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# Frequency of CD4+ regulatory T cells, CD8+ T cells, and human papilloma virus infection in Egyptian Women with breast cancer

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## Abstract

Several subsets of regulatory CD4+ T cells (CD4+ Tregs) have been described in peripheral blood and tumor microenvironment of breast cancer (BC) patients and may play a role in the progression of BC. High-risk human papilloma virus (HR-HPV) has a causal role in cervical, head, and neck tumors but the role of HR-HPV in evoking neoplasia in BC is still unclear. In this study we assessed the prevalence of CD4+CD25+ FOXP3+ regulatory T cells (CD4+Tregs) and CD3+ CD8+ T cells by flow cytometry in peripheral blood from a total of 55 Egyptian women, including 20 treatment-naïve BC, 15 with breast benign lesions (BBL), and 20 healthy volunteers (HV). HR-HPV genotypes type 16, 18, and 31 were investigated in breast tissue from all BC and BBL patients using Real-Time PCR. HR-HPV was detected in 4/20 (20%) and 0/15 (0%) BC and BBL patients respectively. The frequency of CD4+ Tregs was significantly higher in BC compared to BBL and HV, ($P < 0.001$). In addition, we observed a significantly higher frequency of CD3+ CD8+ T cells in peripheral blood of patients with late stage III BC compared to early stage I and II BC ($P = 0.011$). However, there was no significant association between the ratio of CD8+ T cell to CD4+ Tregs frequencies and the expression of Estrogen Receptor (ER), Progesterone Receptor (PR), and Human Epidermal Growth Factor Receptor 2 (HER2). These results lead us to postulate that the association between the frequency of CD4+ Tregs and CD8+ T cells in the peripheral blood may be a prognostic or predictive parameter in Egyptian women with BC. In addition, HR-HPV infection may be implicated in the development of some types of BC in Egyptian women.

## Keywords

breast cancer, HPV, Treg

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## Introduction

Breast cancer (BC) is the second-leading cause of death from cancer in Egyptian women. The overall incidence of cancer is 157.0 per 100,000 Egyptian females with the highest incidence being BC (32%) and a three-fold increase in incidence of cancer is predicted by 2050.[1] While the etiology of BC is still unknown, numerous risk factors have been identified. Increased public awareness and regular screening can play a vital role in the prevention, early detection, and treatment of BC.[2] Virus infections including human parvovirus (B19), Epstein Barr Virus (EBV), and high-risk human papilloma virus (HR-HPV) have a causal role in some thyroid and cervical tumors.[3–6] In addition, HR-HPV infections are associated with many oropharyngeal cancers.[7] However, the role of HR-HPV in evoking neoplasia in BC is still unclear.[8,9]

Human papilloma virus is a small, circular, double-stranded DNA virus that includes more than 120 known subtypes.[10] HR-HPV genotypes 16, 18, and 31 encode a series of early (E1 − E7) and late (L1 and L2) proteins. HR-HPV E6 and E7 early proteins are oncoproteins that stimulate cell cycle progression by inhibiting tumor suppressor genes p53 and p110 RB resulting in cellular transformation and cancer.[11,12] In addition, HR-HPV E5 and E6 early proteins are known to disrupt cytokeratin causing perinuclear cytoplasmic clearing and nuclear enlargement.[13]

The expression of hormone receptors such as estrogen receptor (ER) and progesterone receptor (PR) in BC predicts the response to growth factors, patient prognosis, and response to targeted therapy.[14] Patients positive for these receptors tend to have improved prognosis and better response to hormonal therapy.[15] In contrast, BC patients expressing Human Epidermal Growth Factor Receptor 2 (HER2) have increased disease recurrence and poor prognosis.[16] Triple negative breast cancer is a unique subtype constituting about 20% of breast cancer cases is characterized by the lack of ER, PR, and HER2 expression and poor clinical outcome.[17,18] Metaplastic carcinoma is an aggressive type of BC characterized by low hormone receptor expression and poor prognosis.[19]

Several subsets of CD4+CD25+ FOXP3+ regulatory T cells (CD4+Tregs) have been described in peripheral blood and tumor microenvironment from BC patients with invasive high-grade breast carcinomas, and may play a role in the progression of breast cancer.[20–24] CD4+ Tregs express high levels of FOX P3, CD25, Glucocorticoid-induced TNFR family related protein (GITR), Cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), and CD103.[25–27] In addition, decreased expression of CD127 on human CD4+ Tregs is inversely correlated with FoxP3 expression.[28,29] "Naturally" occurring CD4+ Tregs are derived in the thymus, while "Adaptive" CD4+ Tregs are believed to come from mature T cells in the periphery under specific conditions of persistent antigenic stimulation.[30] CD4+Tregs exert their non-antigen specific inhibitory effects on T cell proliferation and cytokine production through a cell-cell contact-dependent mechanism.[31,32] These cells have also been shown to inhibit the cytotoxic function and decrease the expression of major histocompatibility class I molecules on target cells. CD4+ Tregs were shown to secrete immunosuppressive cytokines interleukin-10 and Transforming Growth Factor-β (TGF-β) that suppress the induction of antitumor immunity.[33,34]

The relationship between the severity of BC and the frequency of CD4+ Tregs in the peripheral blood is not well defined. Quantification of CD4+ Tregs may be valuable for assessing BC progression and as an important therapeutic target. It has been reported that the number of CD4+ Tregs was significantly higher in patients with invasive high-grade BC and may correlate with both shorter relapse-free and overall survival time.[22,35,36] These findings suggest that increased frequency of CD4+ Tregs in BC patients may play a key role in the prognosis of BC.[37,38] However, the frequency of CD4+ Tregs did not directly correlate with the clinical stage of BC in other studies.[39] The aim of this study was to assess the frequency of CD4+ regulatory T cells, CD8+ T cells, and human papilloma virus infection in Egyptian Women with breast cancer compared to women with benign breast lesions (BBL) and healthy volunteers (HV).

## Material and methods

### *Population and source of samples*

All women over the age of 18 visiting the oncology clinic at Al-Azhar university hospital between February 2018 and March 2019 were invited to volunteer in the study. Inclusion criteria included all new treatment naïve cases of histologically confirmed BC at any stage. Women

that were diagnosed with benign breast lesion, as well as women residing in the same areas and admitted to the hospital, for a wide spectrum of acute, non-neoplastic conditions unrelated to known or likely risk factors for BC were invited to volunteer as controls in the study. Exclusion criteria included Patients with systemic therapy prior to surgery, bilateral BC, metastatic or recurrent disease, and cancer of other origin. Peripheral blood and frozen breast tissue samples were collected according to the ethical regulations at Al-Azhar university hospital. Breast tissue samples were not collected from healthy volunteers. Demographic and clinical information was obtained from medical records. The study received approval from the Institutional Review Board at Al-Azhar University and each volunteer gave informed written consent for participation in the study. Specimens were collected from 20 treatment-naïve women with primary BC, 15 benign breast lesions (BBL), and 20 healthy volunteers (HV) after providing informed written consent. To avoid a potential influence of major surgical procedures, as well as of neoadjuvant treatment, blood was drawn after core biopsy but before definitive surgery.

Clinicopathological risk factors were assessed for age, lymph node metastasis, tumor size, histologic grade, tumor stage, and hormonal receptor status. BC is regarded to be hormonal receptor positive if at least 1% stained positive for ER or PR assays as previously described.[40] Expression of ER, PR, and HER2 receptors was assessed by immunohistochemistry staining performed in Al-Azhar University hospital clinical laboratory as previously described.[41] Test results were obtained from the patient medical records. Tumor stages were classified according to the American Joint Committee on Cancer (AJCC)-TNM (tumor, node, metastases) classification.

### *Detection and genotyping of HR-HPV by RT-PCR*

DNA was extracted using the genomic DNA extraction kit for tissues (Invitrogen), following the manufacturer's instructions. The quality of the isolated DNA was checked by polymerase chain reaction (PCR) of control genes with primers generating fragments of 200, 400, and 600 bp as previously described.[42] Specimens were frozen at −70°C for molecular analysis. Three HR-HPV types 16, 18, and 31 were assessed by RT-PCR using the genesis® Advanced Kit specific for each type (Primedesign Ltd, UK) following manufacture protocol. Briefly, genomic DNA is amplified using primers specific to the early E6 gene for types 16, 18, and 31. The β-Actin gene was used as an internal control. The PCR products were analyzed by the "One step Plus™ Real Time PCR System (Applied Biosystem). Several measures were undertaken to avoid DNA contamination, including the use of distinct areas for sample preparation, PCR setup, post-PCR analysis, and equipment. In addition, we used designated sets of pipettes and pipette tips with aerosol filters, lab coats, glove boxes, and waste baskets for the pre-PCR and post-PCR areas.

### *Immunophenotyping of CD4+ Tregs and CD8+ T cells*

Blood samples from patients with primary BC, BBL, and HV were procured from treatment-naïve patients prior to any surgery to avoid a potential influence of major surgical procedures and neoadjuvant treatment. Flow Cytometry testing was performed in a single laboratory using fresh blood samples on the same day of collection as previously described.[43,44]. Two ml peripheral blood sample was collected in EDTA tubes. About 100 µl of whole blood was incubated for 15 min at room temperature with combinations of monoclonal antibodies against CD3 PerCP, CD4 FITC, CD8 PE, CD25 APC, and intracellular transcription factor FOXP3 PE, using immobilization buffer (BD, Biosciences, San Jose, USA). Red cell lysis was performed with FACS Lysing Solution (BD Biosciences). Isotype controls, IgG1 FITC, IgG2 PE, IgG1 PerCP, and IgG1 APC were used for detection of non-specific binding background (BD Biosciences). Compensation settings were established before sample acquisition using color calibrate beads. A minimum of 30,000 CD3+ T cells per sample was acquired using a 4 color FACSCalibur (Beckton Dickenson (BD), USA) and analysis was performed by Cell-Quest Pro software (BD, USA). Gating was performed using the fluorescence-minus-one (FMO) control for each marker. Results were expressed as percent of CD25+ FOXP3+ CD4+ Tregs from the CD4+ lymphocyte gate or CD3+ CD8+ T cells from the lymphocyte gate after subtraction of the isotype control background values ($<0.1\%$).

**Table 1.** Patient demographics and disease characteristics.

| | BC (n = 20) | BBL (n = 15) | HV (n = 20) | *P* |
|---|---|---|---|---|
| Age in years (mean ± SD) | 50.6 ± 11.7 | 55.0 ± 13.7 | 64.3 ± 9.9 | 0.18 |
| HPV | | | | |
| Positive | 4 | 0 | 0 | ND |
| Negative | 16 | 15 | 20 | |
| Percent lymphocyte subsets | | | | |
| CD4+ Tregs (mean ± SD) | 4.39 ± 1.31 | 1.73 ±.74 | 1.73 ± .63 | 0.001 |
| CD8+ (mean ± SD) | 22.51 ± 5.51 | 23.93 ± 6.30 | 23.95 ± 8.08 | 0.8 |
| CD4+ (mean ± SD) | 43.16 ± 8.05 | 43.16 ± 7.39 | 40.17 ± 7.68 | 0.35 |
| Histology type, n (%) | | | | |
| Carcinoma in situ | 1 (5.3%) | | | |
| Invasive duct carcinoma | 14 (73.7%) | | | |
| Invasive mammary carcinoma | 1 (5.3%) | | | |
| Metaplastic carcinoma | 3 (15.8%) | | | |
| Benign mammary lesion | | 4 (33.3%) | | |
| Fibroadenoma | | 3 (25.0%) | | |
| Fibrocystic changes | | 1 (8.3%) | | |
| Benign ductal epithelial cells | | 1 (8.3%) | | |
| Benign mastitis | | 1 (8.3%) | | |
| Benign unspecified | | 2 (16.7%) | | |
| Unknown | 1 | 3 | | |
| Tumor size | | | | |
| <2 cm | 2 (10.5%) | 1 (11.1%) | | |
| 2–5 cm | 12 (63.2%) | 3 (33.3%) | | 0.31 |
| >5 cm | 5 (26.3%) | 5 (55.6%) | | |
| Unknown | 1 | 3 | | |

ND: not done because the group is <5; BBL: breast benign lesions; HV: healthy volunteers; SD: standard deviation.
$P < 0.05$ is significant.

### *Statistical analysis*

Statistical analysis was done using IBM SPSS® Statistics version 23 (IBM® Corp., Armonk, NY, USA). Numerical data were expressed as mean and standard deviation or median and range as appropriate. Qualitative data were expressed as frequency and percentage. For not normally distributed quantitative data, comparison between two groups was done using Mann-Whitney test (non-parametric *t*-test). All tests were two-tailed. When more than two groups of data sets were compared, one-way analysis of variance (ANOVA) was performed as described. A *P*-value <0.05 was considered significant.

## Results

### *Patient demographics and disease characteristics*

The mean age of patients with BC, BBL, and HV was 50.6 ±11.7, 55.0 ±13.7, 64.3 ±9.9 respectively (Table 1). There was no significant difference in age between patients with BC compared to BBL ($P=0.188$). HR-HPV types 16, 18, and 31 were assessed because they are very common and may play a causal role in evoking neoplasia.[3,4,6,45] HR-HPV was detected in breast tissue from 4/20 BC patients compared to 0/15 of BBL patients. The prevalence of HR-HPV genotypes was 3/4 (75%), 1/4 (25%), and 0/4 (0%) for genotypes 16, 18, and 31, respectively. None of the patients were infected with more than 1 HR-HPV genotype.

Metaplastic carcinoma and invasive duct carcinoma were found in 3/4 (75%) and 1/4 HR-HPV infected patients respectively compared to 0/15 and 13/15 HPV negative BBL patients respectively. These results suggest that HR-HPV infection may be associated with more aggressive BC in Egyptian women. Ten of 20 patients with BC (50%) were diagnosed with early stage disease (stage I and II) while 6/20 (30%) were diagnosed with advanced disease (stage III).

The number of BC patients expressing ER, PR, or HER2 was 13/20 (65%), 10/20 (50%), and 4/20 (20%) respectively (Table 2). While 3/20 (15%) BC

**Table 2.** Frequency of CD4+ Tregs and CD8+ T cells in BC patients according to age, HPV, and tumor characteristics.

| | *Item* | *n (%)* | *CD4+ T regs (mean ± SD)* | *P* | *CD8+ T cells (mean ± SD)* | *P* |
|---|---|---|---|---|---|---|
| 1. | Age in years | | | 0.12 | | 0.77 |
| | <50 | 7 (35%) | 3.77 ± 1.08 | | 21.95 ± 6.42 | |
| | >50 | 8 (40%) | 4.76 ± 1.62 | | 20.40 ± 4.13 | |
| | Unknown | 5 (25%) | | | | |
| 2. | HPV | | | ND | | ND |
| | Positive | 4 (20%) | 4.23 ± 1.10 | | 22.57 ± 8.88 | |
| | Negative | 16 (80%) | 4.43 ± 1.39 | | 22.49 ± 4.77 | |
| 3. | Grade | | | ND | | ND |
| | G1 & G2 | 16 (80%) | 4.17 ± 1.35 | | 22.08 ± 6.05 | |
| | G3 | 3 (15%) | 5.04 ± .71 | | 23.74 ± 2.33 | |
| | Unknown | 1 (5%) | | | | |
| 4. | Pathology | | | 0.68 | | 0.5 |
| | IDC | 14 (70%) | 4.37 ± 1.40 | | 22.60 ± 4.91 | |
| | Others | 5 (25%) | 4.14±1.10 | | 21.63 ± 7.91 | |
| | Unknown | 1 (5%) | | | | |
| 5. | LN metastasis | | | 0.19 | | 0.1 |
| | Positive | 8 (40%) | 4.12 ± 1.57 | | 19.74 ± 3.98 | |
| | Negative | 8 (40%) | 4.77 ± .76 | | 24.87 ± 7.06 | |
| | Unknown | 4 (20%) | | | | |
| 6. | Stage | | | 0.79 | | 0.01 |
| | Early (I & II) | 10 (50%) | 4.52 ± 1.41 | | 19.22 ± 4.33 | |
| | Advanced (III) | 6 (30%) | 4.32 ± .97 | | 27.44 ± 5.35 | |
| | Unknown | 4 (20%) | | | | |
| 7. | ER | | | ND | | ND |
| | Positive | 13 (65%) | 4.46 ± 1.40 | | 22.85 ± 6.17 | |
| | Negative | 4 (20%) | 3.68 ± 1.24 | | 20.57 ± 5.54 | |
| | Unknown | 3 (15%) | | | | |
| 8. | PR | | | 0.16 | | 0.19 |
| | Positive | 10 (50%) | 4.74 ± 1.28 | | 24.42 ± 5.76 | |
| | Negative | 7 (35%) | 3.61 ± 1.29 | | 19.30 ± 5.14 | |
| | Unknown | 3 (15%) | | | | |
| 9. | HER2 | | | ND | | ND |
| | Positive | 4 (20%) | 4.04 ± 1.24 | | 22.85 ± 4.94 | |
| | Negative | 13 (65%) | 5.06 ± 1.69 | | 20.56 ± 9.23 | |
| | Unknown | 3 (15%) | | | | |
| 10. | Triple negative | | | ND | | ND |
| | Yes | 3 (15%) | 3.17 ± .87 | | 23.09 ± 2.78 | |
| | No | 14 (70%) | 4.51 ± 1.36 | | 22.14 ± 6.49 | |
| | Unknown | 3 (15%) | | | | |

ND: not done because the group is <5; BBL: breast benign lesions; HV: healthy volunteers; SD: standard deviation.
$P < 0.05$ is significant.

patients lacked expression of all three hormonal receptors (triple negative). All four HR-HPV positive BC patients expressed ER and PR receptors while only 1/4 HR-HPV positive BC patient expressed all three hormonal receptors (Table 3).

### *Prevalence of CD4+ Tregs and CD8+ T cells*

We characterized CD4+ Tregs by the expression of CD4, CD25, and FOXP3.[25] Representative plots of the gating and frequency of frequency of CD4+ Tregs are shown in Figure 1. We observed a significantly higher frequencey of CD4+ Tregs in patients with BC compared to BBL and HV ($P < 0.001$; Table 1). In contrast, there was no significant difference in the total percentage of CD8+ and CD4+ T cells between the three groups (Table 1).

We assessed the histologic grade and disease stage as variables to gain further insight into the association between the frequency of CD4+

**Table 3.** Demographics and characteristics of HR-HPV positive BC patients.

| Patients | HR-HPV genotype | Age (years) | %CD4+ Tregs | %CD8+ T cells | Size[a] | Grade | Stage | Pathology | ER | PR | HER2 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 16 | 45 | 5.53 | 22 | 2–5 | II | II | IDC | + | + | – |
| 2 | 16 | 37 | 3.15 | 22 | >5 | II | III | MC | + | + | – |
| 3 | 16 | 43 | 4.74 | 12.3 | 2–5 | II | II | MC | + | + | – |
| 4 | 18 | 50 | 3.5 | 33.9 | <2 | II | I | MC | + | + | + |

IDC: invasive duct carcinoma; MC: metaloplastic carcinoma; ER: estrogen receptor; PR: progesterone receptor; HER2: human epidermal growth factor receptor 2; (+): positive; (−): negative.
[a]Size in centimeters.

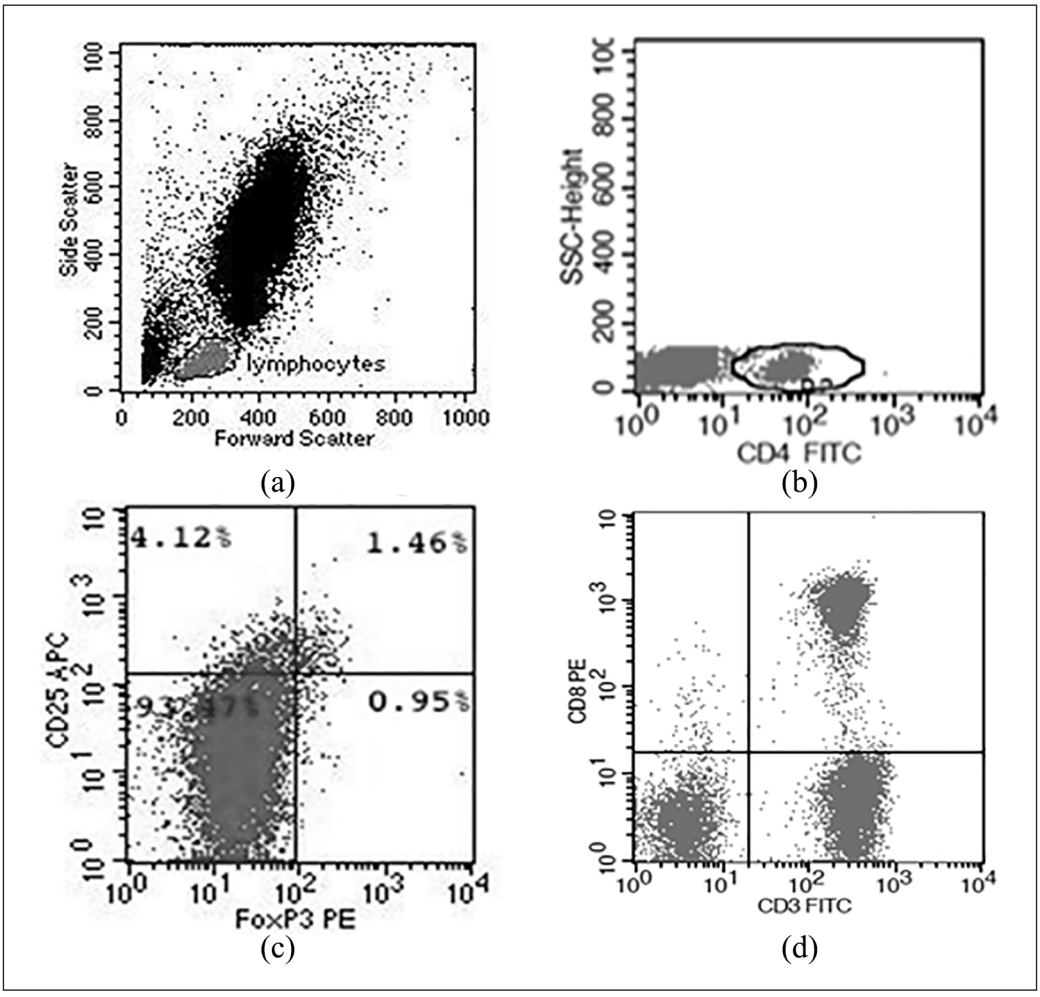


**Figure 1.** Flow cytometric analysis. (a) Using typical forward and side scatter characteristics, a gate was first set on lymphocytes. (b) From the lymphocyte gate, percent CD4+ T cells were determined. (c) From the CD4+ gate, the percent CD25+ FoxP3+ CD4+ Tregs are shown. (d) From the lymphocyte gate, percent CD3+ CD8+ T cells are shown.

Tregs and tumor biology. In addition, we analyzed the frequency of CD4+ Tregs among clinically defined surrogates of BC molecular subtypes for ER, PR, and Her2 expression in order to explore whether any particular clinical disease correlates were associated with increased number of CD4+ Tregs. While invasive duct carcinoma (IDC) was the most prevalent BC subtype, there was no significant difference in frequency of CD4+ Tregs between patients with IDC and other pathological subtypes [$P=0.687$; Figure 2(a)]. In addition, we observed no significant difference in frequency of CD4+ Tregs between BC patients with and without HR-HPV infection [$P=0.750$; Figure 2(b)], lymph node metastases [$P=0.195$; Figure 2(c)], poorly differentiated grade III tumor

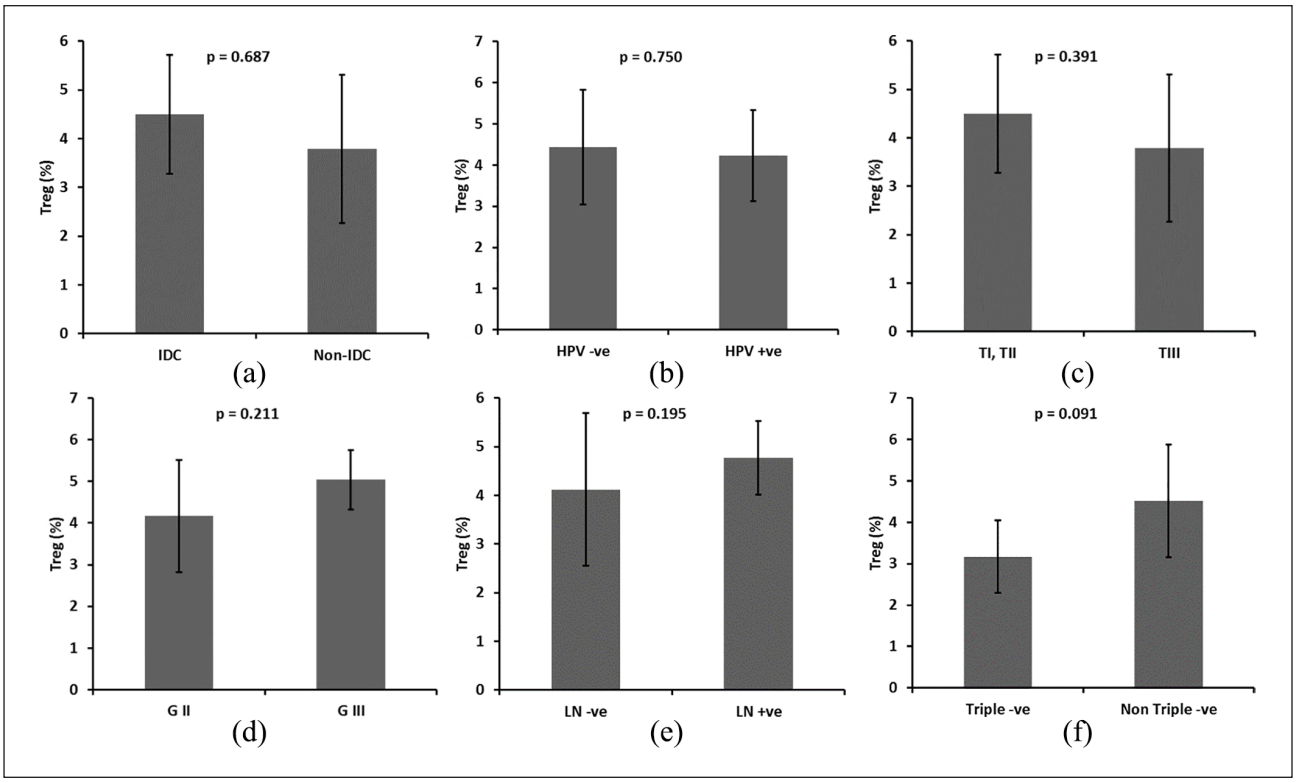


**Figure 2.** Relation between the frequency of CD25+ FOXP3+ CD4+ Tregs in (a) HPV positive (+ve) versus HPV negative (−ve). (b) IDC vs. non-IDC. (c) Small tumor size (TI, TII) versus large tumors (TIII). (d) Well differentiated (G2) versus poorly differentiated (G3). (e) Node negative patients versus node positive patients. (f) Triple −ve versus non-triple −ve patients. Groups were compared using *t* tests (a, b, c, e, f) and ANOVA (d). Bars represent standard deviation.

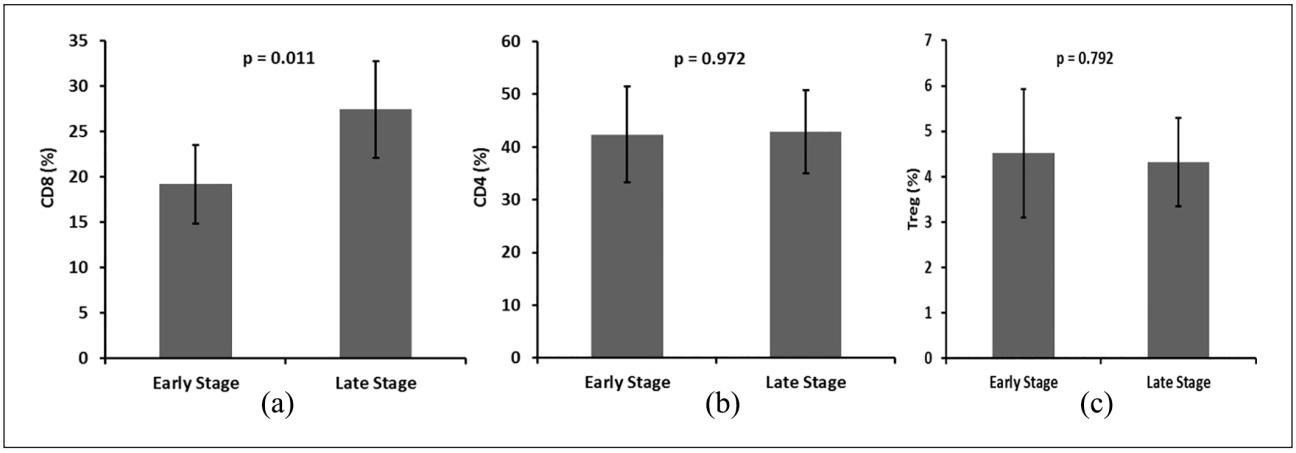


**Figure 3.** Frequency of (a) CD3+ CD8+ T cells. (b) CD4+ T cells. (c) CD25+ FOXP3+ CD4+ Tregs in relation to early (I, II) versus late (III) stage of BC. Mann-Whitney U test was performed to access statistical significance. Bars represent standard deviation. A significant difference in the frequency of CD3+ CD8+ T cells was found between early and late tumor stages ($P=0.011$).

[$P=0.211$; Figure 2(d)], late stage III tumor [$P=0.391$; Figure 2(e)], and triple negative tumor [$P=0.091$; Figure 2(f)].

Tumor-infiltrating CD8+ T lymphocytes in BC patients were reported to have antitumor activity resulting in a favorable effect on patients survival.[46] In this study we observed a significantly higher frequencey of CD8+ T cells in peripheral blood of patients with late stage III compared to early stage I and II BC [$P=0.011$; Figure 3(a)]. In contrast, there was no significant difference in the frequency of CD4+ T cells (Figure 3(b); $P=0.792$) or CD4+

Tregs (Figure 3(c); $P=0.972$) between early compared to late stage BC patients. In addition, there was no significant association between the ratio of CD8+ T cell to CD4+ Tregs frequencies and the expression of ER, PR, or HER2 (data not shown).

## Discussion

While most HPV infections are cleared by the host immune response, a persistent HPV infection is established in some individuals by evading immune-surveillance and generating an anti-inflammatory microenvironment resulting in suppression of Natural Killer cell activity and antigen presentation.[47–49] In addition, HPV infection promotes T-helper cell type 2 and CD4+ Tregs phenotypes and reduces T-helper cell type 1 phenotype, leading to suppression of cellular and humoral immunity and contributes to HPV persistence. In this study, we demonstrated the presence of HR-HPV in the tissues of 20% of BC specimens which is consistent with previously published worldwide data.[8,50–52] The variability in HR-HPV detection rate between previously published studies may be a result of differences in study population, sample source, detection method, and viral load. Our results lead us to hypothesize that HR-HPV may be implicated in the development of some types of BC in Egyptian women and the potential prevention of some breast cancers by HPV vaccination.[8]

It has long been hypothesized that hormone dependent oncogenic viruses may have causal roles in some cancers.[53] Expression of HR-HPV DNA in BC[9] and HR-HPV proteins in cervical cancer[54] has been reported. Meta-analysis of case-control studies suggests that HPV infection is a potential risk factor in BC.[55,56] The degree of risk may be influenced by patient nationality, HPV subtype, and type of tissue infected. Recent studies have demonstrated that exosomes containing microRNAs play a pathogenic role in HR-HPV mediated inflammation and development of cervical cancer.[5,6] In addition, Human Parvovirus infection (B19) and Epstein Barr Virus (EBV) may play an important role in thyroid cancer development and progression via increased inflammation in thyroid tissue.[3,4] The presence of the HR-HPV was shown to be associated with increased inflammatory cytokines and BC progression.[9]

CD4+ Tregs have an immunosuppressive effect that may promote tumor growth and progression of BC. In addition, heterogeneous expression of hormone receptors and growth factors in BC affects patient-specific adaptive immune responses.[57] In this study we assessed the prevalence of CD4+ Tregs in the peripheral blood of Egyptian females with BC. We observed a significantly higher frequency of CD4+ Tregs in patients with BC compared to BBL and HV. These results lead us to postulate that the composition of T cell subsets in peripheral blood of Egyptian patients with BC may favor an immunoregulatory phenotype that is distinct from BBL and HV. Our finding is consistent with previously reported analysis of freshly isolated lymphocytes from normal and malignant breast tissue samples.[58]

A number of studies have demonstrated that increased CD4+ Tregs frequency was associated with poor prognostic characteristics such as higher histological grade, lymph node metastasis.[20,25,59,60] In addition, the mRNA expression of FOXP3, CTLA-4, and GITR in CD4+ Tregs from peripheral blood in BC patients was significantly increased in comparison with healthy individuals.[25] In contrast, a higher level of CD4+ Tregs and a lower ratio of CD4+ Tregs and CD8+ T cells was shown to be associated with better overall survival independent of HPV status and age in patients with oral and oropharyngeal squamous cell carcinomas.[61] In this study, we observed no significant increase in the frequency of CD4+ Tregs in BC patients with poor prognostic characteristics such as higher histological grade, lymph node metastasis, and presence of high-risk HPV which may be due to the relatively small number of study participants. The lack of sample size power calculation is the limitation of this study and may not have permitted discernable differences in the frequency of CD4+ Tregs in BC patients with poor prognostic characteristics. We postulate that circulating CD4+ Tregs may be used as a prognostic or predictive parameter in Egyptian women with BC and should be assessed in a larger longitudinal study with sufficient follow-up time.

A positive correlation was observed in multiple studies between the number of tumor infiltrating cytotoxic CD8+ T cells and clinical outcome in BC that may improve patient survival.[24,37,46,60,62] Decreased number of tumor infiltrating CD8+ T cells in BC was significantly associated with lymph node metastasis and a higher disease stage. These results suggest that cytotoxic CD8+ T cells are heavily involved in antitumor immune responses.

However, CD8+ T cell infiltrates were also shown to be associated with higher histological grade and basal phenotype, and inversely associated with ER and PR expression. Estrogen has been shown to inhibit CD8+ T cell infiltration of tumors in ER+ BC and correlating with decreased overall survival.[63] In addition, CD8+ T cells may play a role in the dissemination of circulating breast cancer cells.[64]

In our study, we performed multivariate least square regression analysis after controlling for age to determine the association between clinical disease stage and the frequency of CD4+ Tregs and CD8+ T cells. We observed a significantly higher frequency of CD8+ T cells, but not CD4+ Tregs, in the peripheral blood from late compared to early stage BC. These results suggest that the total CD8+ T cell counts are not constant during the course of BC and may serve as an important biomarker of disease. In addition, the ratio of CD4+ Tregs and CD8+ T cells was different between early and late stage of BC suggesting that different mechanisms control CD4+ Tregs and CD8+ T cells homeostasis. Our results are consistent with previously reported analysis of freshly isolated lymphocytes from a small set of normal and malignant breast tissue samples.[65]

The association between levels of lymphocyte subsets in peripheral blood and different BC phenotypes has been reported and may be used as a marker for aggressive phenotypes.[66] In addition, assessing the changes in peripheral blood cellular subsets may be a useful tool to evaluate the response of BC patients to therapy.[67] The role of CD8+ T cell frequency in the peripheral blood and the ratio of CD4+ Tregs and CD8+ T cells as a prognostic marker in Egyptian women with BC are not well defined and should be assessed in a large prospective study.

In vitro data often do not adequately address the complexity of in vivo immune processes. However, our study provides important information on the role of various risk factors including HR-HPV in the pathogenesis of BC in Egypt. The cross-sectional design does not permit definitive analysis of the predictive value of CD4+ Tregs and CD8+ T cell frequencies in BC. Nevertheless, we believe that our study provided an important evaluation of CD4+ Tregs and CD8+ T cell frequencies in Egyptian women with BC. Our results support the design of subsequent longitudinal studies to directly examine the association between the frequency of CD4+ Tregs and CD8+ T cells in the peripheral blood and the response to BC treatment.

## Conclusion

Our results lead us to postulate that the association between the frequency of CD4+ Tregs and CD8+ T cells in the peripheral blood may be a prognostic or predictive parameter in Egyptian women with BC. In addition, HR-HPV infection may be implicated in the development of some types of BC in Egyptian women.

### Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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### Ethical approval

Ethical approval for this study was obtained from the Institutional Review Board at Al-Azhar University.

### Informed consent

Written informed consent was obtained from all the subjects before the study.

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