Immunohistochemical Evaluation of $CD3^+$ and $CD8^+$ tumour-Infiltrating Lymphocytes and their Association with Clinicopathological Features in Breast Cancer

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Abstract: *Background*: Breast cancer represents one of the most prevalent malignancies, with progression influenced by both tumour biology and the immune microenvironment. However, current prognostic methods have limited accuracy in predicting tumour aggressiveness and long-term outcomes. Tumour-infiltrating lymphocytes, principally $CD3^+$ and $CD8^+$ T cells, may play an important role in antitumour immunity.

Objective: The primary goal of the study is to assess the immunostaining of CD3 and CD8 proteins in malignant and non- malignant breast tissue and to find their association with breast cancer histopathological data using immunohistochemistry.

Methodology: This research was conducted between December 2025 and March 2026 at Al-Nasiriyah Teaching Hospital in Thi-Qar, Iraq. Immunohistochemical analysis was performed to evaluate $CD3^+$ and $CD8^+$ T-cell expression in both malignant (n=100) and non-malignant (n= 40) tissues of the breast. Statistical analyses were applied using unpaired t-tests, Chi-square and one-way ANOVA to assess differences in immunostaining levels between the studied groups.

Result: The findings demonstrated that $CD3^+$ and $CD8^+$ immunostaining levels were significantly elevated in both stromal and intra-tumoural regions of malignant breast tissues compared to benign tissues. The immunostaining of these potential biomarkers was associated with higher tumour grade, larger tumour size, lymph node metastasis, and the molecular subtypes of breast cancer.

Conclusion: Increased CD3 and CD8 expression in breast cancer is strongly linked to more advanced histopathological parameters. The findings indicate that these immune biomarkers may reflect the tumour immune microenvironment and contribute to a more comprehensive understanding of tumour progression and host immune response in breast cancer.

Keywords: Breast cancer molecular subtype, CD3, CD8, Grade, IHC and stage.

INTRODUCTION

Breast cancer (BC) is a highly diverse group of neoplastic cells disorder characterized by significant differences in clinical manifestation, histopathological architecture, genomic landscape, biological behaviour, and therapeutic responsiveness [1]. Despite tremendous advancements in our knowledge of and ability to treat BC, the disease still has serious global issues and is a major public health concern [2]. Globally, about 2.3 million women were diagnosed with BC, with roughly 670,000 fatalities reported [3]. BC can affect females at any age after puberty worldwide, with incidence increasing as age advances [4]. It also represents the leading cause of mortality withen Iraq female population. The recent Iraqi report found that BC represents the most prevalent cancers among females, approximately one-third of all known female tumour cases [5].

The progression of BC is primarily evaluated using two main histopathological systems: the Nottingham histological grading and the Tumour–Node–Metastasis (TNM) staging systems [6]. The grading system is crucial in clinical prognostication; the standard method for assessing progression of BC, among other factors, involves the visual examination of tissue sections with staining of hematoxylin and eosin (H&E stains). This examination can be conducted using either a microscope or digital whole-slide imaging [7]. It can be used to assess the appearance of aberrant BC cells. Grade I is well-differentiated in this system, while grade III is poorly differentiated [7]. The second system, TNM, is used to predict patient outcomes and guide treatment decisions both before and after surgery. It provides a comprehensive and systematic assessment of cancer development and is classified into four stages. However, it does not take the patient's anti-tumour immune response into account; this limitation can be addressed by integrating an objective, automated immune-based prognostic scoring system with conventional cancer staging methods [8].

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However, these two systems can not accurately distinguish between aggressive and non-aggressive cancers or predict treatment outcomes, such as sustained remission or return, following first treatment [6].

Tumour-infiltrating lymphocytes (TILs) are widely recognised as significant indicators of both prognosis and therapeutic response, especially in triple-negative breast cancer (TNBC) and human epidermal growth factor receptor 2-positive (HER2+) [9]. One of these TIL proteins is CD3, which is necessary for antigen detection, T-cell activation, and the subsequent production of an antigen-specific immunological response. The occurrence of TILs is linked with a more favourable prognosis in BC, further underscoring the significance of CD3 as a biomarker [10]. CD3 may have a greater influence on BC diagnosis and treatment [11]. Another protein is CD8, which predominantly serves as a co-stimulator and, occasionally, as a co-repressor, expressed on the surface of cytotoxic and regulatory T lymphocytes to identify pathogens, cancer, and autoimmunity, facilitating elimination and destruction through antigen recognition in innate and adaptive immune responses [12]. This information provides a compelling rationale for the semi-quantitative assessment of CD3 and CD8 expression levels in breast tissues, as well as for the systematic investigation of their associations with BC histopathological parameters using immunohistochemistry (IHC).

MATERIALS AND METHODS

Human Samples and Ethical Declaration

The study's ethical approval was formally approved by the Ethics Committee of Al-Nasiriyah Teaching Hospital (Approval No. Thi-Qar 243/2025, issued on November 17,11, 2025). The study cohort comprised 140 formalin-fixed, paraffin-embedded (FFPE) breast tissues retrospectively retrieved from the archives of the Histopathology Department, including 40 benign and 100 malignant cases. The histopathology report provided the histological information, including age, grade, stage and molecular subtype of BC. All samples were obtained after receiving ethical approval in compliance with established protocols and institutional requirements. In this investigation,

Immunohistochemistry

Immunohistochemistry (IHC) staining was performed on both benign and malignant breast tissues using a mouse monoclonal anti-CD3 antibody ready to use (Lica, cat no: PA055) and a mouse monoclonal anti-CD8 antibody (ready to use, Lica, cat no: PA0183). This study used the Novolink polymer detection system (RE7140K), with all procedures conducted in accordance with the protocol described by Alghezi *et al*. [13].

Deparaffinization of breast tissue section by using xylenewas first done, and then they were rehydrated by using a frequent series of alcohol ethanol concentrations (100%, 95%, and 70%). They were then rinsed in distilled water for 5 minutes, after which a further wash was performed using phosphate-buffered saline (PBS) for an additional 5 minutes.

An automatic retrieval system (PT Link, Dako) was used to retrieve the antigens. The samples were exposed to heat-induced treatment, 95°C for 20 minutes, in citrate buffer (pH 6), then they were slowly cooled to 60°C, followed by rinsing in PBS for 5 minutes. Ten minutes of incubation with hydrogen peroxide (H_2O_2) was used to stop the activity of endogenous peroxidase. These slides were then rinsed with PBS for five minutes. To reduce nonspecific binding, proteinase K blocking was also used for five minutes.

The primary antibodies, anti-CD3 and anti-CD8, were then applied to the treated tissues, and the slides were incubated overnight at 4°C. The following day, these incubated slides were then submitted to protocol washing using PBS for 30 minutes, and the secondary antibodies were then added for 30 minutes. The sections were subsequently washed for ten minutes in PBS. This study used 3,3'-diaminobenzidine tetrahydrochloride (DAB) for chromogenic detection, which made it possible to see the interaction between the antigen and the antibody. After that, the sections were stained with hematoxylin (Vector Laboratories, UK), mounted with DPX mounting solution (Sigma-Aldrich, UK), and looked at under a light microscope.

IHC Analysis

Tissue sections were manually evaluated by a histopathologist to assess CD3 and CD8 immunostaining in breast tissue samples. Five fields of view were selected to examine stromal and intra tumoural immunostaining, and a semi-quantitative scoring system was applied under a microscope objective for analysis. Both proportion and intensity scores were assessed for CD3 and CD8 expression. The proportion of positively stained cells was

categorised as follows: 0 (<10%), 1 (11–25%), 2 (26–50%), and 3 (>51%). Staining intensity was graded as negative (0), mild (+1), moderate (+2), or strong (+3). The combined intensity and proportion scores range from 0 to 7, yielding a definitive score for each case [14].

Statistical Analysis

Descriptive statistics, including the mean, standard error, and standard deviation, were computed using GraphPad Prism version 8.00 (GraphPad Software, La Jolla, California, USA). Statistical analysis was calculated using the unpaired t-test, one-way ANOVA, followed by Tukey's multiple comparisons test and Chi-square ($\chi 2$). A P-value below 0.05 was considered statistically significant.

RESULT

This study analysed a cohort of 140 participants, significantly distributed into 100 malignant cases (71.4%) and 40 benign cases (22.6%) ($P < 0.001$). The age distribution showed that all benign cases were less than 40 years old, whereas the malignant cases, 70% were aged 41–65 years, 14% were younger than 40 years, and only 8% were older than 65 years. Statistically, there was a significant difference between age and sample types ($P < 0.001$). Regarding histological grading, the cohort had aggressive tumour grades, with 70% of malignant cases as grade 3 (G3) compared to grade 2 (G2) (28%) and grade 1 (G1) (2%) ($P < 0.001$). The tumour stage also showed that T2 was the most prevalent metastatic stage (40%). This study revealed that 48% of cases were classified as N0, while the remaining 52% were distributed

Table 1: The Benign and Malignant Histopathological Data of the Breast

Histopathological features		Number (n)	Percentage%	*P value*
Sample types	Benign	40	28.6	<0.001
	Malignant	100	71.4	
Benign (Age range)	<40	40	100	<0.001
	41-65	0	0	
	<65	0	0	
Malignant (Age range)	<40	14	14	<0.001
	41-65	78	78	
	<65	8	8	
BC grade	G1	2	2	<0.001
	G2	28	28	
	G3	70	70	
Stage T	T1	34	34	<0.001
	T2	40	40	
	T3	18	18	
	T4	8	8	
Stage N	N0	48	48	<0.001
	N1	18	18	
	N2	26	26	
	N3	8	8	
Stage M	M0	0	0	N/A
	M1	0	0	
	MX	100	100	
Molecular subtypes	Luminal A	38	38	0.002
	Luminal B	22	22	
	TNBC	30	30	
	HER2 +	10	10	

across N1, N2, and N3 categories, with significant differences detected between the studied groups (*P* <0.001). Molecular subtype shows significant diversity (*P*=0.0002) with the Luminal A subtype being the most common (38%), triple negative (30%), Luminal B (22%), and HER2 (10%). This parameter provides a baseline for studying the immuno-microenvironment of breast cancer. The histopathological data are presented in Table **1**.

CD3 and CD8 Expression in Breast Tissue Samples

Immunohistochemistry was performed on benign and malignant breast tissues to evaluate the expression levels of CD3 and CD8 proteins. The results established that these potential biomarkers were expressed in both glandular and stromal regions, with expression levels ranging from weak to strong.

The CD3 protein was expressed in the stromal area in some cases of benign tissues (Figure **1A**, arrow). However, the majority of cases had no CD3 expression (Figure **1B**, arrow), In contrast, different levels of CD3 expression ranging as, strong (Figure **1C**, arrow) and strong stromal (Figure **1D**, arrow) to moderate (Figre **1E**, arrow) were found in both intra tumoural and stromal areas of BC tissues.Breast cancer tissues showed no CD3 staining (Negative control), (Figure **1F**, arrow).

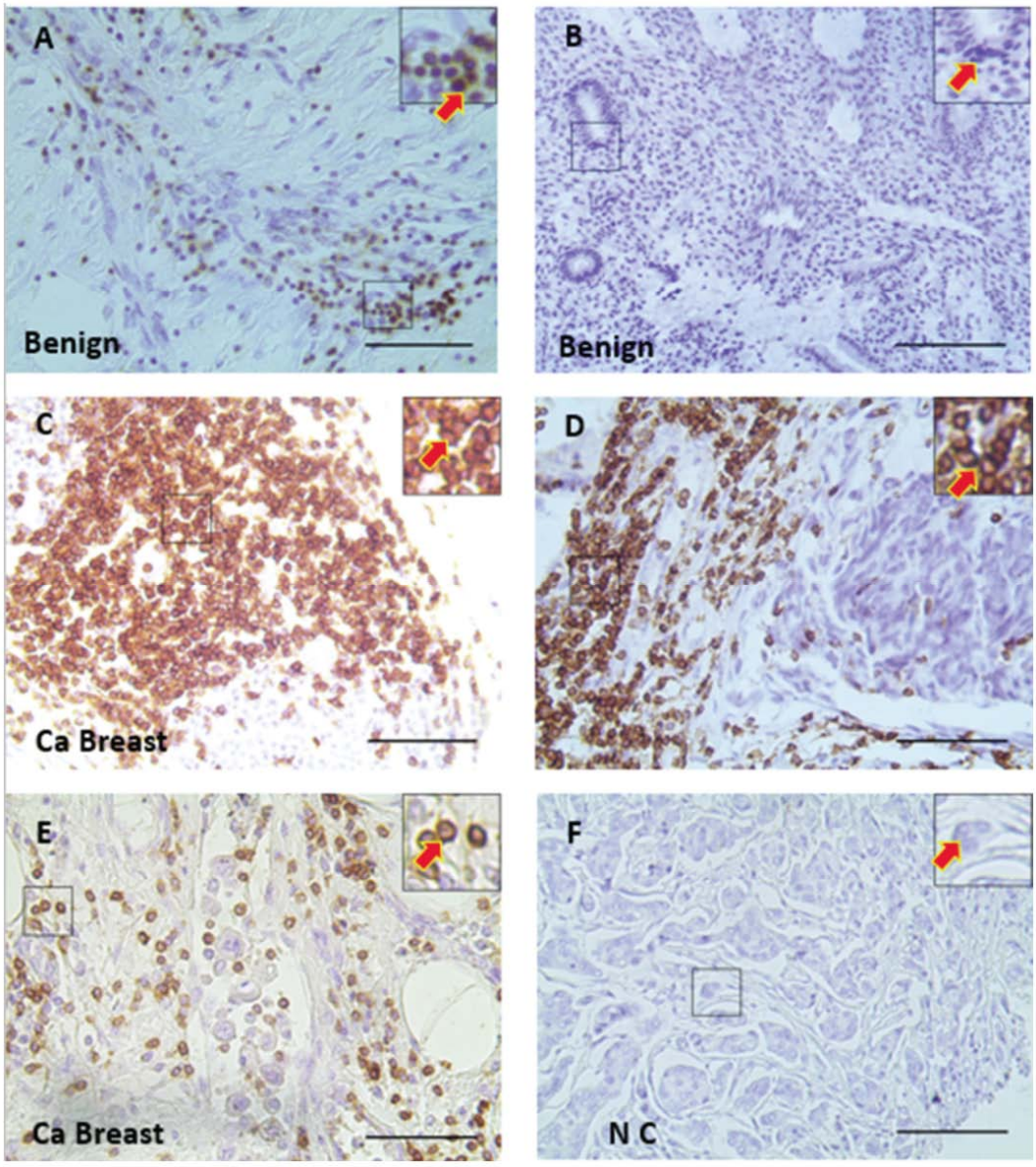

Figure 1: Shows IHC staining of CD3 in benign and malignant breast tissue. (**A**) CD3 expression was seen in benign tissue. (**B**) No CD3 expression was seen in benign tissue. (**C**) Strong CD3 staining was in the malignant tissue. (**D**) Strong CD3stromal expression was in malignant tissue. (**E**) Moderate expression of CD3 was in malignant tissue. (**F**) Breast cancer tissues showed no CD3 staining (Negative control).

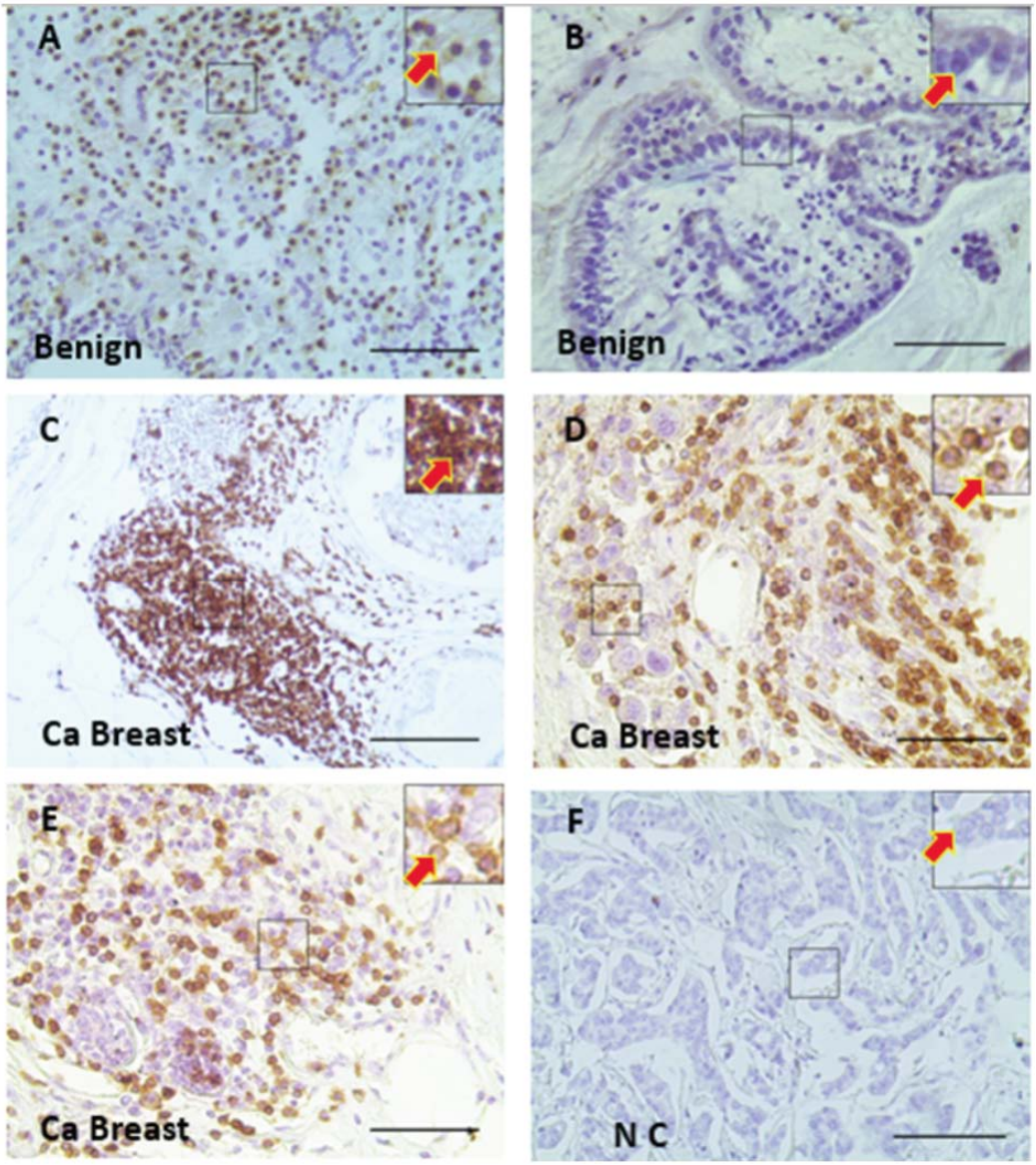

Figure 2: Shows IHC staining of CD8 in benign and malignant breast tissue. (**A**) CD8 staining was seen in benign breast tissue. (**B**) No CD8 expression was in benign tissue. (**C**) Strong stromal CD8 expression was in malignant tissue. (**D**) Strong intratumoural CD8 expression was in malignant tissue. (**E**) Weak expression of CD8 was in malignant tissue. (**F**) Breast cancer tissues showed no CD8 staining (Negative control).

CD8 was also found to be expressed in four benign breast tissues (Figure **2A**, arrow), while the majority of cases had free CD8 staining (Figure **2B**, arrow), In contrast, both intra tumoural and stromal regions of BC had CD8 immunostaining range from strong stromal tissue (Figure **2C**, arrow), and strong intra tumoural (Figure **2D**, arrow) to Weak (Figure **2E**, arrow). Breast cancer tissues showed no CD8 staining (Negative control) (Figure **2F**, arrow).

CD3 and CD8 Expression Associated with BC Clinical Data

This study demonstrated that intra-tumoural and stromal CD3 expression levels were significantly increased in BC compared to benign tissues ($P < 0.0001$) (Table **2** and Figure **3A** and **F**). In addition, CD3 intra-tumoural and stromal staining was negatively linked to BC grade, as determined by ANOVA ($P = 0.048$ and $P = 0.005$, respectively) (Table **2** and Figure **3B** and **G**). Post hoc analysis using the Tukey test showed that stromal CD3 expression was associated with tumour grade when comparing patients with G1 to those with G3 ($P = 0.017$), while no significant differences were observed between G1 and G2 ($P = 0.096$) or between G2 and G3 ($P = 0.096$). Furthermore, CD3 expression in both intertumoural and stromal regions was significantly associated with increased tumour size (T4) ($P = 0.028$ and $P < 0.0001$,

Table 2: Intra-Tumoural and Stromal CD3 Expression in Breast

Comparison	CD3 intra-tumoural immunostaining				CD3 stromal immunostaining			
	Mean ±SD	Findings		P value	Mean ±SD	Findings		*P* value
Benign (40)	0.526±0.506	Increase in malignant		<0.0001	0.850±0.962	Increase in malignant		<0.0001
Cancer (100)	3.50± 1.78				4.07± 1.76			
Grade		Increase in high-grade	Anova	0.048	Grade	Increase in high-grade	Anova	0.005
G1:(2)	1.00±0.00		G1 vs G2	0.237	1.00±0.00		G1 vs G2	0.096
G2:(28)	3.05±1.61		G1 vs G3	0.088	3.57±1.60		G1 vs G3	0.017
G3:(70)	3.63±1.77		G3 vs G2	0.297	4.36±1.72		G3 vs G2	0.096
Stage T		Increase in T4	ANOVA	0.028	Stage T	Increase in T4	ANOVA	<0.0001
T1 (34)	2.91±1.73		T1 vs T2	0.462	3.12±1.98		T1 vs T2	0.018
T2 (40)	3.50±1.75		T1 vs T3	0.021	4.23±1.20		T1 vs T3	0.002
T3 (18)	4.39±1.47		T1 vs T4	0.378	4.78±1.65		T1 vs T4	0.001
T4 (6)	4.00±2.00		T2 vs T3	0.270	5.75±1.16		T2 vs T3	0.611
			T2 vs T4	0.876			T2 vs T4	0.069
			T3 vsT4	0.951			T3 vsT4	0.476
Stage N		Increase in N3	Anova	0.034	Stage N	Increase in N3	Anova	<0.0001
N0 (47)	3.08±1.74		N0 vs. N1	0.380	3.33±1.80		N0 vs. N1	0.0178
N1(17)	3.83±1.85		N0 vs. N2	0.254	4.65±1.58		N0 vs. N2	0.0011
N2 (27)	3.85±1.67		N0 vs. N3	0.054	4.78±1.00		N0 vs. N3	0.0005
N3 (8)	4.75±0.845		N1 vs. N2	0.998	5.75±1.39		N1 vs. N2	0.9929
			N1 vs. N3	0.579			N1 vs. N3	0.3518
			N2 vs. N3	0.551			N2 vs. N3	0.4084
BC subtype		Increase In HER2+	Anova	0.004	BC subtype	Increased In HER2+	Anova	0.001
Luminal A	2.91±1.64		Luminal A vs. luminal B	0.861	3.24±1.91		Luminal A vs. luminal B	0.174
Luminal B	3.25±1.45		Luminal A vs. HER2 positive	0.008	4.14±1.24		Luminal A vs. HER2 positive	0.041
HER2+	4.80±0.974		Luminal A vs. TNBC	0.352	4.80±1.58		Luminal A vs. TNBC	0.001
Triple negative	3.57± 1.88		luminal B vs. HER2 positive	0.067	4.83±1.50		luminal B vs. HER2 positive	0.711
			luminal B vs. TNBC	0.899			luminal B vs. TNBC	0.428
			HER2 positive vs. TNBC	0.169			HER2 positive vs. TNBC	0.991

respectively) (Table **2** and Figure **3C** and **H**), as well as with lymph node involvement (P = 0.034 and P < 0.0001, respectively) (Table **2** and Figure **3 D** and **I**). Additionally, a significant link was noticed between intertumoural and stromal CD3 expression and BC molecular subtypes (P =0.008 and P= 0.001). (Table **2** and Figure **3E** and **H**).

The study also established that the intertumoural and stromal CD8 expression levels were also significantly increased in BC compared to benign breast tissues (P < 0.0001) (Table **3** and Figure **4A** and **F**). In addition, the stromal CD8 expressions were only significantly associated with increased BC grade (P = 0.036) (Table **3** and Figure **4G**), whereas the intertumoural CD8 expression showed no significant

Table 3: Intra-Tumoural and Stromal CD8 Expression in Breast Tissues

Comparison	CD8 intra-tumour immunostaining				CD8 Stromal immunostaining			
	Mean ±SD	Findings		*P* value	Mean ±SD	Findings		*P* value
Benign (40)	0.525±1.01	Increase in malignant		<0.0001	0.625±1.05	Increase in malignant		<0.0001
Cancer (100)	2.11± 1.76				1.85± 1.42			
Grade		No significant difference	Anova	0.233	Grade	Increase in high-grade	Anova	0.036
G1:2	0.00±0.00		G1 vs G2	0.211	0.00±0.00		G1 vs G2	0.298
G2:28	2.18±2.01		G1 vs G3	0.210	1.50± 1.17		G1 vs G3	0.099
G3:70	2.14±1.66		G3 vs G2	0.993	2.04±1.45		G3 vs G2	0.184
Stage T		Increased inT4	ANOVA	0.008	Stage T	Increase inT4	ANOVA	0.0001
T1 (34)	1.85±2.02		T1 vs T2	0.999	1.20±1.15		T1 vs T2	0.095
T2 (40)	1.82±1.60		T1 vs T3	0.053	1.92±1.47		T1 vs T3	<0.0001
T3 (18)	3.17±1.48		T1 vs T4	0.124	3.00±1.28		T1 vs T4	0.018
T4 (6)	3.38±1.69		T2 vs T3	0.037	1.75± 1.75		T2 vs T3	0.026
			T2 vs T4	0.102			T2 vs T4	0.378
			T3 vsT4	0.992			T3 vsT4	0.971
Stage N		Increase in N3	Anova	0.049	Stage N	Increase in N3	Anova	0.026
N0 (47)	1.77±1.74		N0 vs. N1	0.258	1.60±1.39		N0 vs. N1	0.376
N1(17)	2.64±1.92		N0 vs. N2	0.793	2.22±1.42		N0 vs. N2	0.961
N2 (27)	2.16±1.77		N0 vs. N3	0.064	1.77±1.45		N0 vs. N3	0.025
N3 (8)	3.33±0.433		N1 vs. N2	0.797	3.13±0.991		N1 vs. N2	0.711
			N1 vs. N3	0.758			N1 vs. N3	0.422
			N2 vs. N3	0.298			N2 vs. N3	0.081
Breast cancer subtype		Increase In HER2+	Anova	<0.0001	BC subtype	Increase In HER2+	Anova	<0.0001
Luminal A	1.16±1.16		Luminal A vs. luminal B	0.051	1.05±1.01		Luminal A vs. luminal B	0.012
Luminal B	2.14±1.64		Luminal A vs. HER2 positive	<0.0001	2.09±1.40		Luminal A vs. HER2 positive	0.005
HER2+	4.20±1.18		Luminal A vs. TNBC	0.004	3.30±0.789		Luminal A vs. TNBC ve	<0.0001
Triple negative	2.35±1.39		luminal B vs. HER2 positive	0.001	2.21±1.23		luminal B vs. HER2 positive	0.002
			luminal B vs. TNBC	0.945			luminal B vs. TNBC ve	0.034
			HER2 positive vs. TNBC	0.003			HER2 positive vs. TNBC	0.998

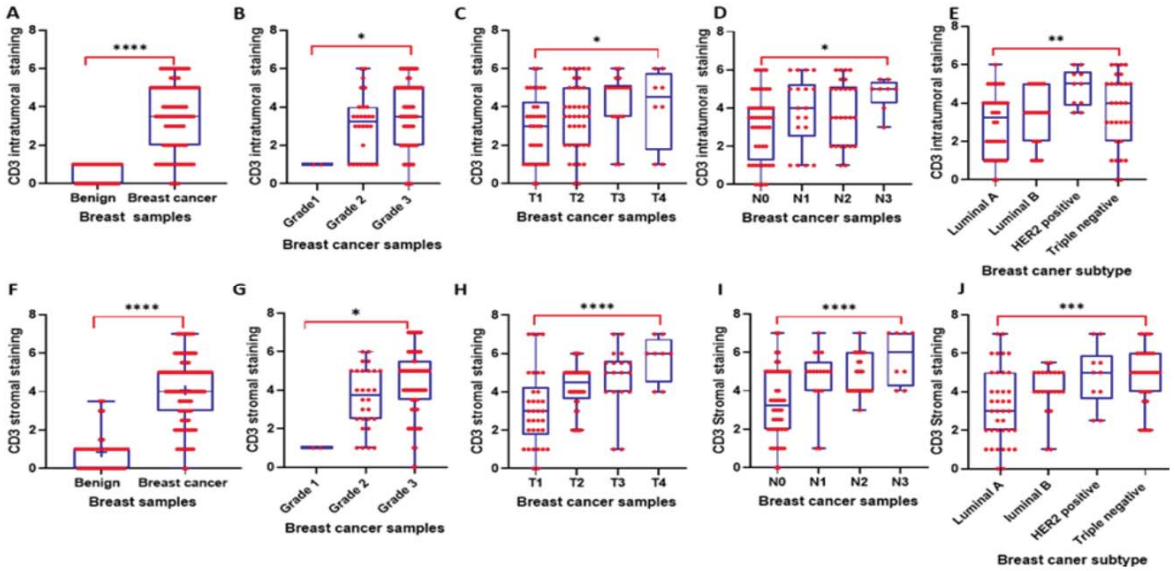

Figure 3: CD3 immunostaining semi-quantification in breast tissue samples using IHC. (**A**) Increased intra-tumoural CD3 staining was in BC (*P* <0.0001). (**B**) High intra-tumoural CD3 was found in high-grade (*P* = 0.048). (**C**) Elevated CD3 intra-tumoural expression was seen in larger tumour size (*P* = 0.028). (**D**) CD3 intra-tumoural immunostaining was increased significantly in N3 (*P* = 0.034). (**E**) There was a significant association between CD3 intra-tumoural immunostaining and BC molecular subtype (*P* = 0.004). (**F**) Increased stromal CD3 in BC (*P*<0.0001). (**G**) Increased stromal CD3 was in high grade (*P*=0.005). (**H**) CD3 stromal expression was higher in a larger tumour size (P <0.0001). (**I**) CD3 stromal staining was linked to lymph node involvement (P <0.0001). (**J**) A significant link was seen between CD3 stromal immunostaining and BC molecular subtype (P = 0.001). Unpaired t-tests or one-way ANOVA tests were used. Benign breast (n=40), BC (n=100), G1 (n=2), G2: (n=28), G3 (n=70), T1 (n= 34), T2 (n=40), T3 (n= 18), T4 (n= 8), N0 (n=48), N1 (n=18), N2 (n=26), N3 (n=8), Luminal A (n=38), Luminal B (n=22), Triple negative (n=30), HER2 + (n=10).

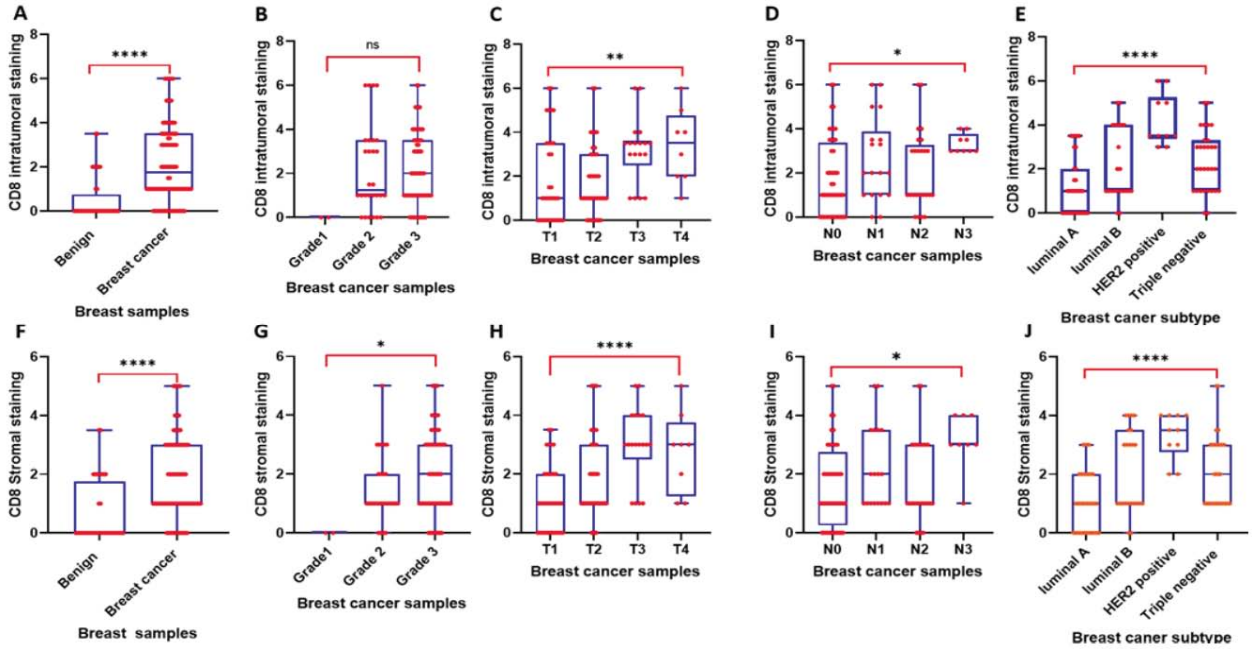

Figure 4: CD8 immunostaining semi-quantification in breast tissue samples using IHC. (**A**) Increased intra-tumoural CD8 was in BC (*P* <0.0001). (**B**) No significant link was seen between intra-tumoural CD8 and BC grade (*P* = 0.233). (**C**) CD8 intra-tumoural expression was significantly associated with larger tumour size (*P* = 0.008). (**D**) CD8 intra-tumoural expression was higher in N3 (*P* = 0.049). (**E**) A significant association was found between CD8 intra-tumoural expression and BC molecular subtype (*P*<0.0001). (**F**) Increased stromal CD8 was in BC (*P*<0.0001). (**G**) Increased stromal CD8 was in high grade (*P*=0.036). (**H**) Increased CD8 stromal immunostaining was found in large tumour size (*P*< 0.0001). (**I**) CD8 stromal immunostaining was linked to lymph node metastasis (*P* = 0.026). (**J**) A significant correlation was demonstrated between CD8 stromal immunostaining and BC molecular subtype (*P*< 0.0001). Unpaired t-tests or one-way ANOVA tests were used. Benign breast (n=40), BC (n=100), G1 (n=2), G2: (n=28), G3 (n=70), T1 (n= 34), T2 (n=40), T3 (n= 18), T4 (n= 8), N0 (n=48), N1 (n=18), N2 (n=26), N3 (n=8), Luminal A (n=38), Luminal B (n=22), Triple negative (n=30), HER2 + (n=10).

correlation with tumour grade of breast using an ANOVA test (Table **3** and Figure **4B**). Furthermore, CD8 expression in both intra-tumoural and stromal regions was significantly associated with increased tumour size (T4) (P = 0.008 and P= 0.0001, respectively) (Table **3** and Figure **4C** and **H**), as well as with lymph node involvement (P = 0.049 and P = 0.026, respectively) (Table **3** and Figure **4D** and **I**). Additionally, a statistical relevance was observed between intra-tumoural and stromal CD8 expression and BC molecular subtypes (P < 0.0001) (Table **3** and Figure **4E** and **J**).

DISCUSSION

An IHC study was done to examine CD3 and CD8 expression levels in malignant and non-malignant breast tissues. These potential biomarkers were detected in intratumoural and stromal compartments of breast tissues, with significantly higher expression observed in malignant tissues compared to benign tissues. This data agrees with the previous finding [15]. However, this result disagrees with another published paper, which reported increased CD3 and CD8 expression levels in benign tissue compared to malignant breast tissues [16]. This may be related to a certain inflammatory condition, mastitis (immune system related to bacterial infection [17]. Another explanation for these differences may be due to the use of different diagnostic methods, different antibodies or different antigen retrievals. Taken together, the existence of strong immune activity within the tumour microenvironment, evidenced by the accumulation and engagement of immune cells, suggests an active attempt by the host immune system to suppress tumour progression.

These findings indicate an inverse relationship between CD3 and CD8 expression and increasing BC grade. However, intra-tumoural CD8 staining did not demonstrate a significant association with tumour grade. This result agrees with the previous findings [18], suggesting these proteins may have a role in tumour differentiation. In contrast, these findings disagree with a previous report [19]. These differences may be attributed to variations in antigen retrieval methods and/or the antibodies used Decreasing CD3 and CD8 levels in poorly differentiated grades may explain their role in tumour progression and differentiation.

Increased CD3 and CD8 staining were also found to be associated with a larger tumour size, suggesting that they might contribute to cancer progression and aggressiveness. This data agrees with previous data [20]. However, this finding disagrees with another study [21], suggesting that when the tumour becomes larger, they become hidden from immune response or creates a physical barrio that prevents infiltration [22]. This may relate to the idea of immune ignorance, where the immune system stops recognising the tumour once it reaches a certain stage of maturity. This association between tumour size and elevated TIL substantiates the hypothesis that a larger tumour mass engenders an inflammatory milieu conducive to cell recruitment. This process entails the aggregation of immune cells, which may prove inadequate to inhibit tumour proliferation in mature stages [22].

Our data demonstrated a significant link between CD3 and CD8 expression and lymph node metastasis. This link suggests that cancer spread to the lymphatic system is associated with a robust immune response at both local and systemic levels, rendering these indicators symptomatic of the tumour's aggressive nature and elevated metastatic behavior this result agree with [23] that noticed increased CD3 stromal expression with high stage of lymph node involvement however its disagree with other studies that noticed by [24] that proposed significant decline in CD3. CD8 expression among patient with extensive lymph node involvement this may result from immune evasion depend on the idea that the tumour that can metastasis have the ability to hide its signal which lead to decline in Til number that lead to safe passage to lymph node in addition to immune exclusions which mean advanced tumour construct physical barrier of dense of stroma (desmoplasia that sequestrated T cell within stromal region preventing their infiltration into the tumour core reporting low CD8 expression anta tumour lead to tumour progression while this phenomenon disagree from our primary quantitively finding its align with specific microscopic observation made during the scoring process. Taken together, these data suggested that both proteins may have a role in tumour formation, differentiation and progression.

Finally, these potential biomarkers were significantly associated with BC molecular subtypes. Triple- TNBC and HER2+ exhibited the highest levels of both CD3 and CD8, whereas Luminal A showed the lowest levels of these proteins. These findings align with current knowledge of cancer biology, which categorises HER2+ and TNBC tumours as immunologically "hot," thereby enhancing their responsiveness to modern immunotherapies in contrast to hormone-positive

tumours. Tumours in the Luminal A group are "cold" to the immune system. However, this data agrees with [25] that noticed an increase in TIL in (TNBC, HER2) molecular subtype. In contrast, our data disagrees with [22].

This discrepancy may be attributed to the limited sample size and the subjective nature of the manual scoring approach. Overall, both CD3 and CD8 may have a significant role in the molecular characterisation of BC.

One important limitation of this study is the lack of follow-up and survival data, which makes it difficult to determine whether CD3 and CD8 can serve as independent prognostic markers, despite their clear association with tumour features. In addition, the use of IHC may introduce variability due to differences in tissue processing, antigen retrieval, staining techniques, and interpretation of results. Such variability can influence the consistency and reproducibility of CD3 and CD8 expression across different samples and studies [13]. Another limitation is the age difference observed between the benign and malignant groups, which may represent a potential confounding factor. This variation reflects the actual distribution of cases encountered during the sample collection period, where benign breast lesions were more frequently diagnosed in younger patients. The selection of cases was based on sample availability and predefined inclusion criteria rather than intentional age matching, as selective recruitment of older benign cases could have introduced sampling bias and affected the statistical validity of the study. To minimize the potential impact of this confounding factor, multivariate analysis was performed with adjustment for age, tumour grade, and tumour stage. Nevertheless, this limitation should be considered when interpreting the findings of the study. In addition, Multivariate analysis was not performed because the small sizes of certain subgroups, particularly G1 (n=2) and T4 (n=8), may yield statistically unreliable estimates. Therefore, larger studies are required to determine whether CD3 and CD8 expression are independently associated with clinicopathological parameters after adjustment for potential confounding factors.

CONCLUSION

This study demonstrates a highly significant correlation ($p < 0.0001$) between the expression of TILs, specifically CD3 and CD8, and the clinicopathological features of BC in a cohort of 140 samples. A marked increase in CD3 and CD8 expression was detected in BC compared to benign tissues, reflecting an active host immune response to tumour development. Furthermore, CD3 and CD8 expression levels were significantly associated with molecular subtypes, with higher immune cell densities observed in high-grade tumours, particularly in HER2-positive and TNBC. These findings suggest that CD3 and CD8 may contribute to the immunological characterisation of BC and could serve as useful immune-associated markers in future studies. However, additional longitudinal investigations with survival analysis and larger cohorts are required to determine their independent prognostic significance

AUTHORS CONTRIBUTIONS

Shahad and Dhafer hypothesised the study, executed the experimental protocols, authored the preliminary report, and conducted the statistical analysis. Dhafer and Rasha conducted the data collection. Shahad, Dhafer, and Rasha contributed to the drafting, critical review, and revision of the manuscript. All authors have read and approved the final version.

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CONFLICTS OF INTEREST

The Author(s) declare(s) that there are no relevant financial or non-financial competing interests to report

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