

# EXPRESSION OF CD4, CD8 BIOMARKERS IN INVASIVE CARCINOMA OF BREAST WITH CLINICOPATHOLOGICAL CORRELATION

## ABSTRACT

**Introduction:** Tumor infiltrating lymphocytes (TILs) are widely considered a key sign of the immune interaction between host and tumor, and potentially prognostic biomarkers of good or bad outcome in various cancers, including invasive breast cancer (IBC). **Aim and Objectives:** To correlate the expression of CD4, CD8 T-lymphocytes in invasive carcinoma breast with established markers of prognosis like tumour size, grade, lymph node status and molecular subtypes mainly ER, PR, Her 2Neu, Ki67 status, mainly the triple negative breast cancers(TNBC). **Methodology:** 58 Invasive breast carcinoma proven tissue blocks were subjected to immunohistochemistry and morphometric analysis for positive CD4, CD8 T-lymphocytes were done. **Results:** Triple negative breast cancer subtype shows high TILs than other pathologic subtypes. Tumor interface CD8+ cells very well correlated with the pathological higher nodal stage. Majority CD4, CD8 positive cells were populated more towards the stromal and interface of the tumor microenvironment rather than intratumoral. **Conclusion:** CD4+ and CD8+ counts may be a valuable independent prognostic tool in predicting the outcome in invasive breast cancer.

**Keywords:** Breast cancer, Invasive breast Cancer, CD4 and CD8 Markers, Clinico Pathological Correlation, Tumour infiltrating lymphocytes.<sup>1</sup>

## INTRODUCTION:

Breast cancer (BC) is a neoplasm characterized by molecular and cellular heterogeneity. It is the leading cause of mortality among women globally<sup>1</sup>. Recent advances in diagnosis and treatment of the breast cancer showed the importance of the role of the new markers in prognosis and target therapies will help in patient survival<sup>2</sup>. Many studies have showed the importance of the microenvironment focusing on the function and interaction between immune cells and cancer cells in its role in cancer progression including Breast cancer<sup>3</sup>. It is generally known that regional lymph nodes are a significant immunological defense for tumor expansion<sup>4</sup>.

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Tumor infiltrating lymphocytes (TILs) have higher specific immunological reactivity against tumor cells. They play dual role i.e., either suppress or promote growth of the tumor<sup>5</sup>. Tumor infiltrating lymphocytes are part of adaptive immunity that migrate into the tumor and stroma, are considered a manifestation of host antitumor response. Moreover, its role in tumor progression or regression is been studied in various solid tumors<sup>6,7</sup>. Tumor cells can express antigens and become targets for a T cell-mediated adaptive immune response. The ability of CD4+, CD8+ lymphocytes to recognize tumor antigens has been well documented, and tumor regression is observed when tumor-reactive T lymphocytes invade cancers more so by CD8 CTLs<sup>8-10</sup>. The anti- and pro-tumor function of TILs in breast cancer is dependent on the TIL subtype. Definitely cytotoxic T cells (CD8+ T cells) have been described to be associated with better clinical outcome in different phenotypes of Breast Cancer<sup>11-14</sup>. The clinical significance of TILs in Breast Cancer is still debatable and the conclusions remain controversial. Generally, assessing the immunogenicity of the neoplasm, and its distribution may determine if an immune response will be prognostically favorable mainly for TNBC and HER2neu overexpressed tumors<sup>15, 16</sup>. With successive hormonal and chemotherapeutics, the next modality aims to be immunotherapy in breast cancer. TNBCs are said to have more mutations compared to other subtypes, which could be paramount targets for immunotherapy.

## MATERIALS AND METHODS

58 formalin-fixed and paraffin embedded tissue blocks along with histopathological slides were retrieved from the archives of the Department of pathology, Chettinad Academy of Research and Education. The study protocol was approved by the Ethics committee of this tertiary care hospital. Core biopsies, lumpectomy specimens, neoadjuvant chemotherapy patients were completely exempted from the study. Estrogen receptor, progesterone receptor, Her2 and Ki67 immunopositive staining for each case was done and evaluated as per American Society of Clinical Oncology/College of American Pathologists Guidelines (ASCO/CAP guidelines). Parameters like pTNM staging, lymph node status, morphological and molecular subtypes, Modified Scarff Bloom Richardson grading. All the recorded data was entered on a detailed proforma designed for this study. Immunohistochemistry:- Formalin-fixed, paraffin embedded tissue sections of 3.5 microns thick. Sections were deparaffinised in xylene followed by hydration in graded ethanol. Antigen retrieval was performed by heating process at 100deg Celsius for 20 mins in Tris-EDTA buffer (pH 7.4). Endogenous peroxidase block in the tissue sections was done for 10 minutes. Sections were covered with appropriate primary antibody for 45 minutes at room temperature. After adequate buffers washes. DAB(3,3'-diaminobenzidine tetrahydrochloride) chromogen added. Sections were counterstained with hematoxylin, dehydrated with ethanol and xylene, and mounted with DPX(Di-n-butylphthalate in xylene). Tonsillar tissue sections served as positive control for markers CD4+, CD8+.

**Microscopic assessment of CD4, CD8 T cells and scoring:** In our study, the CD4 and CD8 T cells were assessed in immunohistochemical (IHC) slides adapted from Ankita et al study, the scoring of immune stained positive CD4, CD8 TILs was done independently by two pathologists. CD4+, and CD8+ TILs were counted in five randomly selected high power fields (40X magnification) and the counts were averaged.

1. + (1-25 cells)
2. ++ (26-50 cells)
3. +++ (≥51 cells) in the tumour and the stroma separately
4. Positive TILs up to 25 cells were considered as low TIL count
5. > 25 cells (i.e. ++, +++) were considered as high TIL count.

## RESULTS

In this study, total 58 tissue blocks of patients who underwent modified radical mastectomy (MRM) were only taken and about 63.8% (37/58) had right MRM, 36.2% (21/58) had left MRM. Majority in the study group were females (96.6%). Only 2 male subjects and were diagnosed with invasive ductal carcinoma NST. The mean patient's age was 50.03 years (ranging from 35-74 years) Among the study population, majority 20 (34.5%) of them were belonging to 41 - 50 years. The molecular subtypes showed high triple negative subtype cases (48.3%) than others. Morphological diagnosis of invasive ductal carcinoma NST [77.5%] were predominant cases, followed by invasive carcinoma with medullary like features [15.50%] followed by invasive papillary carcinoma [3.40%], and mucinous carcinoma [3.40%] were observed. Of all

cases, (94.8%) were relapse free survivors, 1 patient had recurrence, and 2 patients did not survive. 53 patients of lymph node positive cases observed. The descriptive statistics of all 58 patients were compiled with tabulation.

**Correlation between Intratumoral, stromal, tumor interface CD4+, CD8+ expression with pathological variables and molecular subtypes:-**

Stromal CD4 count when correlated with negative hormonal receptor status(ER,PR) there was significant( $p = 0.05$ ) value. When compared to the tumor stage, low intratumoral CD4 and high stromal and interface CD4 count was found in T1, T2 stages of tumor and in all positive nodal status as well. Collectively CD4 intratumoral expression was low in relation to all the parameters like tumor size, lymph nodes and all molecular subtypes. Majority of TNBC showed low intratumoral CD4 count and high stromal and interface CD4 count. Low tumor stage (T1, T2) showed high intratumoral CD8 positive expression and also significant correlation was observed with triple negative subtype (ER-, PR-, Her2-) of  $p$  value ( $< 0.05$ ) which was statistically significant. Whereas stromal CD8+ didn't show any significant difference compared to any of the parameters, but high stromal and interface CD8 count is seen compared to intratumoral CD8 count. Tumor interface CD8+ cells very well correlated with the pathological higher nodal stage. Again, high CD8 count is seen more in triple negative breast cancers. Also noted that both stromal and interface CD4, CD8 infiltrate were high in Her2 and TNBC cases than the intratumoral sites. CD8 T cells were high compared to CD4 T cells around the normal breast TDLU. Moreover suggests immune response in these two subtypes is different from hormonal receptor subtype.

**Table 1. Cross tabulation between Tumour interface CD4 count (High & Low) and other variables:**

		Tumor interface CD4		Chi sq p value
		High	Low	
pTNM Tumour Size	T1 & T2	31 (83.8%)	6 (16.2%)	0.295
	T3 & T4	17 (85%)	3 (15%)	
pTNM Nodal Status	N0 & Nx	20 (76.9%)	6 (23.1%)	0.115
	N1 & above	28 (90.3%)	3 (9.7%)	
pTNM Metastasis	M0	40 (85.1%)	7 (14.9%)	0.315
	Mx	8 (80%)	2 (20%)	
ER	Positive	16 (76.2%)	5 (23.8%)	0.17
	Negative	32 (86.5%)	5 (13.5%)	
PR	Positive	16 (76.2%)	5 (23.8%)	0.17
	Negative	32 (86.5%)	5 (13.5%)	

Her2Neu	Positive	11 (73.3%)	4 (26.7%)	0.159
	Negative	37 (86%)	6 (14%)	
Ki67	1+ & 2+	28 (73.7%)	10 (26.3%)	0.009
	3+	20 (100%)	0 (0%)	
Pathological Subtype	TNBC	24 (85.7%)	4 (14.3%)	0.196
	Luminal A	12 (85.7%)	2 (14.3%)	
	Luminal B	4 (57.1%)	3 (42.9%)	
	Her2 / Neu	8 (88.9%)	1 (11.1%)	

Table2.Cross tabulation between Intratumoral CD8 count (High & Low) and other variables:

		Intratumoral CD8		Chi sq p value
		High	Low	
pTNMTumour Size	T1 & T2	20 (54.1%)	17 (45.9%)	0.77
	T3 & T4	10 (50%)	10 (50%)	
pTNM Nodal Status	N0 & Nx	13 (50%)	13 (50%)	0.716
	N1 & above	17 (54.8%)	14 (45.2%)	
pTNM Metastasis	M0	24 (51.1%)	23 (48.9%)	0.241
	Mx	6 (60%)	4 (40%)	
ER	Positive	6 (28.6%)	15 (71.4%)	0.008
	Negative	24 (64.9%)	13 (35.1%)	
PR	Positive	6 (28.6%)	15 (71.4%)	0.008
	Negative	24 (64.9%)	13 (35.1%)	
Her2Neu	Positive	8 (53.3%)	7 (46.7%)	0.885
	Negative	22 (51.2%)	21 (48.8%)	
Ki67	1+ & 2+	16 (42.1%)	22 (57.9%)	0.043

	<b>3+</b>	14 (70%)	6 (30%)	
<b>Pathological Subtype</b>	<b>TNBC</b>	16 (57.1%)	12 (42.9%)	<b>0.011</b>
	<b>Luminal A</b>	5 (35.7%)	9 (64.3%)	
	<b>Luminal B</b>	1 (14.3%)	6 (85.7%)	
	<b>Her2 / Neu</b>	8 (88.9%)	1 (11.1%)	

**Table3. Cross tabulation between Stromal CD8 count (High & Low) and other variables:**

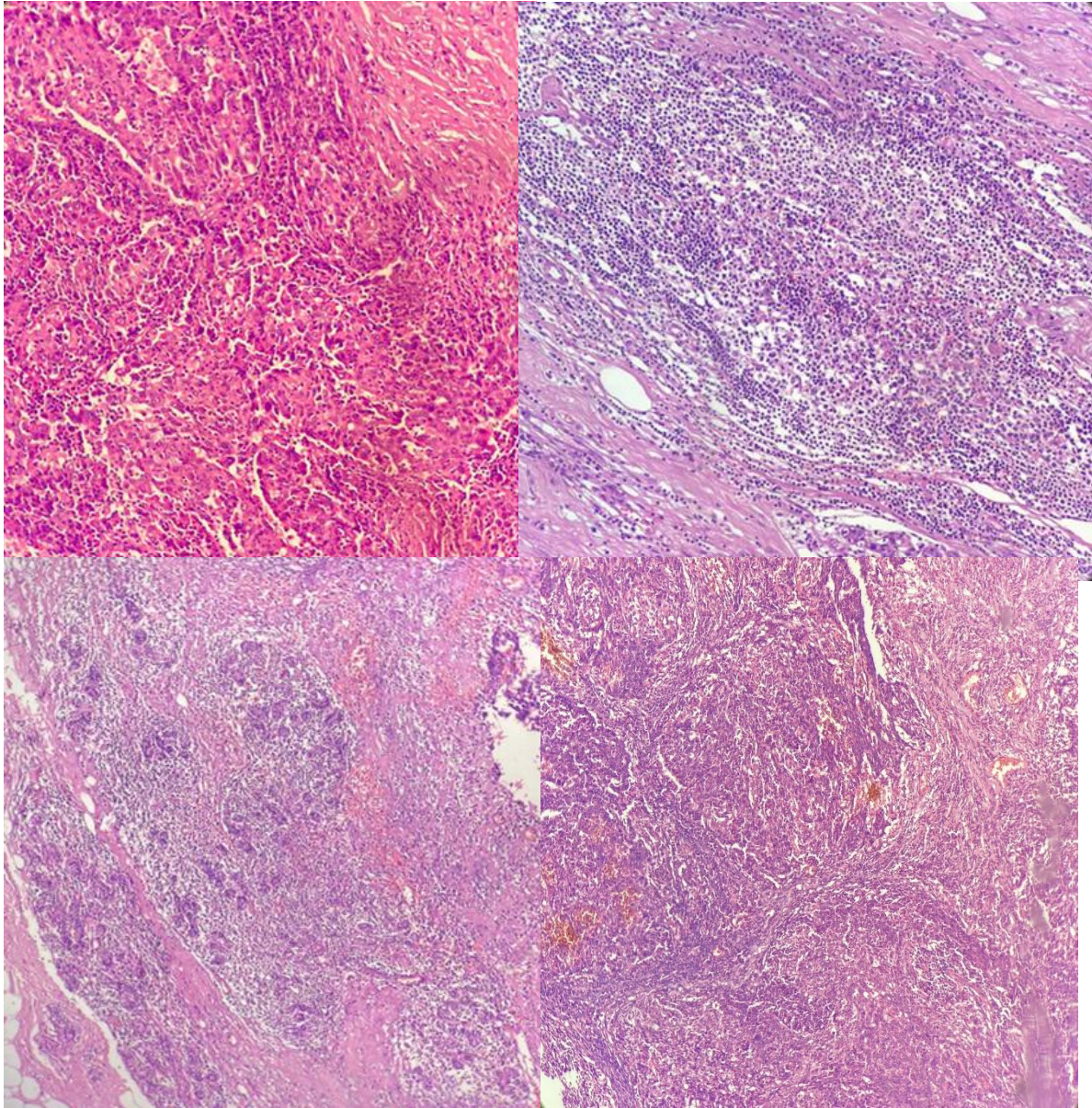
		<b>Stromal CD8</b>		<b>Chi sq p value</b>
		<b>High</b>	<b>Low</b>	
<b>pTNMTumour Size</b>	<b>T1 &amp; T2</b>	34 (91.9%)	3 (8.1%)	0.393
	<b>T3 &amp; T4</b>	19 (95%)	1 (5%)	
<b>pTNM Nodal Status</b>	<b>N0 &amp;Nx</b>	23 (88.5%)	3 (11.5%)	0.204
	<b>N1 &amp; above</b>	30 (96.8%)	1 (3.2%)	
<b>pTNM Metastasis</b>	<b>M0</b>	43 (91.5%)	4 (8.5%)	0.452
	<b>Mx</b>	10 (100%)	0 (0%)	
<b>ER</b>	<b>Positive</b>	19 (90.5%)	2 (9.5%)	0.356
	<b>Negative</b>	34 (91.9%)	3 (8.1%)	
<b>PR</b>	<b>Positive</b>	19 (90.5%)	2 (9.5%)	0.356
	<b>Negative</b>	34 (91.9%)	3 (8.1%)	
<b>Her2Neu</b>	<b>Positive</b>	14 (93.3%)	1 (6.7%)	0.404
	<b>Negative</b>	39 (90.7%)	4 (9.3%)	
<b>Ki67</b>	<b>1+ &amp; 2+</b>	33 (86.8%)	5 (13.2%)	0.11
	<b>3+</b>	20 (100%)	0 (0%)	
<b>Pathological Subtype</b>	<b>TNBC</b>	25 (89.3%)	3 (10.7%)	0.14
	<b>Luminal A</b>	13 (92.9%)	1 (7.1%)	

	<b>Luminal B</b>	6 (85.7%)	1 (14.3%)
	<b>Her2 / Neu</b>	9 (100%)	0 (0%)

**Table4. Cross tabulation between Tumour Interface CD8 count (High & Low) and other variables:**

		Tumor interface CD8		Chi sq p value
		High	Low	
<b>pTNMTumour Size</b>	<b>T1 &amp; T2</b>	34 (91.9%)	3 (8.1%)	0.266
	<b>T3 &amp; T4</b>	20 (100%)	0 (0%)	
<b>pTNM Nodal Status</b>	<b>N0 &amp; Nx</b>	23 (88.5%)	3 (11.5%)	<b>0.049</b>
	<b>N1 &amp; above</b>	31 (100%)	0 (0%)	
<b>pTNM Metastasis</b>	<b>M0</b>	44 (93.6%)	3 (6.4%)	0.554
	<b>Mx</b>	10 (100%)	0 (0%)	
<b>ER</b>	<b>Positive</b>	19 (90.5%)	2 (9.5%)	0.33
	<b>Negative</b>	35 (94.6%)	2 (5.4%)	
<b>PR</b>	<b>Positive</b>	19 (90.5%)	2 (9.5%)	0.33
	<b>Negative</b>	35 (94.6%)	2 (5.4%)	
<b>Her2Neu</b>	<b>Positive</b>	13 (86.7%)	2 (13.3%)	0.223
	<b>Negative</b>	41 (95.3%)	2 (4.7%)	
<b>Ki67</b>	<b>1+ &amp; 2+</b>	34 (89.5%)	4 (10.5%)	0.174
	<b>3+</b>	20 (100%)	0 (0%)	
<b>Pathological Subtype</b>	<b>TNBC</b>	26 (92.9%)	2 (7.1%)	0.175
	<b>Luminal A</b>	14 (100%)	0 (0%)	
	<b>Luminal B</b>	5 (71.4%)	2 (28.6%)	
	<b>Her2 / Neu</b>	9 (100%)	0 (0%)	

## MICROSCOPIC IMAGES

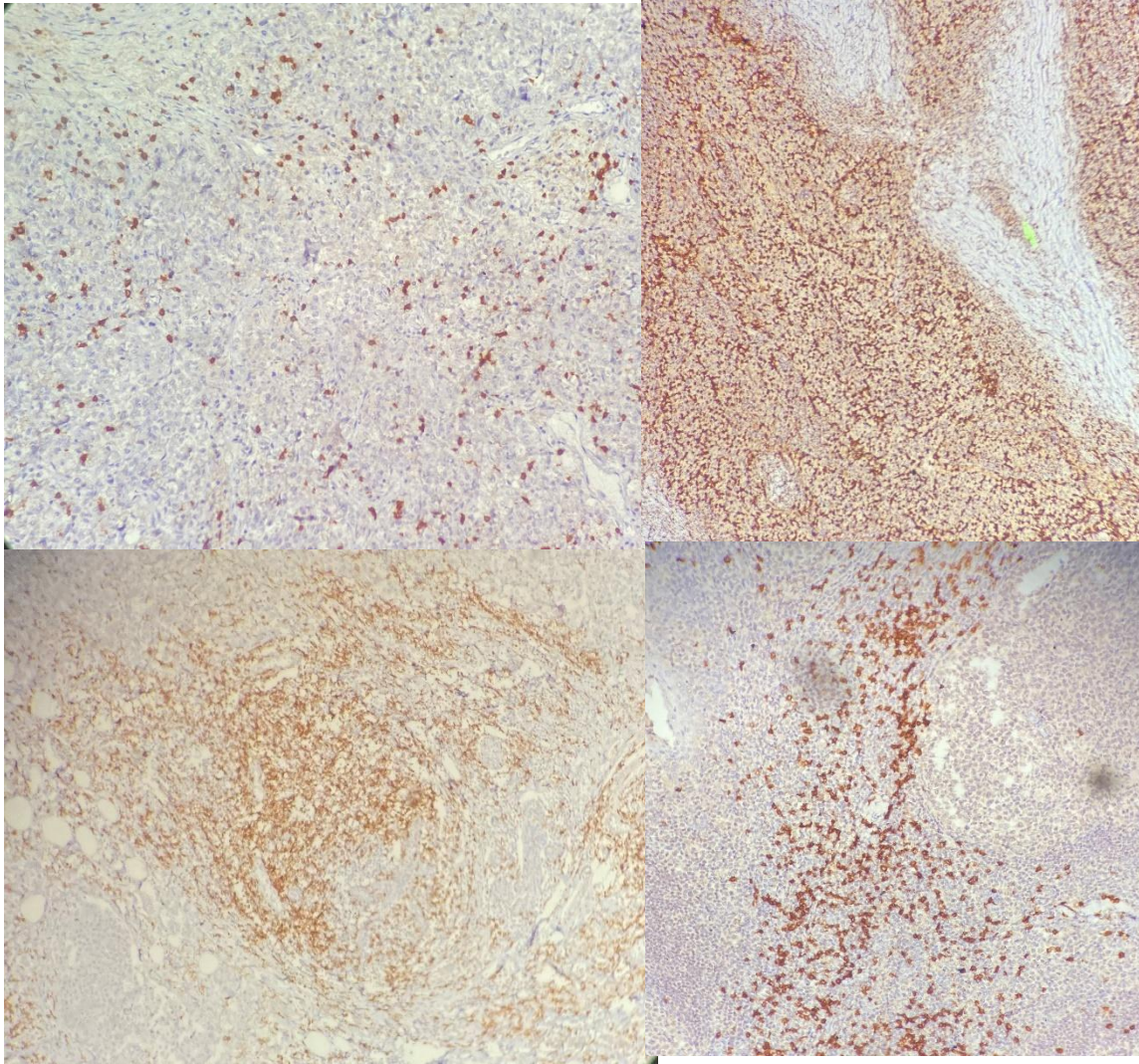


**Fig(a)- Intratumoral lymphocytes(x40 magnification, H&E)**

**Fig(b)-Tumor interface lymphocytic infiltrate(x40 magnification, H&E)**

**Fig(c)-Stromal lymphocytic infiltrate see extending to the adjacent normal breast terminal duct lobular unit(x20),**

**fig(d) extensive lymphoplasmacytic infiltrates in the stroma and intratumoral(x40)**



**Fig(e,f)- Intratumoral CD8, CD4 immunopositive T-lymphocytes(x40,x20)**

**Fig(g)- Tumor interface CD8 positive Tcells(x20)**

**Fig(h)- Stromal CD8 positive Tcells (x40)**

## **DISCUSSION**

**RafalMatkowski et al (2009)** studied 88 breast cancer patients with ductal histology. Along with the CD4 and CD8 expression, the type, density, localization and distribution of tumour infiltrating lymphocytes (TILs) was studied. The patients with high expression of CD4 or CD8 had lymph node involvement and worse cancer specific overall survival in this study<sup>17</sup>.

**Sahar M A Mahmoud et al (2011)** analysed 1334 tissue microarray cores . The study results revealed that the total number of CD8(+) cells was positively correlated with tumour grade, patient's age during the diagnosis, molecular markers expression. They observed that the Stromal CD8+ T cell lymphocytes were independently associated with better prognosis and patient survival<sup>18</sup>.

**Ankita Singh Rathore et al (2014)** CD3, CD4 and CD8 antigens from sections of 150 breast cancer patients. They observed that the Intra tumour counts, the high (++/+++ ) CD4+ count (P<0.001) displayed the highest survival stromal counts, the high CD8+ count (P<0.001) showed a better survival. They concluded that each of them are better independent predictors of favourable survival outcome in infiltrating ductal breast carcinoma<sup>19</sup>.

**Ezzeldin M Ibrahim et al (2014)** studied the prognostic value of tumour-infiltrating lymphocytes in triple-negative breast cancer by a meta-analysis. They extracted data of 2,987 patients with early stage BC from 8 studies. They concluded that the Rich TILs were significantly associated with improved survival outcome in early TNBC and further they recommended that they can be considered as a strong prognostic factor in this subtype<sup>20</sup>. Invasive ductal histology is the predominant type to be involved<sup>21</sup>.

**Hirofumi Matsumoto et al** study found that high levels of CD8+ iTILs and CD4+ sTILs were significantly associated with better clinical outcomes in TNBC. The role of CD4+ TILs in antitumour responses is often to stimulate the CD8+ TILs to attack tumor cells. The CD4+ T-cells benefit the CD8+ CTLs in maintaining the tumour immunity which happens in three phases, viz. by primary induction, effector maintenance, and memory of CD 8+ CTL responses<sup>22</sup>. **Kim et al** reported that declined number of CD8+ TILs in breast tumours were significantly related with lymph node metastasis, high stage and higher proliferative index<sup>23</sup>.

Triple negative breast cancers (TNBC) are most likely to have tumors with >50 % lymphocytic infiltrate, has the greatest survival benefit from each 10 % increase in TIL. ER+ve,PR+ve,HER2- tumors tend to have the least immune infiltrate<sup>24</sup>. T cells were the predominant immunophenotype being noted in 81% of tumors, compared to B cells in predicting the biological behavior. They came to a conclusion that, probably favors neoplastic progression rather than acting as an antitumor immune response<sup>25</sup>.

**Kurozumi et al.** recently investigated the relationship between TILs and prognosis in 294 cases and reported that high stromal TILs expression was a good prognostic marker in ER-negative cancer subtype<sup>26</sup>.

**CONCLUSION:** In conclusion of this study, it is to highlight that immune markers like CD4,CD8T cells expression might benefit patients particularly in triple negative breast cancer and Her2 overexpressed subtypes which is more immunogenic than the hormonal subtypes. Understanding the impact of specific subsets of immune cells that infiltrate tumors and its microenvironment is important for making rational decisions in the development of targeted therapies in invasive breast carcinomas. As shown in our study, lymph node involvement was seen in cases with high CD8 cells in the tumor interface. So, in addition to ER,PR,Her2 and Ki67, CD8 can also be used in patients to assess the prognosis.

**ETHICAL CLEARANCE:** Ethical Clearance for this study was got approved from the Institutional Human Ethical Committee.

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