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Evaluation of stromal tumor-infiltrating lymphocytes and immune checkpoint expression in triple-negative breast cancer treated with neoadjuvant chemotherapy with or without carboplatin

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Abstract: **Background**: Triple-negative breast cancer (TNBC) is predominant among younger Pakistani women with advanced and biologically aggressive disease. Ethnic, genetic and environmental factors, as well as neoadjuvant chemotherapy (NACT), may affect the tumor immune microenvironment, including stromal tumor-infiltrating lymphocytes (TILs), immune checkpoint proteins expression like lymphocyte activation gene-3 (LAG-3), programmed cell death-1 (PD-1) and programmed cell death ligand-1 (PD-L1). **Objectives**: To investigate the expression of LAG-3, PD-1 and PD-L1 immune checkpoint proteins and evaluate their association with TILs in patients with TNBC receiving NACT with or without carboplatin. **Methods**: This quasi-experimental study included 54 patients with TNBC aged 24 to 45 years treated at two tertiary care hospitals in Karachi, Pakistan. Thirty-nine patients received anthracycline-taxane-carboplatin therapy (A+T+C group), whereas fifteen received anthracycline-taxane therapy (A+T group). Clinical response was assessed using Response Evaluation Criteria in Solid Tumor (RECIST) based on mammography and breast ultrasonography before and after NACT. TILs were assessed on hematoxylin and eosin-stained (H&E) post-NACT resection sections and categorized as low (0-10%), intermediate (11-40%), or high (>40%) according to international recommendations. Immunohistochemistry was performed for PD-1, PD-L1 and LAG-3 expression in tumor cells (TC) and TILs using compartment-specific proportional scoring with a 1% positivity cutoff. Associations were analyzed using chi-square/Fisher's exact tests. **Results**: The A+T+C group attained a higher RECIST-based clinical response rate than the A+T group (92.31% vs. 60.0%) with a reduction in tumor size (2.67 cm, 95% CI: 2.14–3.23; p=0.001). High TILs density was significantly associated with PD-L1 expression on TC (χ^2 = 18.72; p < 0.001), LAG-3 expression on immune cells (IC) (χ^2 = 13.67; p < 0.001) and PD-1 expression on TC (χ^2 = 12.38; p = 0.002). No significant associations were observed with LAG-3 expression on TC, PD-L1 and PD-1 expressions on IC. **Conclusion**: Post-NACT TILs density was significantly associated with PD-L1 expression on TC and LAG-3 expression on IC in TNBC. Carboplatin improved the clinical response without significantly affecting the TIL density or immune checkpoint expression.

Keywords: Lymphocyte activation gene-3; Programmed cell death-1; Programmed cell death ligand-1; Triple negative breast cancer; Tumor infiltrating lymphocytes

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INTRODUCTION

Breast cancer (BC) is a heterogeneous malignancy with an increased mortality and incidence rate among all female cancers. (Sung *et al*., 2020) Triple-negative breast cancer (TNBC) is one of the most challenging subtypes, representing approximately 10–15% of all diagnosed breast cancers. The overall survival of TNBC patients is worse, with a high risk of relapse within the first 3 to 5 years (Mehrotra *et al*., 2025).

TILs are a vital component of the cellular anti-cancer immune response and are present within the tumor microenvironment. Their role is not only prognostic but also predictive of response to immunotherapy, thereby increasing their potential utility in precision medicine for TNBC in patients receiving adjuvant and neoadjuvant chemotherapy (NACT) (Gao G *et al*., 2020; Brummel *et al*., 2023). Over the past few decades, NACT for breast cancer treatment has increased and is now becoming a backbone modality in clinical practice worldwide. In TNBC, a reduction in tumor size is beneficial for assessing response to NACT. Certain biomarkers are strong prognostic factors, showing a positive relationship with better survival and treatment response, potentially allowing for treatment de-escalation strategies. Therefore, identifying reliable biomarkers to predict patient response is crucial. Higher TILs often signal a better prognosis, identifying "immune-rich" tumors that may show a favorable prognosis and benefit from checkpoint inhibitors (Gao G *et al*., 2020). Cancer cells take advantage of this natural mechanism by elevating PD-L1 expression on their surface. This interaction with PD-1 on

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T cells leads to T cell deactivation, allowing the tumor to evade immune surveillance. Various research focuses on developing new ways to disrupt this interaction and improve patient outcomes by blocking the immune checkpoint proteins programmed cell death-1 (PD-1) and its ligand, PD-L1. PD-1 and PD-L1 are expressed on IC and TC, respectively. The interaction of PD-1 to PD-L1 leads to immune cell exhaustion and impairs their ability to target cancer cells. Several studies have shown the efficacy of anti–PD-1 or anti–PDL-1 immunotherapy in different cancers, including TNBC (Wu *et al*., 2022; Javed *et al*., 2024; Yang *et al*., 2023). Additionally, previous study has shown that TILs are a source of other immune markers, such as PD-L1 (Gonzalez-Ericsson *et al*., 2020). However, it is noted that despite the significance of anti–PD-1/PD-L1 therapy, resistance to treatment remains a major challenge (Mandal *et al*., 2025). This highlights the importance of further research into other immune checkpoints and their association with TILs to enable more patients to benefit from immunotherapy. Therapeutic resistance involves phenotypic changes in cancer cells and/or in the tumor microenvironment (Said and Ibrahim, 2023). Lymphocyte-activating gene 3 (LAG-3) is another clinically relevant immune checkpoint; it belongs to the immunoglobulin superfamily and is found on the membranes of B cells, TILs, natural killer cells and dendritic cells (Maruhashi *et al*., 2020; Chocarro *et al*., 2021). LAG-3 is particularly notable as it is frequently co-expressed with PD-1 on exhausted T-cells. Preclinical studies have demonstrated that dual blockade of these pathways by the drugs produces synergistic anti-tumor effects (Liu *et al*., 2021). However, data on TILs and immune checkpoint expression in TNBC from South Asian populations, particularly young Pakistani women, are limited.

In this study aimed to assess the proportion of TILs and the expression of the immune checkpoint proteins (LAG-3, PD-1 and PD-L1) in TNBC among young women from Karachi, Pakistan, thereby generating a baseline immunological and molecular profile that may facilitate future biomarker discovery and support the development of region-specific immunotherapeutic approaches. In addition, clinical response to neoadjuvant chemotherapy with and without carboplatin was evaluated.

MATERIALS AND METHODS

This quasi-experimental study included 54 consecutive female patients (aged 24–45 years) with TNBC, classified by negative immunohistochemical expression of ER, PR and HER-2. The individual patient was the primary unit of assignment and analysis (N=54). Fourteen (n=14) and fourty (n=40) patients were recruited from Ziauddin Hospital and Jinnah Postgraduate Medical Centre (JPMC) respectively. All TNBC patients underwent surgery following NACT according to a standard regimen. Clinical tumor stage and nodal status were determined using standard bilateral mammography and breast ultrasonography before initiation of NACT and after NACT (Thomassin-Naggara *et al*., 2014). The completion of at least four NACT cycles and the histological confirmation of negative immunohistochemical expression of ER, PR and HER-2 were the inclusion criteria for TNBC patients. Incomplete clinical records, ongoing autoimmune illness, or a history of cancer were among the exclusion criteria.

NACT regimen

All patients received at least four cycles of NACT and were divided into two treatment groups: anthracycline, taxane (A+T) and anthracycline, taxane and platinum-based drug carboplatin (A+T+C) groups according to the treatment regimen determined as part of routine clinical care; allocation was non-randomized. Treatment completion and cycle adherence were verified by patients medical records.

Clinical response assessment before and after NACT

Tumor size for clinical response was evaluated using RECIST (Response Evaluation Criteria in Solid Tumor) criteria based on mammography and breast ultrasonography (Kitajima *et al*., 2018). Clinical response was evaluated following four cycles of chemotherapy (A+T and A+T+C) and after the therapy. The absence of any detectable primary tumor was considered as complete response (CR), whereas a partial response (PR) was defined as tumor size reduction of at least 30%. Progressive disease (PD) was defined as an increase in tumor size of at least 20% and stable disease (SD) was defined as disease that did not meet the criteria for either PR or PD.

Pathological evaluation after NACT

Following surgery, tumor resection specimens underwent routine gross pathological examination. Residual tumor size was assessed using calipers and representative sections were prepared for hematoxylin and eosin (H&E) staining and immunohistochemical analysis.

Histopathological evaluation of tumor-infiltrating lymphocytes (TIL)

Tissue specimens were obtained during surgical resection following NACT and tissue blocks were embedded in paraffin. TILs were analyzed on 4-µm sections by two experienced pathologists. All tissues were fixed in buffered formalin (10%). The Hematoxylin–eosin-stained (H&E) sections were evaluated to confirm the histological diagnosis using a light microscope at × 100–200 magnification. Histopathological assessment of TILs was performed according to international consensus recommendations in TNBC (Salgado *et al*., 2015; Dieci *et al*., 2018). https://www.tilsinbreastcancer.org/what-are-tils/). The area of necrosis and technical artifacts was excluded from analysis. TIL levels were evaluated as percentages and three predefined categories were used:

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low TIL scores (0-10%), intermediate (11-40%) and high TIL (> 40%).

Immunohistochemical evaluation of PD-1, PD-L1 and LAG3

Tissue sections (4-µm thick) were obtained from the resection tissue sample following NACT treatment. These sections were deparaffinized using xylene and subsequently rehydrated through a series of graded alcohols. Antigen retrieval was done using a citrate buffer at pH 6.0 in a steamer, heated at 95–98 °C for 20 minutes and then allowed to cool at room temperature for an additional 20 minutes. Blocking of endogenous peroxidase activity was achieved using 3% hydrogen peroxide; then the slides were preincubated with 5% BSA blocking solution for 5 minutes and incubated overnight with the primary antibodies. Primary antibodies against PD-1 (1:100 dilution; Monoclonal antibody (J121), Invitrogen), PD-L1 (1:100 dilution; polyclonal antibody, Invitrogen) and LAG-3 (1:100 dilution; CD223 polyclonal antibody, Invitrogen) were used. Sections were rinsed with PBS and then incubated with a secondary antibody conjugated to HRP (EnVision FLEX DAKO) for 45 minutes. DAB was added as a substrate for 10 min, followed by washing and staining with hematoxylin. The sections were mounted and the expression of immune checkpoint proteins was evaluated separately in ICs and TCs. However, PD-1, PD-L1 and LAG-3 expressions in tumor cells TCs was evaluated as the proportion of TC with membrane stain of any intensity; expression in ICs was evaluated as the proportion of IC with any intensity that contained PD-1, PD-L1 or LAG-3 (Vennapusa *et al*., 2019). Tumor cells (TC) were defined as PD-L1-, PD-1- and LAG-3-negative or -positive if the tumor proportion score (TPS) was <1% or >1%, respectively. For immune cells (IC), the immune proportion score (IPS) was used with the same cutoff value (Ulas *et al*., 2023).

Statistical analysis

Fisher's exact test was applied to evaluate the relationship of TILs with other significant biomarkers, namely LAG-3, PD-1 and PD-L1 using IBM SPSS Statistics, Version 24.0 (IBM Corp., Armonk, NY, USA). Tumor size before and after treatment was compared using a paired t-test. Statistical significance was set at $p < 0.05$ and 95% confidence intervals (CIs) were calculated where applicable.

RESULTS

Demographics of TNBC patients

The clinicopathological profiles of the TNBC patients involved in this study are presented in table 1. Among the 54 female TNBC patients, age ranged from 24 to 40 years in 37 patients (68.51%) and 41 to 45 years in 17 patients (31.48%); the mean age was 36.13 ± 6.18 years. Before NACT, 29 TNBC patients (53.70%) presented with cT3. After NACT, tumor stage was ypT2 in 35 patients (64.81%), ypT1 in 7 patients (12.96) and ypT3 in 3 patients (5.56%). Lymph node stages were ypN0 in 7 patients (12.96%), ypN1 in 44 patients (81.48%), ypN2 in 1 (1.86%) and ypN3 in 2 patients (3.70%). All patients had infiltrating ductal carcinoma (IDC) (54;100%) and 42 patients (77.77%) had grade III tumors. The primary surgical treatment was modified radical mastectomy (MRM) in 50 patients (92.59%), followed by breast conserving treatment in 4 patients (7.40 %).

Clinical response

As shown in Table 2, Clinical response using RECIST-based evaluation to neoadjuvant chemotherapy with and without carboplatin was favorable. Among the 39 patients in the A+T+C group, 10 (25.64%) achieved a CR, 26 (66.67%) showed partial remission, 2 patients (5.13%) attained SD and 1 patient (2.56%) showed PD. The response rate was higher in the A+T+C group (92.31%) compared with the A+T group. A significant difference in tumor size (2.67 cm) was also noticed in the A+T+C group, as shown in table 3.

Association between stromal TILs proportions and immune checkpoint protein expression in TNBC

A highly significant association was observed between high TILs proportions (Fig.1; A1-A2; B1-B2; C1-C2) and PD-L1 expression on TCs ($\chi^2 = 18.724$; $p < 0.0001$) with PD-L1-positive tumors demonstrating a high proportion of TILs (65%). LAG-3 expression on ICs ($\chi^2 = 13.67$, $p < 0.001$) was significantly associated with TILs and with LAG-3-positive cases predominantly exhibiting intermediate to high TIL levels. PD-1 expression on TCs was also significantly associated with TIL proportions ($\chi^2 = 12.386$, $p = 0.002$). In contrast, no significant associations were observed between TIL proportions and PD-1 expression on ICs ($p = 0.105$), PD-L1 expression on ICs ($p = 0.688$), or LAG-3 expression on TCs ($p = 0.065$; Table 4). Lymph node, tonsil and ovary tissues were used as positive control for PD-1, PD-L1 and LAG-3 expression as shown in Supplementary figure S1.

Comparison of TILs proportions and immune checkpoint protein expression between the two groups in TNBC

No significant differences were observed in the expression of immune checkpoint proteins PD-L1, PD-1 and LAG-3 (Fig. 2; A1-A4; B1-B4; C1-C4) on either tumor cells or immune cells between the two groups (A+T and A+T+C). Furthermore, the distribution of low, intermediate and high TIL categories did not show significant differences (p = 0.601) between the two groups (A+T and A+T+C; Table 5).

DISCUSSION

Research on immune checkpoint proteins in breast cancer has increased in recent years, highlighting their clinical significance and potential to improve patient management strategies.

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Table 1: Baseline demographics of TNBC patients.

Characteristics	N = 54 (%)
Age (Years)	
24 – 40	37 (68.51)
41 – 45	17 (31.48)
T prior to NACT	
cT1	1 (1.86)
cT2	18 (33.33)
cT3	29 (53.70)
cT4	6 (11.11)
N prior to NACT	
cN0	5 (9.25)
cN1	43 (79.60)
cN2	4 (7.40)
cN3	2 (3.70)
T Stage after surgery	
ypT0	9 (16.67)
ypT1	07 (12.96)
ypT2	35 (64.81)
ypT3	3 (5.56)
N Stage after surgery	
ypN0	7 (12.96)
ypN1	44 (81.48)
ypN2	1 (1.86)
ypN3	2 (3.70)
M Stage	
M0	51 (94.44)
M1	3 (5.55)
Tumor subtype	
IDC	54 (100)
Histological grade	
I	1 (1.85)
II	11 (20.37)
III	42 (77.77)
Surgery	
MRM	50 (92.59)
BCS	4 (7.40)

TNBC: Triple-negative breast cancer; MRM: Modified radical mastectomy; IDC: Infiltrating ductal carcinoma; BCS: Breast conservative surgery; NACT: Neoadjuvant chemotherapy; Data expressed as number (percentage).

Table 2: Clinical response to neoadjuvant therapy with and without carboplatin.

NACT regimen	N	RECIST evaluation	Frequency (%)	Response (%)	*p*-value
A+T	15	CR	1 (6.67%)	Positive (60.00)	
		PR	8 (53.33%)		
		SD	2 (13.33%)	Negative (40.00)	0.01*
		PD	4 (26.67%)		
A+T+C	39	CR	10 (25.64%)	Positive (92.31)	
		PR	26 (66.67%)		0.01*
		SD	2 (5.13%)	Negative (7.69)	
		PD	1 (2.56%)		

CR: Complete response; PR: Partial response; SD: Stable Disease; PD: Progressive Disease; NACT: Neoadjuvant chemotherapy; A+T: Anthracycline-taxane regimen; A+T+C: Anthracycline-taxane-carboplatin regimen; RECIST:

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Table 3: Tumor size comparison before and after NACT with and without carboplatin.

NACT regimen	Tumor size (cm) before NACT Mean ±SD	Tumor size (cm) after NACT Mean ±SD	Mean difference (95% CI)	*p*-value
A+T	4.76 ± 1.28	3.56 ± 1.53	1.20 (95% CI: 0.59 – 1.80)	0.001*
A+T+C	5.20 ± 1.43	2.53 ± 1.65	2.67 (95% CI: 2.14 – 3.23)	0.001*

NACT: Neoadjuvant chemotherapy; A+T: Anthracycline-taxane regimen; A+T+C: Anthracycline-taxane-carboplatin regimen. * $p < 0.05$ was considered statistically significant.

Table 4: Association of immune checkpoint and tumor-infiltrating lymphocytes (TILs) in TNBC

Immune-checkpoints	TILs proportions	Negative		Positive		Chi-Square value	*p-value*
		n	%	n	%		
PD-1-IC	High	2	22.20	21	46.70	4.50	0.105
	Intermediate	4	44.50	20	44.40		
	Low	3	33.30	4	8.90		
PD-1-TC	High	14	33.33	9	75.00	12.38	0.002*
	Intermediate	24	57.14	0	0.00		
	Low	4	9.53	3	25.00		
PD-L1-IC	High	16	40.00	7	50.00	0.74	0.688
	Intermediate	18	45.00	6	42.90		
	Low	6	15.00	1	7.10		
PD-L1-TC	High	2	9.09	21	65.60	18.72	0.0001*
	Intermediate	14	63.64	10	31.30		
	Low	6	27.27	1	3.10		
LAG-3-IC	High	8	47.10	15	40.50	13.67	0.001*
	Intermediate	3	17.60	21	56.80		
	Low	6	35.30	1	2.70		
LAG-3-TC	High	9	29.00	14	60.90	5.47	0.065
	Intermediate	17	54.84	7	30.40		
	Low	5	16.13	2	8.70		

TILs: Tumor-infiltrating lymphocytes; PD-1: Programmed cell death protein 1; PD-L1: Programmed death-ligand 1; LAG-3: lymphocyte activation gene-3; High, intermediate, and low indicate the relative proportion of TILs expressing each immune checkpoint. * $p < 0.05$ was considered statistically significant.

Table 5: Immune checkpoint proteins expression and TILs density among the two groups.

Immune-checkpoints	Protein expression status	A+T group	A+T+C group	*p-value*
PD-L1-TC	Negative	06 (40.00)	16 (41.03)	0.59
	Positive	09 (60.00)	23 (58.97)	
PD-L1-IC	Negative	12 (80.00)	28 (71.79)	0.40
	Positive	03 (20.00)	11 (28.21)	
PD-1-TC	Negative	10 (66.66)	30 (76.92)	0.56
	Positive	05 (33.33)	09 (23.08)	
PD-1-IC	Negative	01 (6.66)	06 (15.38)	0.48
	Positive	14 (93.33)	33 (84.61)	
LAG-3-TC	Negative	07 (46.67)	24 61.54)	0.24
	Positive	08 (77.42)	15 (38.46)	
LAG-3-IC	Negative	05 (33.33)	12 (30.77)	0.55
	Positive	10 (66.67)	27 (69.23)	
TILs	Low	03 (20.00)	04 (10.26)	0.60
	Intermediate	05 (33.33)	19 (48.72)	
	High	07 (46.67)	16 (41.03)	

TNBC: Triple-negative breast cancer; TC: Tumor cell; IC: Immune cells; A+T: Anthracycline-containing-taxane; A+T+C: Anthracycline-taxane-carboplatin; TILs: Tumor Infiltrating Lymphocytes. Data expressed as number (percentage).

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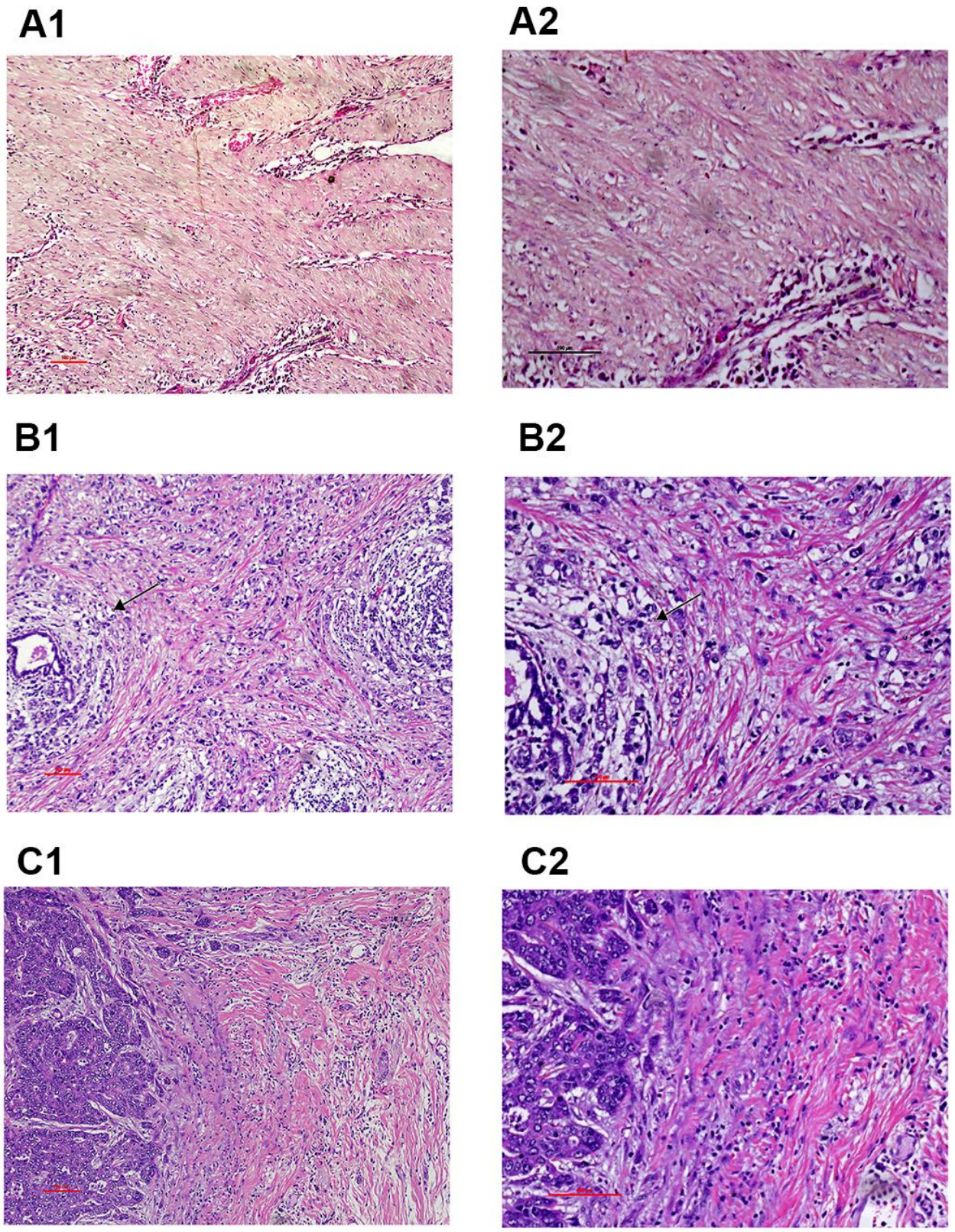


Fig. 1: Evaluation of tumor infiltrating lymphocytes in TNBC. TILs evaluation area was analyzed at 100X and 200X magnification. Representative H & E-stained images showing three different stromal TILs levels: A1 (100X) and A2 (200X): Low level (0-10%), B1 (100X) and B2 (200X): Intermediate level (11-40%) C1 (100X) and C2 (200X): high level (>40%); arrow indicates the distribution of the tumor area. Scale bar 100 µm.

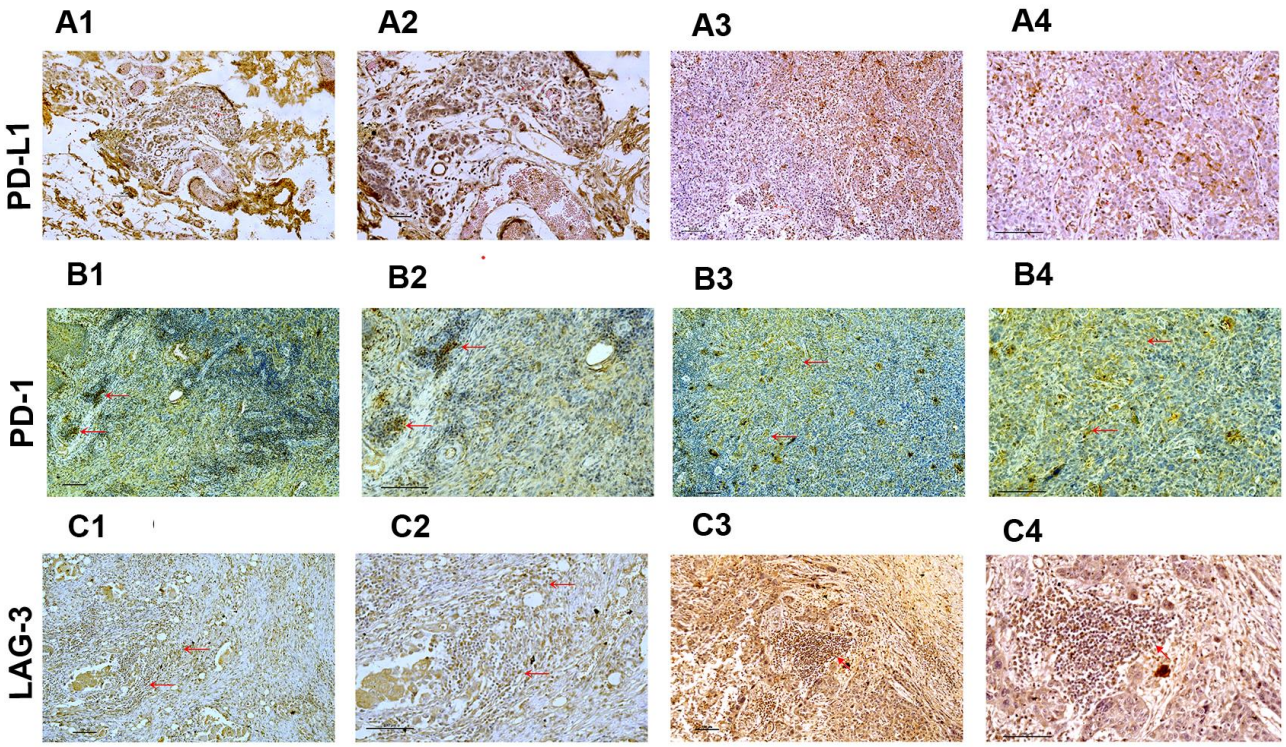


Fig. 2: Immunohistochemical features of PD-1, PD-L1 and LAG-3 at 100X and 200X: PD-L1: A1 (100X) & A2 (200X). Tumor cells; A3 (100X) & A4 (200X). Immune cells; PD-1: B1 (100X) & B2 (200X). Tumor cells; B3 (100X) & B4 (200X). Immune cells; LAG-3: C1 (100X) & C2 (200X). Immune cells; C3 (100X) & C4 (200X). Tumor cells; Scale bar; 100 µm.

In the tumor microenvironment (TME), comprising both ICs and TC, LAG-3, PD-1 and PD-L1 play a role in resistance to anti-tumor therapy (Wu *et al.*, 2022; Rivoltini *et al.*, 2024). Despite advancements in this evolving field, only a small number of immune markers have been explored as potential therapeutic targets in TNBC.

The present study investigates the associations among the expression of three immune checkpoint proteins (LAG-3, PD-1 and PD-L1) and TILs density in TNBC patients undergoing NACT.

According to the RECIST criteria, current study demonstrated the effectiveness of a carboplatin-based neo-adjuvant regimen, with an overall clinical response rate of 92.3% (CR and PR) and an average tumor size reduction of 2.67 cm in the A+T+C group. Similarly, Loibl *et al.*, 2018 demonstrated that carboplatin significantly enhanced the rate of pathological complete response (pCR) in patients with TNBC. Prihantono similarly indicated a total response rate (both CR and PR) of 93.33% among seventy TNBC patients, which closely align with the findings of the present study (Faruk, 2023). Kunnuru *et al, 2020* reported a favorable clinical and pathological response (78%) in 15% of TNBC patients. However, these findings should be interpreted as indicators of clinical response rather than pathological response, as pCR was not consistently documented across the study cohort.

The two groups (A+T and A+T+C) did not significantly alter the expression of PD-1, PD-L1, or LAG-3 on either TC or IC, nor did it significantly affect TILs proportions (Table 5). Therefore, these findings suggest that carboplatin may enhance treatment efficacy through mechanisms other than direct modulation of these immune biomarkers.

In contrast, the phase III KEYNOTE-522 trial demonstrated that the addition of the PD-1 inhibitor pembrolizumab to platinum-containing neoadjuvant chemotherapy significantly improved pCR, event-free survival and overall survival in patients with early-stage TNBC (Schmid *et al.*, 2020). Unlike the phase III KEYNOTE-522 trial, the current study did not designed to evaluate pCR or survival but rather to examine differences in immune checkpoint protein expression and stromal TIL proportions between chemotherapy regimens. Similarly, Sharma *et al.* (phase II trial) reported that baseline immune biomarkers, including stromal TILs and PD-L1 expression, were associated with pathological

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complete response. In contrast, the study did not demonstrate that the addition of carboplatin itself directly altered these immune biomarkers (Sharma *et al*, 2024).

The young women with TNBC in the Karachi subset population exhibit a high prevalence of PD-L1 positive tumors (65%; p =0.0001). This suggests that a larger fraction of this cohort might respond favorably to immune checkpoint inhibitors (ICIs) compared to other populations. Berckelaer *et al*. 2019 reported 1.9% PD-L1 expression in tumor cells, which is quite uncommon, whereas TILs exhibited a significantly higher expression rate (43%). Amin *et al*., 2022 found PD-L1 expression and large tumor size in a younger age group (≤50 years) . It is well established that PD-L1 expression on TC enhance the efficacy of anti-PD-1/PD-L1 therapies and it may be served as a biomarker to identify individuals who are most likely to benefit from immune checkpoint inhibition (Cortes *et al*., 2024). A meta-analysis has shown that the TILs as long-term prognostic indicators for HER2-positive and TNBC breast tumors, but not for the HR + HER2-subtype (Li *et al*., 2022). PD-L1 may promote survival, epithelial-mesenchymal transition (EMT) and chemotherapy resistance by exerting its effects through tumor-intrinsic signaling, PI3K/Akt and mTOR pathways (Yadollahi *et al*., 2021). These findings suggest that TNBC tumors in the present study may depend heavily on PD-L1-mediated signaling both as a shield against immune evasion and as a direct proliferative advantage, making PD-1/PD-L1 blockade a compelling therapeutic avenue. Although a strong association was seen with PD-L1-positive TC (p=0.0001), no significant association was seen between TIL density and PD-L1 expression on IC (p = 0.688; Table 4). This difference might suggest that PD-L1 expression on tumor cells is more closely associated with adaptive immune pressure, whereas PD-L1 expression on IC could represent a more heterogeneous and treatment-influenced stromal environment.

The expression of PD-L1 on IC may be influenced by macrophage-rich infiltrates, cytokine exposure, stromal heterogeneity and treatment-induced remodeling after NACT (Wang *et al*., 2026). PD-L1 expression on TC might signify a more direct adaptive response to localized cytotoxic immune stress. This disparity might have been due to variation in method for compartment-based scoring. Other considerations include environmental exposures, post-NACT immune remodeling, younger patient age, later-stage disease biology and technical variations in staining interpretation and cutoff application.

Patients with increased PD-L1 expression on IC demonstrated a better prognosis for breast cancer (Cirqueira *et al*., 2021). Interestingly, compared to individuals with PD-L1-negative tumors, these data unequivocally demonstrate that patients with PD-L1-positive TNBC respond better to neoadjuvant chemotherapy and have better survival outcomes. (Azim *et al*., 2022). Similar studies also confirmed that PD-L1 was associated with high TILs in the TNBC subtype and improved patients' outcomes (Alkhayyal *et al*., 2022). In TNBC, where tumors are often ER-, PR- and HER2-negative, high tumor-infiltrating lymphocyte (TIL) numbers and PD-L1 expression are recognized as prognostic biomarkers of response to immunotherapy (Angelico *et al*., 2023).

Evaluation of TILs and PD-1/PD-L1 expression, along with other co-inhibitory immune checkpoints, is warranted in TNBC patients (Qiu *et al*, 2021). LAG-3 expression varies significantly across cancer types, with high expression reported in melanoma (50%), neuroendocrine malignancies (47%) and uterine cancers (42%), whereas colorectal cancer demonstrates comparatively low frequencies of LAG-3 expression (15%) (Adashek *et al*., 2023). A high LAG-3 expression was found in 52.7% of patients under the age of 55 years and LAG-3 expression was strongly associated with T-cell infiltration, suggesting its close association with the tumor immune microenvironment and a potential active but immunosuppressed antitumor immune response (Liu *et al*., 2021).

A recent study revealed that LAG-3 expression was more common in TNBC, with more than 70% of patients showing positive expression compared with luminal and HER2-positive breast cancer subtypes (Balestrieri *et al*., 2026). The presence of both intermediate (56.8%) and high (40.5%) TILs in the LAG-3 positive group (Table 4) supports a more accurate statement that LAG-3 positivity was associated with intermediate-to-high TIL proportions (p = 0.001). Thus, present study showed that their existence in the tumor microenvironment might be associated to a potential advantage from immunomodulatory therapies in TNBC patients.

Recently, LAG-3 targeted therapy has emerged as novel therapeutic option for cancer patients with Relatlimab, an anti-LAG-3 monoclonal antibody, receiving FDA approval for use in combination with PD-1 inhibitors (Ibrahim *et al*., 2023).

Several limitations of this study should be acknowledged. First, the relatively small sample size limited the statistical power and generalizability of the findings. Second, inclusion of patients from two centers may have introduced selection bias and the prognostic significance of immune checkpoint proteins could not be assessed because long-term follow-up data for overall and disease-free survival were unavailable. Third, only post-NACT resection specimens were subjected to immunohistochemical analysis, precluding comparison with pre-treatment biomarker expression and limiting the

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evaluation of pathological complete response (pCR) prediction. Fourth, biomarker scoring was based on pathologist interpretation rather than H-scores or digital image analysis, introducing the potential for observer-related variability. Finally, treatment response was assessed using RECIST clinical criteria without centralized radiological review and therefore cannot be considered a substitute for standardized pathological response assessment.

Larger, multicenter prospective studies should be conducted with standardized treatment allocation and longer follow-up to validate the observed associations between TIL density, PD-1, PD-L1 and LAG-3 in TNBC. Furthermore, the predictive value of these immune markers for pathological complete response, recurrence and survival outcomes should be evaluated.

CONCLUSION

In TNBC patients receiving NACT, PD-1, PD-L1 and LAG-3 were expressed in both TC and IC. Post-NACT TIL density was significantly associated with PD-L1 and PD-1 expression on TC and LAG-3 expression on IC. No significant associations were observed between TIL density and PD-1 or PD-L1 expression on IC or LAG-3 expression on TC. Carboplatin-containing NACT was associated with a higher clinical response rate but did not significantly alter TIL density or immune checkpoint protein expression.

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Author's contributions

Conception/design: Shamim Mushtaq as PI won a research grant from the Higher Education Commission (HEC), Karachi, Pakistan. Provision of study material or patients: Ghulam Haider diagnosed cases and provided TNBC biopsies and supported in assembly of data, Moazzam Ali Shahid; Performed the experiments, Serajudaulla Syed; slides interpretation. Shamim Mushtaq; Manuscript writing: Bushra Wasim; manuscript review, Javeria Samee; assist in sampling and experiments all authors approved the final draft of the manuscript.

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Data availability statement

All data generated or analyzed during this study are included in this published article and its supplementary information files.

Ethical approval

All procedures were conducted in line with the Declaration of Helsinki. Written informed consent was obtained from all participants and/or their legal guardians. The study was granted ethical approval from the Research Ethics Committee (ERC) of Ziauddin University (Reference code:1730120SMBIO). This quasi-experimental, non-randomized study was conducted and reported in accordance with the TREND (Transparent Reporting of Evaluations with Nonrandomized Designs) statement. This study was performed in adherence with the TREND guidelines. See supplementary file for the TREND checklist.

Conflict of interest

The authors declare no conflicts of interest.

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