral lymphocytic infiltration with focal strong nuclear immunoreactivity for FOXP3. (IHCX400 for all).

FOXP3 H scores ranged from 0 to 240 with ± SD of 113.83 ± 79.43 and a median of 135.

***3) IHC expression of FOXP3 in peri-tumoral infiltrating lymphocytes:*** Six (10.3%) were of score 0, 12 (20.7%) cases were of score 1+, 16 (27.6%) cases were of score 2+ and 24 (41.4%) cases were of score 3+ (Figure 3: D). FOXP3 H. score ranged from 0 to 255 with ± SD of 123.53 ± 78.87 and a median of 140.

***4) Relationship of FOXP3 H. scores in tumor cells with the clinicopathological parameters of BC cases:*** Higher FOXP3 H. scores in epithelial cells were significantly associated with younger age group, low grades BC cases, and a non-triple negative group of cases (Table 1).

**Table (1):** Correlation between FOXP3 H. score in tumor cells with the clinico–pathological parameters of BC cases.

| | n=66 | FOXP3 H.score in tumor cells Mean ± SD | Test of Significance | p value |
|---|---|---|---|---|
| **Age (years)** | | | | |
| <50 | 35 | 131.57 ± 55.46 | Z = 2.143* | 0.032* |
| ≥50 | 31 | 103.06 ± 63.49 | | |
| **Grade** | | | | |
| I | 4 | 191.25 ± 29.55 | | |
| II | 51 | 110.39 ± 55.20 | KW=6.511* | 0.039* |
| III | 11 | 127.73 ± 76.46 | | |
| **N (45 case)** | | | | |
| N0 | 10 | 125.50 ± 61.93 | KW=3.780 | 0.437 |
| N1 | 17 | 129.12 ± 51.97 | | |
| N2 | 26 | 116.54 ± 67.33 | | |
| N3 | 12 | 96.67 ± 58.67 | | |
| **Vascular Invasion** | | | | |
| Absent | 17 | 114.90 ± 54.81 | Z=0.602 | 0.547 |
| Present | 49 | 127.65 ± 76.10 | | |
| **Intra-tumoral lymphocytes in H&E** | | | | |
| Absent | 2 | 115.0 ± 77.78 | KW =0.950 | 0.813 |
| Low | 18 | 108.06 ± 57.47 | | |
| Moderate | 16 | 122.81 ± 63.14 | | |
| High | 30 | 122.0 ± 62.76 | | |
| **Peri-tumoral lymphocytes in H&E** | | | | |
| Absent | 8 | 116.25 ± 39.62 | | |
| Low | 15 | 137.67 ± 73.12 | KW =3.390 | 0.335 |
| Moderate | 25 | 122.20 ± 59.55 | | |
| High | 18 | 97.22 ± 56.31 | | |
| **ER** | | | | |
| Negative | 49 | 113.16 ± 60.24 | Z=1.292 | 0.196 |
| Positive | 17 | 132.65 ± 61.19 | | |
| **Her2neu** | | | | |
| Negative | 49 | 111.53 ± 62.61 | Z=1.498 | 0.134 |
| Positive | 17 | 137.35 ± 51.42 | | |
| **Molecular classification (1)** | | | | |
| Triple negative | 37 | 104.05 ± 60.10 | | |
| Her2 positive | 12 | 141.25 ± 53.60 | KW =5.049 | 0.080 |
| Luminal | 17 | 132.65 ± 61.19 | | |
| **Molecular classification (2)** | | | | |
| Non triple negative | 29 | 136.21±57.33 | Z=2.232* | 0.026* |
| Triple negative | 37 | 104.5 ± 60.10 | | |

$\bar{x}$: Mean **SD**: Standard deviation **H. score:** Histo score **n**: number
**N:** lymph node **KW**: Kruskal Wallis test **Z:** Mann Whitney test **rs**: Spearman coefficient **ER**: estrogen receptor **PR:** progesterone receptors **FOXP3**: Forkhead Box P3 *: Statistically significant.

***5) Relationship of FOXP3 H. score in tumor cells and its expression in both peri and Intra-tumoral infiltrating lymphocytes of BC cases:*** High H. score of FOXP3 expression in tumor cells was significantly associated with positive FOXP3 expression and its higher scores in intra-tumoral lymphocytes (p=<0.001 & p=0.002respectively). It was also associated with positive FOXP3 expression and its higher H. score in peri-tumoral lymphocytes (Table 2).

**Table (2):** Correlation between FOXP3 H. score in tumor cells and its expression in both peri and intratumoral infiltrating lymphocytes.

| | FOXP3 H. score in tumor cells Mean ± SD | Test of Significance | P value |
|---|---|---|---|
| **FOXP3 expression in intra-tumoral lymphocytes** | | | |
| Zero/ 1+ | 77.39 ± 38.52 | Z= 4.127* | <0.001* |
| 2+/3+ | 141.22±59.19 | | |
| **FOXP3 H. score in intra- tumoral lymphocytes** | ---------- | rs = 0.383* | 0.002* |
| **FOXP3 expression in peritumoral lymphocytes** | | | |
| Zero/ 1+ | 78.33 ± 49.26 | Z=3.266* | |
| 2+/3+ | 136.50±60.89 | | 0.001* |
| **FOXP3 H. score in peri-tumoral lymphocytes** | ------ | rs =0.427* | 0.001* |

**FOXP3:** Forkhead Box P3 **H. score:** Histo score **n:** number **Z:** Mann Whitney test **rs:** Spearman coefficient $\bar{X}$**:** Mean **SD:** Standard deviation, *: Statistically significant.

***6) Relationship of FOXP3 H. scores in intra-tumoral lymphocytes and the clinicopathological parameters of BC cases:*** There was no significant relationship between FOXP3 H. scores in intra-tumoral lymphocytes and any of the clinicopathological parameters of BC cases.

***7) Relationship of FOXP3 H. scores in peri-tumoral lymphocytes and the clinicopathological parameters of BC cases:*** High H. score of FOXP3 expression in peri-tumoral lymphocytes was significantly associated with a younger age group of cases (Table 3).

**Table (3):** Correlation between FOXP3 H. score in peri-tumoral lymphocytes and the clinico–pathological parameters of BC cases.

| | n=58 | FOXP3 H. scores in peri-tumoral lymphocytes (Mean ± SD) | Test of Significance | P value |
|---|---|---|---|---|
| **Age group** | | | | |
| <50 | 31 | 150.32 ± 65.55 | Z=2.568* | 0.010* |
| ≥50 | 27 | 92.78 ± 82.72 | | |
| **Menopausal status** | | | | |
| No | 35 | 142.42 ± 75.42 | Z=1.767 | 0.077 |
| Yes | 31 | 101.85 ± 78.50 | | |
| **T** | | | | |
| T1 | 7 | 141.43 ± 89.52 | | 0.147 |
| T2 | 37 | 106.49 ± 77.43 | KW=5.361 | |
| T3 | 10 | 150.0 ± 70.87 | | |
| T4 | 4 | 183.75 ± 60.47 | | |
| **N(45 case)** | | | | |
| N0 | 9 | 106.11 ± 90.06 | | |
| N1 | 14 | 142.14 ± 75.36 | Z=3.005 | 0.391 |
| N2 | 24 | 131.67 ± 75.80 | | |
| N3 | 11 | 96.36 ± 81.52 | | |
| **Vascular invasion** | | | | |
| No | 41 | 123.17 ± 77.34 | Z=0.241 | 0.810 |
| Yes | 17 | 124.41 ± 84.89 | | |
| **ER** | | | | |
| Negative | 43 | 116.86 ± 79.63 | Z=1.118 | 0.263 |
| Positive | 15 | 142.67 ± 76.01 | | |
| **PR** | | | | |
| Negative | 48 | 119.06 ± 76.71 | Z=1.182 | 0.237 |
| Positive | 10 | 145.0 ± 89.72 | | |
| **Her2neu** | | | | |
| Negative | 44 | 115.57 ± 79.92 | Z=1.227 | 0.220 |
| Positive | 14 | 148.57 ± 72.52 | | |
| **Molecular classification (1)** | | | | |
| Triple negative | 33 | 105.91 ± 80.75 | KW=3.558 | 0.169 |
| Her2 positive | 10 | 153.0 ± 67.17 | | |
| Luminal | 15 | 142.67 ± 76.01 | | |
| **Molecular classification (2)** | | | | |
| Non-triple negative | 25 | 146.80 ± 71.34 | Z=1.875 | 0.061 |
| Triple negative | 33 | 105.91 ± 80.75 | | |

$\bar{X}$: Mean **SD**: Standard deviation **H. score:** Histo score **n**: number, **N:** lymph node **KW**: Kruskal Wallis test **Z:** Mann Whitney test, **ER**: estrogen receptor **PR:** progesterone receptors **FOXP3**: Forkhead Box P3 *: Statistically significant.

***8) No significant difference was found when comparing intra-tumoral and peri-tumoral FOXP3 positive lymphocytes with clinicopathological parameters of BC cases.***

**Immunohistochemical profile of Cox2:**

Assessment of IHC expression of COX2 was done in malignant epithelial cells only. Twenty-seven out of 66 cases (40.9%) showed positive expression of COX2 (Figure 2: D, E, F). The COX2 H. score ranged from 0 to 270 with ± SD of 34.09 ± 55.31and a median of 0.

***1) Relationship of COX-2 expression and its H.score with the clinicopathological parameters of BC cases.*** A significant association was found between higher H. scores of COX2 expression and negative PR expression in tumor cells (p=0.025).

Furthermore, higher H. scores of COX2 expression were related to negative ER expression and a non-luminal group of BC cases although the statistical significance was not reached (p=0.069 for both).

***2) Relationship of COX-2 expression and its H. scores in tumor cells and FOXP3 expression in tumor cells and both intra-tumoral & peri-tumoral infiltrating lymphocytes of BC cases:*** Negative COX-2 in malignant epithelial cells tended to be associated with FOXP3 positivity in peri-tumoral infiltrating lymphocytes although not reaching the statistical significance was not reached (**P** =0.063) (Table 4).

**Table (4):** Correlation between COX-2 expression and H. score in epithelial cells and FOXP3 expression in tumor cells as well as both intra-tumoral and peri-tumoral infiltrating lymphocytes of BC cases

| | **COX-2 expression in tumor cells** | | **Test of Sig. &P value** | **COX-2 H. score in tumor cells** | |
|---|---|---|---|---|---|
| | **Negative (n=39)** | **Positive (n=27)** | | **Mean ± SD** | **Test of Sig. &P value** |
| | **n (%)** | **n (%)** | | | |
| **FOXP3 H.score in tumor cells** | 116.54 ±58.72 | 120.56 ±64.34 | Z=0.287<br>**P** =0.774 | ---------- | rs = 0.169<br>**P** =0.174 |
| | **(n=38)** | **(n=26)** | | | |
| **FOXP3 expression in intra-tumoral lymphocytes** | | | | | |
| Zero/1+ | 12(31.6) | 11(42.3) | $\chi^2$=0.772 | 22.39 ±31.62 | Z=0.016 |
| 2+/3+ | 26 (68.4) | 15(57.7) | **P** =0.380 | 40.085±65.19 | **P** =0.988 |
| **FOXP3 H score in intra-tumoral lymphocytes** | | | | | |
| Min. – Max. | 0.0 – 240.0 | 0.0 – 240.0 | | | |
| Mean ±SD | 126.05 ± 76.70 | 95.96 ± 81.44 | Z=1.306 | | rs = -0.064 |
| Median | 150.0 | 70.0 | **P** =0.192 | ---- | **P** =0.617 |
| | **(n=36)** | **(n=22)** | | | |
| **FOXP3 expression in peri-tumoral lymphocytes** | | | | | |
| Zero/1+ | 8(22.2) | 10(45.5) | $\chi^2$=3.444 | 28.89 ± 36.92 | Z= 0.904 |
| 2+/3+ | 28(77.8) | 12(54.5) | **P** =0.063 | 35.50 ± 65.09 | **P** =0.366 |
| **FOXP3 H score in peri-tumoral lymphocytes** | | | | | |
| Min. – Max. | 0.0 – 255.0 | 0.0 – 210.0 | | | |
| Mean ±SD | 134.58 ± 78.50 | 105.45±77.87 | Z=1.372 | | rs = -0.074 |
| Median | 150.0 | 120.0 | **P** =0.170 | ----- | **P** =0.579 |

**FOXP3**: Forkhead Box P3 **COX-2**: cyclooxygenase 2 **H. score:** Histo score **$\chi^2$:** Chi square test **Z:** Mann Whitney test **rs:** Spearman coefficient **n**: number

**Survival Analysis:**

The median OS for the studied group was 23 months (Range: 10-52). Univariate Kaplan-Meier survival analysis revealed improved OS in patients with high infiltration by peri-tumoral infiltrating lymphocytes on H&E and high FOXP3 positive intra-tumoral lymphocytes scores (p=0.018 and 0.014 respectively) (Figure 4).

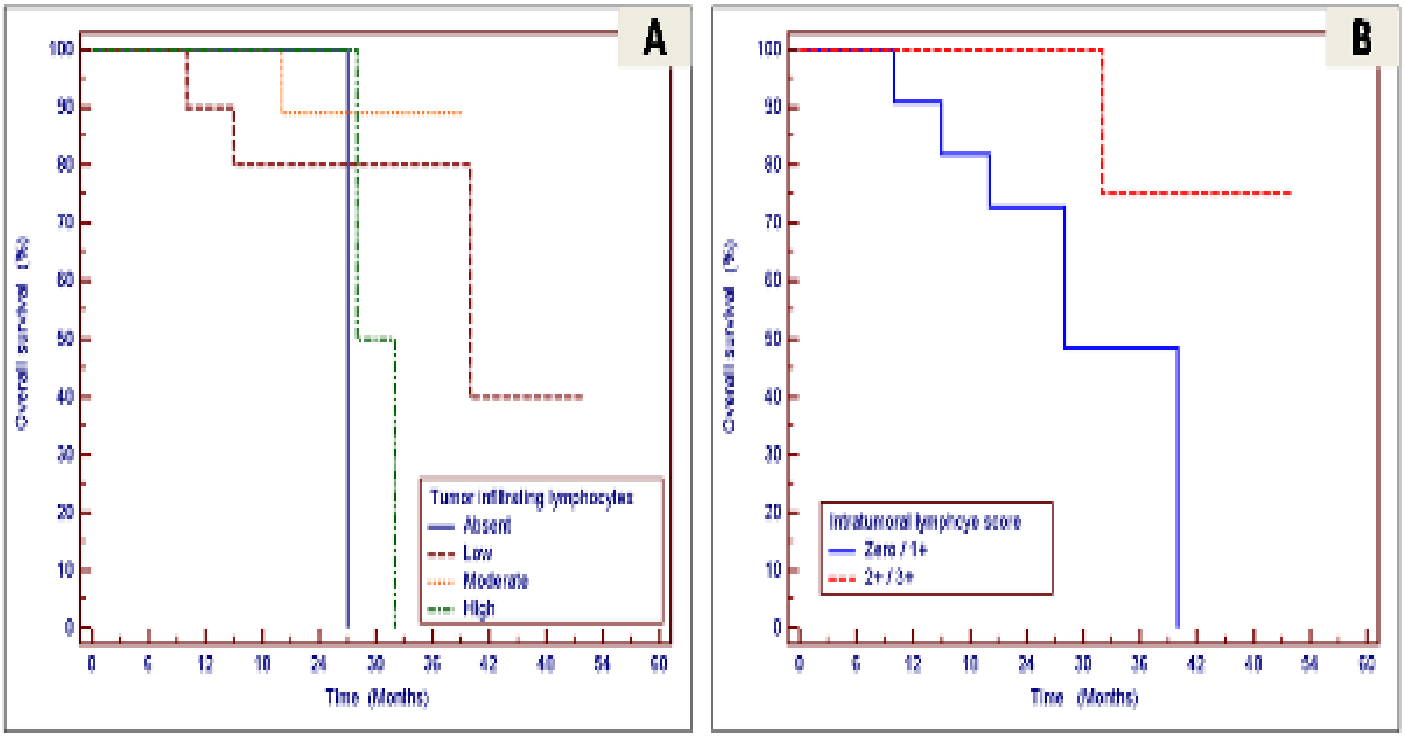


**Figure (4):** Kaplan-Meier Overall survival curve for; A: Peri-tumoral infiltrating lymphocytes on H&E (P=0.018), B: FOXP3 expression in intra-tumoral infiltrating lymphocytes (P=0.014).

Multivariate Cox regression analysis revealed FOXP3 positive intra-tumoral lymphocytes score as independent prognostic factors affecting patients' OS (p= 0.043) (Table 5).

**Table (5):** Multivariate COX regression analysis for Overall survival.

| Variable | P | OR | 95%C.I | |
|---|---|---|---|---|
| | | | Lower | Upper |
| **FOXP3 positive intra-tumoral lymphocytes score** | 0.043* | 9.280* | 1.073 | 80.186 |
| **Peri-tumoral lymphocytes in H&E** | 0.188 | 1.590 | 0.797 | 3.171 |

**BC:** Breast carcinoma OR **:** Odds ratio **CI**: Confidence interval
*: Significant **FOXP3**: Forkhead Box P3

## DISCUSSION

The infiltration of tumors by regulatory T cells (defined as FOXP3+ TILs) has been reported to be associated with patient survival in a variety of cancers, but its prognostic value remains controversial [(19)]. In the current study, we investigated the prognostic effect of FOXP3 +tumor infiltrating lymphocytes adjusted with conventional clinicopathological data in the breast cancer cohort population. In this study, FOXP3 was expressed in tumor cells of all positive cases as cytoplasmic and that was similar to the results of several other studies in different sites [(20)]. **Zuo *et al.*** [(21)] demonstrated that ~80% of normal breast samples expressed FOXP3 in the epithelial cell nuclei, whereas only 20% of cancer tissues expressed nuclear FOXP3. Similarly, H.score of FOXP3 expression in tumor cells and peritumoral infiltrating lymphocytes of the current work was higher in younger age and this is similar to results of one study in thyroid carcinoma [(22)].This study revealed higher H. scores of FOXP3 expression in tumor cells of lower grade tumors consistently with studies done in gastric and endometrial cancers [(23)]. Furthermore, increasing H. scores of FOXP3 in tumor cells was associated with non-triple negative tumors. Non-triple negative tumors have a better prognosis and better varieties of treatments such as hormone receptor-targeted therapies with or without chemotherapy than triple-negative cases [(24)].

Additionally, the loss of FOXP3 expression in mammary and prostatic epithelial tissues leads to tumor formation. Therefore, FOXP3 expression in tumor cells has been hypothesized to represent a favorable prognostic factor in human cancers [(25)].

Multivariate Cox regression analysis; using overall survival time in months and the co-varieties which showed significant association with overall survival; revealed that high FOXP3 In the current work, FOXP3 positivity in tumor cells was significantly associated with an increase in intratumoral and peritumoral positively stained FOXP3+ lymphocytes score (p=<0.001) and this is consistent with **Takenaka *et al.*** [(26)] who reported that a high infiltration of FOXP3+ lymphocytes was accompanied by FOXP3+ tumor expression.

According to univariate survival analysis done in the current study, we found that patients with increased infiltration of their breast cancers by peritumoral

lymphocytes had a significant association with improved overall survival (p=0.018) and patients with high FOXP3 expression scores in intratumoral lymphocytes had also a significant association with improved overall survival (p=0.014). Moreover, expression score in intratumoral infiltrating lymphocytes was an independent prognostic factor affecting patients' overall survival (P=0.043).

Many other studies found that an increase in peritumoral infiltration by lymphocytes is associated with better survival especially in triple-negative (TN) breast cancers which represent more than half of our cases (56%) and can explain our results [(27)].

Tregs can induce immune tolerance and lead to tumor progression by the following mechanisms: secretion of immunosuppressive molecules such as transforming growth factor-beta (TGFβ) and IL-10; direct cytolysis of NK cells and CD8+ cells; metabolic disruption; and promoting angiogenesis [(28)].

Similar to the results of the current study, other reports showed no difference between intratumoral infiltrating lymphocytes or peritumoral infiltrating lymphocytes as a prognostic indicator [(29)].

The present study revealed that there is no association between COX2 expression in tumor cells and different clinicopathologic parameters of the studied cases. Some reports showed that COX2 expression was associated with larger tumor size, high nuclear grading, vascular invasion, poor differentiation, positive lymph nodes, and distant metastases [(30)].

Similar to published data, our study revealed higher H scores of COX2 expression in PR negative cases (p= 0.025) and tend to be associated with ER-negative cases (p= 0.069) [(31)].

This study showed no significant correlation between COX2 and FOXP3 expression in tumor cells. High expression of FOXP3 in peritumoral lymphocytes was noticed in COX2 negative cases with a trend for statistical significance (p=0.063).

Many other studies in lung cancer, renal cancer, and melanoma, revealed that FOXP3 Tregs infiltration was significantly associated with high COX2 expression and that may be due to the important role of COX2 derived prostaglandins (PGE2) in the transformation of Tregs[(32)].

The National Cancer Institute (NCI) Surgery Branch has developed an experimental therapy that involves taking white blood cells from patients' tumors, multiplying them, and then giving the cells back to the patient or TIL is given to over 200 patients with melanoma. Researchers want to know if TIL shrinks tumors in people with the digestive tract, urothelial, breast, or ovarian/endometrial cancers. This study is still recruiting [(33)].

## CONCLUSION

High FOXP3 expression in peritumoral and intratumoral infiltrating lymphocytes is considered a good independent prognostic factor in the studied population as it was noticed to be associated with low-grade tumors and non-triple negative subtype and showed improvement on overall survival. On the other hand, COX2 might be considered to have a poor prognostic role in studied breast cancer cases as it was associated with the PR negative group.

**Conflict of Interest**: The authors declare that they have no Conflict of Interest.

**Funding:** No fund was contributed to the current study.

## REFERENCES

1. **Desantis C, Fedewa S, Goding Sauer A *et al.* (2016):** Breast cancer statistics, 2015: Convergence of incidence rates between black and white women. CA Cancer J Clin., 66: 31-42.
2. **El Bolkainy M, Nouh M, Farahat I *et al.* (2013):** Epidemiology of cancer in Pathology of cancer. Chapter 2, 4th ed. Cairo: Cairo press. Pp.15-31. http://www.elbolkainy.net/wp-content/uploads/2016/03/Appendix_28122015.pdf
3. **Ibrahim A, Khaled H, Mikhail N *et al.* (2014):** Cancer incidence in Egypt: Results of the national population-based cancer registry program. Journal of Cancer Epidemiology, 14: 437971.
4. **Sakaguchi S (2005):** Naturally arising Foxp3-expressing CD25 + CD4+ regulatory T cells in immunological tolerance to self and non-self. Nat Immunol., 6: 345–52.
5. **Pardoll D (2012):** The blockade of immune checkpoints in cancer immunotherapy. Nat Rev Cancer, 12:252–64.
6. **Liu F, Lang R, Zhao J *et al.* (2011):** CD8(+) cytotoxic T cell and FOXP3(+) regulatory T cell infiltration in relation to breast cancer survival and molecular subtypes. Breast Cancer Res Treat., 130:645–55.
7. **Mahmoud S, Paish E, Powe D *et al.* (2011):** An evaluation of the clinical significance of FOXP3+ infiltrating cells in human breast cancer. Breast Cancer Res Treat., 127:99–108.
8. **Yang B, Jia L, Guo Q *et al.* (2016):** Clinicopathological and prognostic significance of cyclooxygenase-2 expression in head and neck cancer: A meta-analysis. Oncotarget., 7: 47265–77.
9. **Mahic M, Yaqub S, Johansson C *et al.* (2006):** FOXP3 CD4 CD25 adaptive regulatory T cells express cyclooxygenase-2 and suppress effector T cells by a prostaglandin E2-dependent mechanism. J Immunol., 177: 246-54.
10. **Yuan X, Chen L, Li M (2010):** Elevated expression of Foxp3 in tumor-infiltrating Treg cells suppresses T-cell proliferation and contributes to gastric cancer progression in a COX-2-dependent manner. Clin Immunol., 134:277-88.
11. **Sicking I, Rommens K, Battista M *et al.* (2014):** Prognostic influence of cyclooxygenase-2 protein and mRNA expression in node-negative breast cancer patients. BMC Cancer, 14: 952-56.
12. **Elston C, Ellis I (1991):** Pathological prognostic factors in breast cancer. The value of histological grade in breast

cancer: experience from a large study with long-term follow-up. Histopathology, 19: 403-10.

13. **Giuliano A, Connolly J, Edge S *et al.* (2017):** Breast cancer-major changes in the American Joint Committee on Cancer eighth edition cancer staging manual. CA Cancer J Clin., 67: 290–303.
14. **Miyoshi Y, Shien T, Ogiya A *et al.* (2019):** Associations in tumor-infiltrating lymphocytes between clinicopathological factors and clinical outcomes in estrogen receptor-positive/ human epidermal growth factor receptor type 2 negative breast cancer. Oncology Lett., 17: 2177-86.
15. **Hammond M, Hayes D, Dowsett M *et al.* (2010):** American Society of Clinical Oncology/College of American Pathologists guideline recommendations for immunohistochemical testing of estrogen and progesterone receptors in breast cancer. Arch Pathol Lab Med., 134: 48-72.
16. **Wolff A, Hammond M, Allison K *et al.* (2018):** Human epidermal growth factor receptor 2 testings in breast cancer: American Society of Clinical Oncology/College of American Pathologists clinical practice guideline focused update. J Clin Oncol., 36: 2105–22.
17. **Merlo A, Casalini P, Carcangiu M *et al.* (2009):** FOXP3 expression and overall survival in breast cancer. J Clin Oncol., 27: 1746-52.
18. **Takenaka M, Seki N, Toh U *et al.* (2013):** FOXP3 expression in tumor cells and tumor-infiltrating lymphocytes is associated with breast cancer prognosis. Mol Clin Oncol., 1: 625-32.
19. **Liu S, Foulkes W, Leung S *et al.* (2014):** Prognostic significance of FOXP3+ tumor-infiltrating lymphocytes in breast cancer depends on estrogen receptor and human epidermal growth factor receptor-2 expression status and concurrent cytotoxic T-cell infiltration. Breast Cancer Res., 16(5):432-37.
20. **Tao H, Mimura Y, Aoe K *et al.* (2012):** Prognostic potential of FOXP3 expression in non-small cell lung cancer cells combined with tumor-infiltrating regulatory T cells. Lung Cancer, 75(1):95-101.
21. **Zuo T, Liu R, Zhang H *et al.* (2007):** FOXP3 is a novel transcriptional repressor for the breast cancer oncogene SKP2. J Clin Invest., 117(12):3765-3773.
22. **Cunha L, Morari E, Nonogaki S *et al.* (2012):** Foxp3 expression is associated with aggressiveness in differentiated thyroid carcinomas. Clinics (Sao Paulo), 67(5):483-488.
23. **Gupta S, Joshi K, Wig J *et al.* (2007):** Intratumoral FOXP3 expression in infiltrating breast carcinoma: Its association with clinicopathologic parameters and angiogenesis. Acta Oncol., 46(6):792-797.
24. **Miyashita M, Sasano H, Tamaki K *et al.* (2015):** Prognostic significance of tumor-infiltrating CD8+ and FOXP3+ lymphocytes in residual tumors and alterations in these parameters after neoadjuvant chemotherapy in triple-negative breast cancer: a retrospective multicenter study. Breast Cancer Res., 17:124.
25. **Iurchenko N, Glushchenko N, Buchynska L (2014):** Comprehensive analysis of intratumoral lymphocytes and FOXP3 expression in tumor cells of endometrial cancer. Exp Oncol., 36(4):262-266.
26. **Adams S, Goldstein L, Sparano J *et al.* (2015):** Tumor infiltrating lymphocytes (TILs) improve prognosis in patients with triple negative breast cancer (TNBC). Oncoimmunology, 4(9):930-35.
27. **Yu X, Zhang Z, Wang Z *et al.* (2016):** Prognostic and predictive value of tumor-infiltrating lymphocytes in breast cancer: a systematic review and meta-analysis. Clin Transl Oncol., 18(5):497-506.
28. **Devaud C, Darcy P, Kershaw M (2014):** Foxp3 expression in T regulatory cells and other cell lineages. Cancer Immunol Immunother., 63(9):869-876.
29. **Dieci M, Mathieu M, Guarneri V *et al.* (2015):** Prognostic and predictive value of tumor-infiltrating lymphocytes in two phase III randomized adjuvant breast cancer trials. Ann Oncol Off J Eur Soc Med Oncol., 26(8):1698-1704.
30. **Kelly L, Hill A, Kennedy S *et al.* (2003):** Lack of prognostic effect of Cox-2 expression in primary breast cancer on short-term follow-up. Eur J Surg Oncol., 29(9):707-710.
31. **Jana D, Sarkar D, Ganguly S *et al.* (2014):** Role of Cyclooxygenase 2 (COX-2) in Prognosis of Breast Cancer. Indian J Surg Oncol., 5(1):59-65.
32. **Mougiakakos D, Johansson C, Trocme E *et al.* (2010):** Intratumoral forkhead box P3-positive regulatory T cells predict poor survival in cyclooxygenase-2-positive uveal melanoma. Cancer, 116(9):2224-2233.
33. **Rosenberg S (2010):** A Phase II study using short-term cultured, autologous tumor-infiltrating lymphocytes following a lymphodepleting regimen in metastatic cancers plus the administration of pembrolizumab. ClinicalTrials.gov. https://clinicaltrials.gov/ct2/ show/NCT01174121