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Prognostic Significance of CD68 and CD163 Tumor-Associated Macrophages in Hormone Receptor-Positive Vs Triple-Negative Invasive Breast Carcinoma: A Retrospective IHC Study

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ABSTRACT

Background: Tumor-associated macrophages (TAMs) influence tumor progression within the microenvironment. M1-like macrophages ($CD68^+$) exhibit tumoricidal properties, whereas M2-like macrophages ($CD68^+/CD163^+$) promote tumor growth through hypoxia-driven angiogenesis. This study assessed the localization and density of $CD68^+$ and $CD163^+$ TAMs in hormone receptor-positive and triple-negative invasive breast carcinomas and evaluated their association with clinicopathological parameters.

Methods: A retrospective cross-sectional study was conducted on histopathologically confirmed invasive breast carcinoma cases. Formalin-fixed paraffin-embedded tissues underwent immunohistochemical staining for ER, PR, Her2/neu, CD68, and CD163. Descriptive statistics were calculated, and associations with clinicopathological variables were analyzed using the χ^2 test. Survival outcomes were assessed by Kaplan-Meier and log-rank tests, and prognostic significance was evaluated using univariate Cox regression analysis.

Results: The study showed association of $CD68^+$ and $CD163^+$ TAMs in ER, PR positive and Her2/neu negative cases in TS (tumor stroma) and TN (tumor nest) with higher histologic grades. High infiltration of both $CD68^+$ and $CD163^+$ TAMs were seen in triple negative invasive breast carcinomas (TNBC). There was significant correlation of increased $CD163^+$ TAMs in TN and TS with relapse free survival and overall survival rates based on log rank tests.

Conclusion: Breast cancer remains the leading malignancy contributing to cancer-related mortality among women globally. Conventional treatment strategies have primarily focused on directly targeting tumor cells. However, increasing evidence highlights the importance of addressing the tumor microenvironment as a therapeutic approach to overcome treatment resistance and enhance clinical outcomes.

Keywords: Breast carcinoma, Densities, Invasive, Triple negative, Tumor Associated Macrophages

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INTRODUCTION

Breast cancer is the most common cancer among women worldwide and remains a major public health concern. According to the World Health Organization (WHO), approximately 2.3 million new cases of breast cancer were diagnosed globally in 2022, making it the most common cancer among women in 157 of 185 countries.[1] Tumor-associated macrophages (TAMs) play a pivotal role in regulating tumor cells and the tumor microenvironment. The microenvironment of tumor is a complex ecosystem surrounding tumor cells and include fibroblasts, adipocytes, vascular tissues and immune cells.[2] The immune microenvironment in breast carcinomas consist of tumor associated macrophages predominantly as much as in 50% of the cells.[3] TAMs have recently been identified as significant contributors to tumor development and progression. Based on activation mode, they are of two types: TAMs are broadly classified into classically activated (M1-like) and alternatively activated (M2-like) phenotypes. M1-like TAMs are identified by CD68 expression, secrete pro-inflammatory cytokines, and stimulate a Th1-mediated immune response, thereby exerting cytotoxic effects on tumor cells.[4] In contrast, M2-like macrophages, characterized by co-expression of CD68 and CD163, facilitate tumor progression through hypoxia-driven angiogenesis and subsequent enhancement of tumor cell proliferation. TAMs may show different prognostic indicators based on different regions and at different densities.[5] The objective of this study was to evaluate the distribution and density of $CD68^+$ and $CD163^+$ TAM and compare the expression of densities of CD68 and CD163 with clinicopathological variables in both ER, PR positive, Her2/neu negative cases and triple negative (ER, PR, Her2/neu negative) invasive breast carcinoma cases.

Although numerous studies have suggested that increased TAM density particularly M2-like macrophage is associated with poor prognosis, lymph node metastasis, higher tumor grade, and reduced survival, the prognostic significance of TAMs remains controversial. Several studies have demonstrated that high $CD68^+$ macrophage infiltration correlates with adverse outcomes and decreased survival in breast carcinoma.[6-9] Conversely, other investigators have reported no significant association between $CD68^+$ macrophage density and survival, and in certain molecular subtypes, macrophage infiltration has even been linked to improved prognosis.[10-12] Furthermore, the prognostic impact appears to vary depending on macrophage localization (intratumoral versus stromal), tumor molecular subtype, and the markers used to identify macrophage subsets.[13,14] Differences in methodology, scoring systems, and cut-off values, along with the functional plasticity of macrophages that challenges the traditional M1/M2 dichotomy, have contributed to conflicting results in the literature.[15,16]

MATERIALS AND METHODS

A retrospective cross-sectional study was carried out in a tertiary care center for a period of 5 years. Complete history, clinical details like age, size of the tumour and preoperative investigation findings like fine needle aspiration cytology (FNAC) and ultrasonography (USG breast) wherever available was retrieved from past records from 2019 onwards. Total 72 cases of confirmed invasive breast carcinomas were retrieved from the record along with their follow up. Paraffin embedded blocks of histo-pathologically confirmed invasive breast carcinomas were collected. Immunohistochemistry (IHC) for ER, PR and Her2/neu was done on representative paraffin embedded blocks along with CD68 and CD163. The primary antibodies used were: ER (clone SP1, rabbit monoclonal, Quartett, ready-to-use), PR (clone 1E2, rabbit monoclonal, Quartett, ready-to-use), Her2/neu (clone 4B5, rabbit monoclonal, Quartett ready-to-use), CD68 (clone KP1, mouse monoclonal, Quartett, dilution 1:100), and CD163 (clone 10D6, mouse monoclonal, BioGenex, dilution 1:100). Antigen retrieval was performed using heat-induced epitope retrieval (HIER) with citrate buffer (pH 6.0) for ER, PR, CD68, and CD163, and EDTA buffer (pH 9.0) for Her2/neu, according to manufacturer recommendations. ER, PR showing nuclear staining was considered to be positive when the breast tumor contained at least 1% of the positive cells. Her2/neu was scored using guidelines defined by American college of pathologists. Appropriate internal and external controls were included for each IHC run. Normal breast ductal epithelium served as an internal positive control for ER and PR. Known Her2/neu-positive breast carcinoma tissue was used as an external control. Reactive tonsillar tissue and macrophage-rich areas were used as positive controls for CD68 and CD163. Negative controls were obtained by omitting the primary antibody.

Inclusion criteria comprised histopathologically confirmed cases of primary invasive breast carcinoma (Stage I-III) with no evidence of distant metastasis at diagnosis, availability of adequate formalin-fixed paraffin-embedded tissue for immunohistochemical analysis, and complete clinicopathological and follow-up data. Patients who had received neoadjuvant chemotherapy, radiotherapy, hormonal therapy, or targeted therapy prior to surgical excision were excluded to avoid treatment-related alteration in TAM density. Exclusion criteria also included male breast carcinoma, carcinoma in situ lesions, Stage IV disease at presentation, recurrent tumors at the time of diagnosis, and cases with incomplete clinical or follow-up records. All histopathologically confirmed cases of invasive breast carcinoma that met the pre-defined inclusion and exclusion criteria and had adequate formalin fixed paraffin embedded tissue available for immunohistochemical evaluation were included using convenience sampling method. A formal a priori sample size calculation was not done as the study was exploratory in nature and based on the availability of archived cases during the study period. Total 72 cases were collected which were further stratified into two biologically distinct cohorts based on ER, PR and Her2 status of their biopsy samples. ER, PR positive with Her2/neu negative cases were labelled as Cohort A. ER, PR, Her2/neu negative

cases were labelled as Cohort B. Ethical approval (Approved No: 1592, Dated 23/11/2023) was obtained from the Institutional ethics committee of our institution. Patients were followed up for a minimum duration of 6 months and a maximum of 60 months, with follow-up data obtained from medical records and telephonic communication where necessary. Recurrence-free survival (RFS) was defined as the time from initial surgery to documented local or distant recurrence, and overall survival (OS) was defined as the time from diagnosis to death due to breast carcinoma or last follow-up. TAMs in the tumor nest (TN) refer to intraepithelial macrophages located within tumor cell clusters, whereas tumor stroma (TS) refers to macrophages present in the surrounding desmoplastic stromal tissue. TAMs were quantified using the hot-spot method. TAMs have been calculated by hot-spot quantitative method. Each slide was initially scanned at low magnification (100×) to identify areas with the highest density of positively stained macrophages. Five non-overlapping hotspot fields were selected in both TN and TS separately. Counting was performed at high power (400×) using a microscope with a field diameter of 0.5 mm, corresponding to a field area of approximately 0.196 mm^2. Only cytoplasmic staining was considered positive for CD68 and CD163, as both are cytoplasmic macrophage markers. The mean of positively stained macrophages across the five fields was calculated for each case. All slides were independently evaluated by two experienced pathologists who were blinded to clinicopathological data and patient outcomes. In cases of discrepancy, a consensus was reached by joint review.

Statistical analysis: For statistical analyses, the mean, median, standard error, range of TAMs of both ER, PR positive and triple negative carcinomas (TNC) were calculated. Then number of tumor positive macrophage count was divided into lower and higher groups based on cut-off points according to the median for both cohorts. $\chi 2$ test was done to compare CD68 and CD163 expression with clinico-pathological variables. Kaplan-Meier analysis and log rank tests were used to demonstrate the survival rates based on recurrence and breast mortality rates. Cox regression proportional hazard models were used to analyze the deaths from breast carcinomas according to CD68 and CD163 TAMs expression in univariate analysis. A p-value of ≤ 0.05 was considered as statistically significant. Statistical analysis was performed using IBM SPSS Statistics 21 version software.

RESULTS

The expression of CD68 and CD163 TAMs markers was studied in TN and TS of all the 72 cases. Cohort A consisted of 33 cases and cohort B consisted of 39 cases .The CD68 and CD163 positive expression was noted in both the TS and TN. [Fig.3a-e,4a-e] In Cohort A, mean value of CD68 in TN was 20.2 [Fig.3b], CD68 in TS was 49.78 [Fig.3c], CD163 in TN was 12.45 [Fig 3d], CD163 in TS was 32.42[Fig.3e], CD163/CD68 in TS was 0.77 and CD163/CD68 in TN was 1.15. The cut-off of median CD68 in TS was 42, CD68 in TN was 20, CD163 in TS was 28, CD163 in TN was 8, CD163/CD68 in TS was 0.65 and in TN was 0.6. Similarly, the standard error (SE) expression of CD68 in TS was 5.4, CD68 in TN was 2.64, CD163 in TS was 3.52, CD163 in TN was 1.79, CD163/CD68 in TS was 0.095 and CD163/CD68 in TN was 0.31 [Table 1]. In cohort B, mean value of CD68 in TN was 36.38 [Fig.4b], CD68 in TS was 81.38 [Fig.4c], CD163 in TN was 21.74 [Fig.4d], CD163 in TS was 46.1 [Fig.4e], CD163/CD68 in TS was 0.64 and CD163/CD68 in TN was 0.73. The cut-off of median CD68 in TS was 80, CD68 in TN was 38, CD163 in TS was 46, CD163 in TN was 20, CD163/CD68 in TS was 0.58 and in TN was 0.6. Similarly, the standard error (SE) expression of CD68 in TS was 5.04, CD68 in TN was 2.24, CD163 in TS was 3.34, CD163 in TN was 2.06. CD163/CD68 in TS was 0.064 and CD163/CD68 in TN was 0.08 [Table 2].

The localization and densities of TAMs in association with clinicopathological variables like age, tumor size, and histological grading and lymph node status have been shown in Table 3a & 3b. Cohort A in our study showed that there was no statistically significant association of CD68 and CD163 with clinicopathological variable. In cohort B, CD68 showed positive association with higher age group (p value <0.01) and positive lymph node status (p value = 0.037) in TN with no association seen in TS. A high infiltration of CD68 was significantly associated in TN with higher breast cancer mortality rates (p value=0.003). No other significant association was found of CD68, CD163, and CD163/68 in our present study.

Table 1: Distribution pattern of TAMs in Cohort A (n=33)

Variables in cohort A	Mean ± SE	Median (Range)
CD68+ TAMs		
Tumor stroma	49.78 ± 5.4	42 (18 - 170)
Tumor nest	20.2 ± 2.64	20 (3 - 50)
CD163+ TAMs		
Tumor stroma	32.42 ± 3.52	28 (2 - 80)
Tumor nest	12.45 ± 1.79	8 (5 - 52)
Ratio of CD163 to CD68		
Tumor stroma	0.77 ± 0.095	0.65 (0.03 - 2.66)
Tumor nest	1.15 ± 0.31	0.6 (0.12 - 10.4)

SE- Standard error

Table 2: Distribution pattern of TAMs in Cohort B (n=39)

Variables in cohort B	Mean ± SE	Median (Rage)
CD68+ TAMs		
Tumor stroma	81.38 ± 5.04	80 (28 – 160)
Tumor nest	36.38 ± 2.24	38 (10 – 60)
CD163+ TAMs		
Tumor stroma	46.1 ± 3.34	46 (2 – 80)
Tumor nest	21.74 ± 2.06	20 (5 – 52)
Ratio of CD163 to CD68		
Tumor stroma	0.64 ± 0.064	0.58 (0.07 - 2)
Tumor nest	0.73 ± 0.08	0.6 (0.08 - 2.8)

SE- Standard error

Table 3a: TAMs association with clinicopathological variables in Cohort A

Variable	CD68						CD163						CD163/68					
	Tumor stroma			Tumor nest			Tumor stroma			Tumor nest			Tumor stroma			Tumor nest		
	Low	High	P value	Low	High	P value	Low	High	P Value	Low	High	P value	Low	High	P value	Low	High	P value
Age (in years)																		
<50	9	6	0.227	9	6	0.227	7	8	0.898	5	10	1.0*	5	10	0.112	5	10	0.202
≥50	7	11		7	11		8	10		5	13		11	7		10	8	
Tumor size																		
≤2cm	7	8	0.849	5	10	0.112	7	8	0.898	3	12	0.283*	7	8	0.849	8	7	0.407
>2cm	9	9		11	7		8	10		7	11		9	9		7	11	
Histological grading																		
1 - 2	14	16	0.509	14	16	0.509	14	16	1.0*	9	21	1.0*	15	15	1.0*	14	16	1.0*
3	2	1		2	1		1	2		1	2		1	2		1	2	
Lymph node status																		
Positive	7	3	0.141*	6	4	0.465*	4	6	0.722*	3	7	1.0*	5	5	1.0*	4	6	0.722*
Negative	9	14		10	13		11	12		7	16		11	12		11	12	
Recurrence rate																		
Yes	2	4	0.656*	4	2	0.398*	2	4	0.665*	2	4	1.0*	2	4	0.656*	2	4	0.665*
No	14	13		12	15		13	14		8	19		14	13		13	14	
Breast cancer mortality																		
Yes	2	2	1.0*	2	2	1.0*	2	2	1.0*	1	3	1.0*	2	2	1.0*	1	3	0.607*
No	14	15		14	15		13	16		9	20		14	15		14	15	

[* Fisher's exact test]

Table 3b: Clinicopathological variables association with TAMs in cohort B (TNC)

Variable	CD68						CD163						CD163/68					
	Tumor stroma			Tumor nest			Tumor stroma			Tumor nest			Tumor stroma			Tumor nest		
	Low	High	P value	Low	High	P value	Low	High	P value	Low	High	P value	Low	High	P value	Low	High	P value
Age (years)																		
<50	9	8	0.643	14	3	<0.01	8	9	0.855	8	9	0.701	8	9	0.855	6	11	0.232
≥50	10	12		5	17		11	11		9	13		11	11		12	10	
Tumor size																		
≤2cm	7	8	0.839	6	9	0.389	7	8	0.839	9	6	0.102	8	7	0.648	9	6	0.170
>2cm	12	12		13	11		12	12		8	16		11	13		9	15	
Histological grading																		
1 - 2	8	8	0.894	8	8	0.894	6	10	0.242	6	10	0.522	7	9	0.605	6	10	0.366
3	11	12		11	12		13	10		11	12		12	11		12	11	
Lymph node status																		
Positive	8	8	0.894	11	5	0.037	9	7	0.433	8	8	0.501	9	7	0.433	5	11	0.119
Negative	11	12		8	15		10	13		9	14		10	13		13	10	
Recurrence rate																		
Yes	4	2	0.407*	4	2	0.407*	3	3	1.0*	1	5	0.206*	2	4	0.407*	1	5	0.190*
No	15	18		15	18		16	17		16	17		15	18		17	16	
Breast cancer mortality																		
Yes	2	11	0.003	6	7	0.821	7	6	0.651	5	8	0.648	8	5	0.257	6	7	1.0
No	17	9		13	13		12	14		12	14		11	15		12	14	

[* Fisher's exact test]

Univariate cox regression analysis of RFS and OS were performed for clinicopathological variables in both cohorts for recurrence (Table 4a, Table 4b) and mortality (Table 5a, Table 5b). CD68 was independent prognostic factor in TS (HR = 6.69, CI = 1.48 - 30.29, p value = 0.014) in Cohort B. High CD68 expression in tumor stroma was significantly associated with poor overall survival. Patients with high CD68 expression had a 6.69-fold increased hazard of mortality compared to those with low expression. The high CD68 tumor stroma in cohort B demonstrated a median survival of 4 years with 95% CI was 2.945 - 5.05 years as depicted in fig 2 survival plot.

Cohort A consisting of 33 cases experienced 6 recurrence and 4 mortalities while in Cohort B comprising 39 cases experienced 6 recurrence and 13 mortalities. Median survival time could not be estimated in certain groups as the Kaplan-Meier survival curves did not reach the 50% event threshold during the follow-up period. This may be attributable to the low number of events and substantial censoring. But the high CD68 tumor stroma group demonstrated a median survival of 4 years with 95% CI was 2.945 - 5.05 years. Median follow up duration for cohort A in experiencing recurrence and mortality was 5years. Similar for cohort B were 5 years for both the event.

Table 4a: Univariate Cox Proportional Hazards Regression Analysis for Recurrence in Cohort A (ER+, PR+, HER2/neu Negative Invasive Breast Carcinoma)

Variables	No. of Patient (No. of event)	HR	95%CI	P value
Age (in years)				
≥ 50	18 (3)	0.79	0.16-3.91	0.77
< 50	15 (3)			
Tumor Size (in cm)				
> 2	18 (3)	0.93	0.19 - 4.62	0.93
<=2	15 (3)			
Histological Grade				
1&2	30 (5)	0.27	0.31 - 2.46	0.249
3	3 (1)			
Nodal Status				
Yes	10 (3)	2.45	0.49 - 12.15	0.272
No	23 (3)			
CD68 TN				
High	17 (2)	0.4	0.075 - 2.238	0.303
Low	16 (4)			
CD68 TS				
High	17 (4)	1.77	0.33 - 9.63	0.513
Low	16 (2)			
CD 163 TN				
High	23 (4)	0.91	0.17 - 4.99	0.916
Low	10 (2)			
CD163 TS				
High	18 (4)	1.7	0.31- 9.24	0.543
Low	15 (2)			
CD68 / CD 163 TN				
High	18 (4)	1.7	0.31- 9.24	0.543
Low	15 (2)			
CD68 / CD 163 TS				
High	17 (4)	2.36	0.43 - 12.91	0.324
Low	16 (2)			

HR: Hazard Ratio

Table 4b: Univariate Cox Proportional Hazards Regression Analysis for Recurrence in Cohort B (ER-ve, PR-ve, HER2/neu Negative Invasive Breast Carcinoma)

Variables	No. of Patient (No. of event)	HR	95%CI	P value
Age				
≥ 50	22 (4)	1.42	0.26-7.79	0.681
< 50	17 (2)			
Tumor Size				
> 2	24 (5)	3.19	0.37 - 27.38	0.289
<=2	15 (1)			
Histological Grade				
1&2	16 (4)	3.03	0.556 - 16.59	0.2
3	23 (2)			
Nodal Status				
Yes	16 (2)	0.75	0.13 - 4.13	0.747
No	23 (4)			
CD68 TN				
High	20 (2)	0.43	0.79 - 2.34	0.33
Low	19 (4)			
CD68 TS				
High	20 (2)	0.46	0.08 - 2.53	0.374
Low	19 (4)			
CD 163 TN				
High	22 (5)	3.71	0.434 - 31.82	0.231
Low	17 (1)			
CD163 TS				
High	20 (3)	0.962	0.194 - 4.76	0.962
Low	19 (3)			
CD68 / CD 163 TN				
High	21 (5)	4.65	0.54 - 39.86	0.16
Low	18 (1)			
CD68 / CD 163 TS				
High	20 (2)	0.48	0.08 - 2.62	0.398
Low	19 (4)			

HR: Hazard Ratio

The present study yielded 12 recurrence events and 17 mortality events. Based on Schoenfeld's approximation, the overall power to detect moderate hazard ratios (HR ≈ 2.0) was limited, indicating that the study may be underpowered for small to moderate effects. However, the significant associations observed in Cohort B were associated with relatively large effect sizes (HR > 4), which increases statistical power.

A post hoc power analysis for the Cox proportional hazards model was performed using Schoenfeld's method. The statistical power was calculated using the formula:

$$Power = \Phi\left(\sqrt{E \cdot p(1-p)} \left| \ln(HR) \right| - z_{\alpha/2}\right)$$

where E represents the total number of events and p the proportion of subjects in the exposed group. For the association between CD68 expression in tumor stroma and mortality in Cohort B (HR = 6.69; E = 13), the post hoc power was estimated to be 92.7% at α = 0.05. But in cohort A it is lower.

DISCUSSION

Breast neoplasm is one of the most common malignant tumors in females, second only to cervical carcinoma in incidence.[1] Traditional treatment strategies have primarily focused on directly targeting tumor cells through radiotherapy or chemotherapy. However, recent advancements in cancer biology have introduced the concept of the tumor microenvironment (TME) as a critical player in tumor progression and response to therapy. Targeting the cellular components of the TME, particularly tumor-associated macrophages (TAMs), has emerged as a promising approach to improve patient prognosis and survival outcomes.

TAMs are broadly classified into two functional subtypes based on their mode of activation. Classically activated macrophages (M1 subtype) enhance immune responses by stimulating MHC class I-restricted immune cells, which play a role in defending against viral, bacterial, and parasitic infections. M1 macrophages also secrete pro-inflammatory cytokines that exert cytotoxic effects on tumor cells, and are therefore referred to as pro-inflammatory or tumoricidal macrophages. In contrast, alternatively activated macrophages (M2-like subtype) are involved in immune suppression and tissue repair. These macrophages contribute to tissue remodelling, hypoxia-induced angiogenesis, wound healing, and facilitate tumour cell proliferation and migration.[17]

Table 5a: Univariate Cox Proportional Hazards Regression Analysis for Mortality in Cohort A (ER+, PR+, HER2/neu Negative Invasive Breast Carcinoma)

Variables	No. of patient (No. of event)	HR	95%CI	P value
Age				
≥ 50	18 (4)			
< 50	15 (0)			
Tumor Size				
> 2	18 (3)	2.68	0.28- 25.83	0.393
<=2	15 (1)			
Histological Grade				
1&2	30 (4)			
3	3 (0)			
Nodal Status				
Yes	10 (2)	2.68	0.38 - 19.12	0.323
No	23 (2)			
CD68 TN				
High	17 (2)	0.08	0.11 - 5.7	0.825
Low	16 (2)			
CD68 TS				
High	17 (2)	0.8	0.11 - 5.7	0.825
Low	16 (2)			
CD 163 TN				
High	23 (3)	1.5	0.15 - 14.34	0.73
Low	10 (1)			
CD163 TS				
High	18 (2)	0.86	0.12- 6.12	0.882
Low	15 (2)			
CD68 / CD 163 TN				
High	18 (3)	2.68	0.28- 25.83	0.393
Low	15 (1)			
CD68 / CD 163 TS				
High	17 (2)	1.09	0.15 - 7.76	0.93
Low	16 (2)			

HR: Hazard Ratio

Table 5b: Univariate Cox Proportional Hazards Regression Analysis for Mortality in Cohort B (ER -ve, PR -ve, HER2/neu Negative Invasive Breast Carcinoma)

Variables	No. of patient (No. of event)	HR	95%CI	P value
Age				
≥ 50	22 (9)	1.9	0.58 - 6.205	0.283
< 50	17 (4)			
Tumor Size				
> 2	24 (7)	0.64	0.214 - 1.89	0.417
<=2	15 (6)			
Histological Grade				
1&2	16 (4)	0.57	0.17 - 1.87	0.36
3	23 (9)			
Nodal Status				
Yes	16 (7)	1.98	0.66 - 5.89	0.22
No	23 (6)			
CD68 TN				
High	20 (7)	1.11	0.37 - 3.31	0.849
Low	19 (6)			
CD68 TS				
High	20 (11)	6.69	1.48 - 30.29	0.014*
Low	19 (2)			
CD 163 TN				
High	22 (8)	1.17	0.38 - 3.59	0.776
Low	17 (5)			
CD163 TS				
High	20 (6)	0.81	0.27 - 2.43	0.718
Low	19 (7)			
CD68 / CD 163 TN				
High	21 (7)	0.99	0.33 - 2.94	0.985
Low	18 (6)			
CD68 / CD 163 TS				
High	20 (5)	0.54	0.17 - 1.66	0.284
Low	19 (8)			

*P value <0.05; HR: Hazard Ratio

Despite the well-documented functions of M1 and M2 macrophages, the expression patterns and prognostic significance of $CD68^+$ and $CD163^+$ tumor-associated macrophages (TAMs) in breast cancer remain incompletely understood. CD68 serves as a pan-macrophage marker that labels both M1 and M2 macrophage populations. The prognostic significance of $CD68^+$ TAMs appear to vary across different malignancies, with studies reporting favorable outcomes in certain cancers such as colorectal and prostate cancer, whereas increased $CD68^+$ macrophage infiltration has been associated with poor prognosis in bladder, breast, ovarian, and cervical cancers. [17-19]

In the present study, a significant association was observed between $CD68^+$ TAMs in triple negative cases in both tumor stroma (TS) with mortality and tumor nests (TN) with age and lymph node status, consistent with findings reported by Pelekanou V et al. [20] high infiltration and density of $CD68^+$ TAMs were associated with several adverse clinicopathological features, including older age, positive lymph node status, and higher breast cancer-specific mortality. These associations align with previous study conducted by Medrek C et al.[8]. There was significant correlation of increased CD68 TAMs in TS with overall survival rates based on log rank tests.

Several studies have demonstrated that high TAM density correlates with aggressive tumor features. Research by Gwak JM et al[21], Jeong H et al[22], Ni C et al[23], and Sousa S et al[24] reported that increased infiltration of TAMs in both tumor nests (TN) and tumor stroma (TS) was significantly associated with higher tumor grade and elevated Ki-67 expression. Similarly, Yang M et al[25] identified a significant association between $CD68^+$ TAM density and tumor histological grade. However, findings from Ch'ng ES et al[26] and Yuan ZY et al[27] indicated that the accumulation of TAMs in TS was linked solely to higher tumor grade, with no observed correlation to relapse-free survival (RFS) or overall survival (OS).

In contrast, Mahmoud SM et al[9] found that high $CD68^+$ TAM density was associated with poorer survival outcomes, whereas Medrek C et al[8] reported no significant association between $CD68^+$ TAMs and survival.

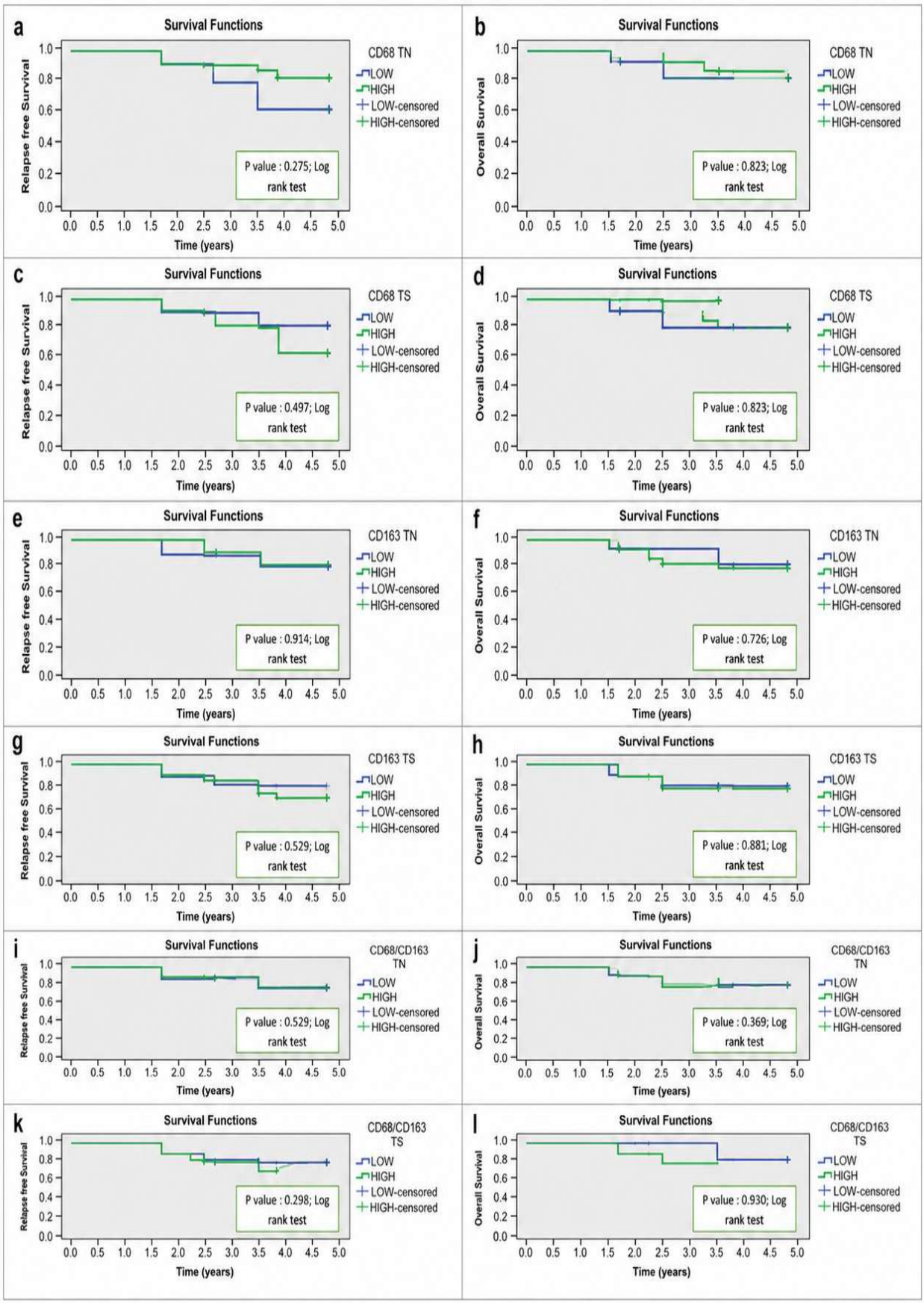

Figure 1 (a-l): Prognostic significance of TAMs in Cohort A. Kaplan-Meier curves for relapse-free survival (RFS) and overall survival; a) P value=0.275 log rank test, b) P value=0.823 log rank test, c) P value=0.497 log rank test, d) P value=0.823 log rank test, e) P value=0.914 log rank test, f) P value=0.726 log rank test, g) P value=0.529 log rank test, h) P value=0.881 log rank test, i) P value=0.529 log rank test, j) P value=0.369 log rank test, k) P value=0.298 log rank test , l) P value=0.930 log rank test

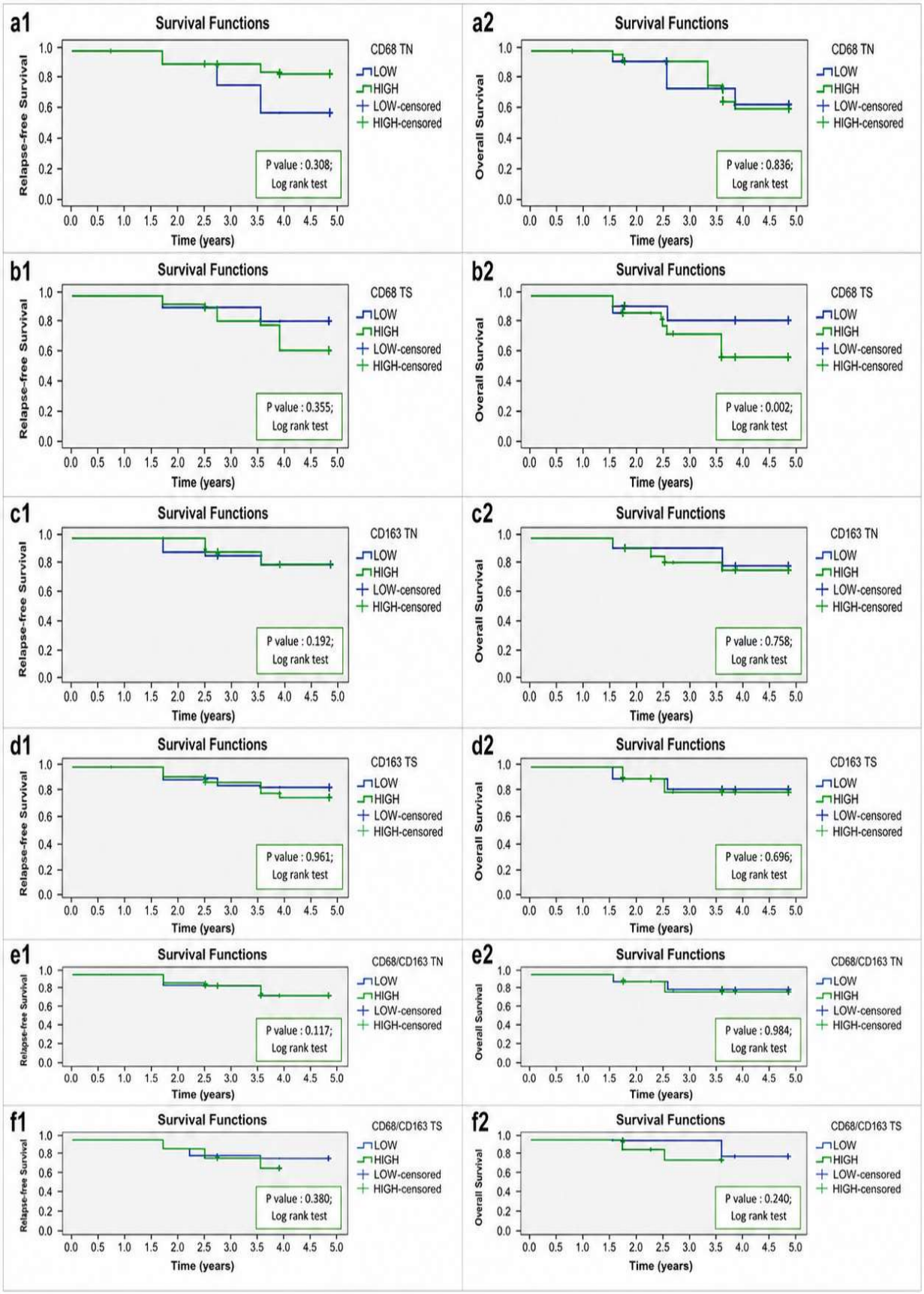

Figure 2 (A1-F2): Prognostic significance of TAMs in Cohort B. Kaplan-Meier curves for relapse-free survival (RFS) and overall survival; A1) P value=0.308 log rank test, A2) P value=0.836 log rank test, B1) P value=0.355 log rank test, B2) P value=0.002 log rank test, C1) P value=0.192 log rank test, C2) P value=0.758 log rank test, D1) P value=0.961 log rank test, D2) P value=0.696 log rank test, E1) P value=0.117 log rank test, E2) P value=0.984 log rank test, F1) P value=0.380 log rank test , F2) P value=0.240 log rank test

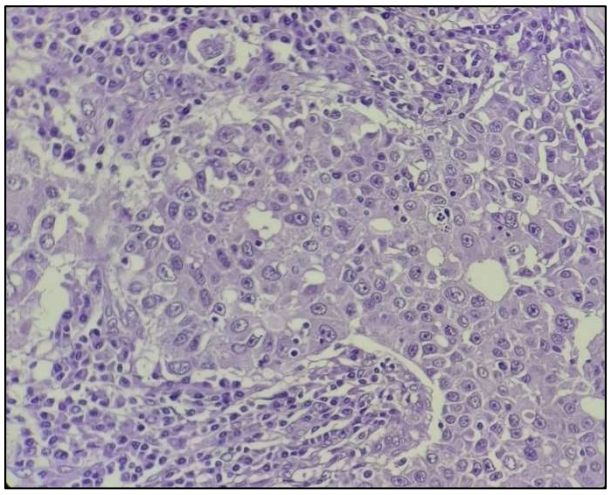

Figure 3a

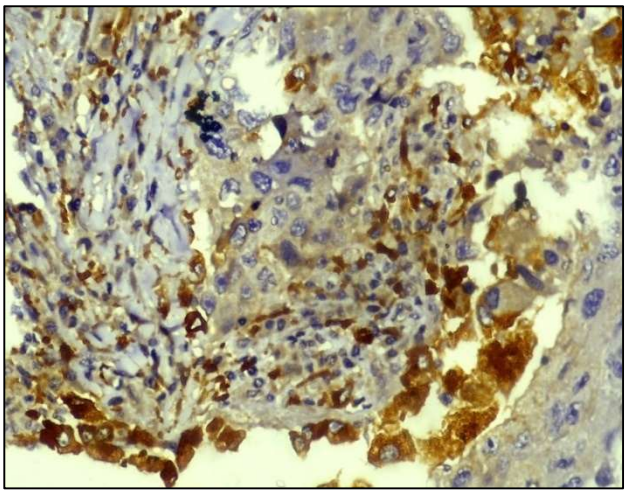

Figure 3b

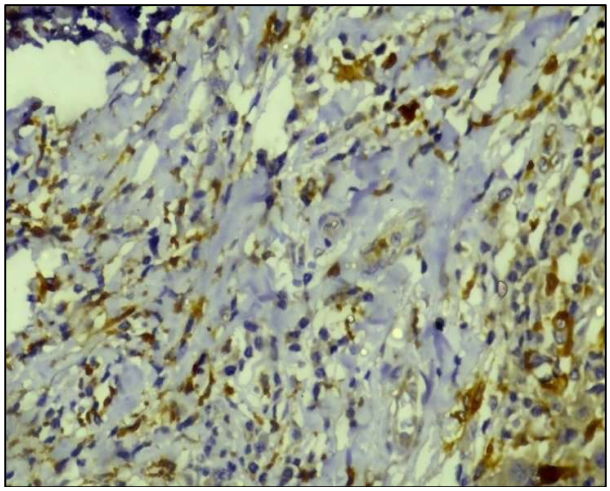

Figure 3c

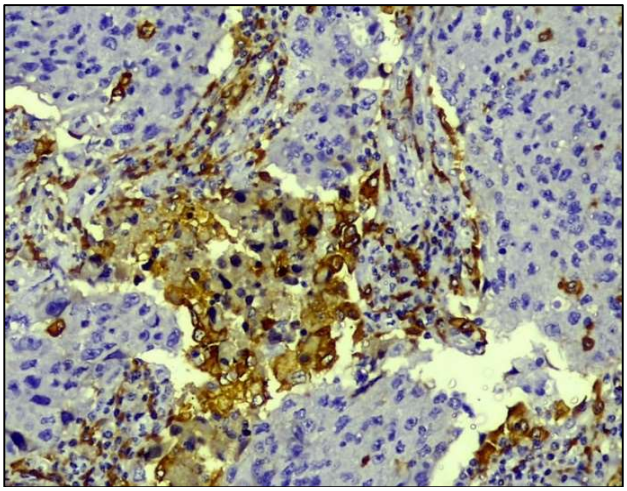

Figure 3d

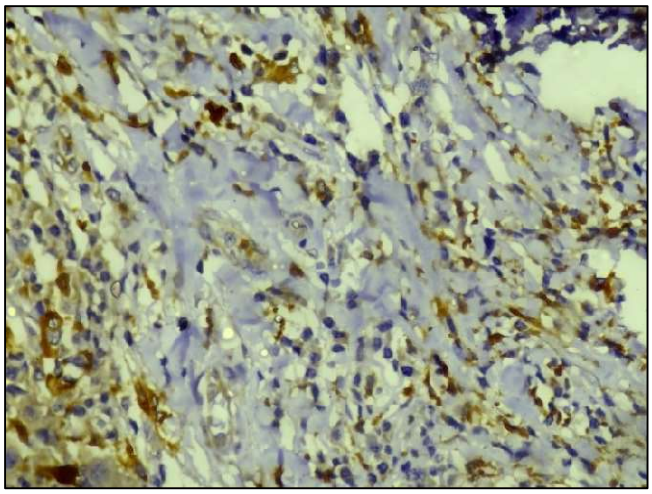

Figure 3e

Figure 3; COHORT A: a) Microphotograph shows poorly differentiated invasive duct carcinoma, ER, PR +, Her2neu -ve. (HPE, 400x), b) Positive CD68 expression in TN (IHC, 400x), c) Positive CD68 expression in TS (IHC, 400x), d) Positive CD163 expression in TN (IHC, 400x), e) Positive CD163 expression in TS (IHC, 400x)

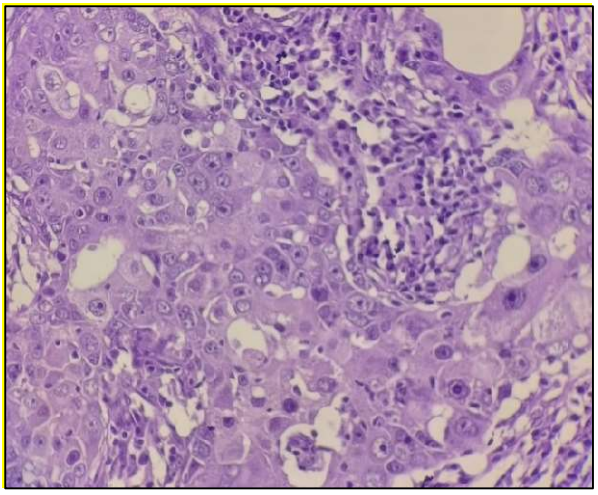

Figure 4a

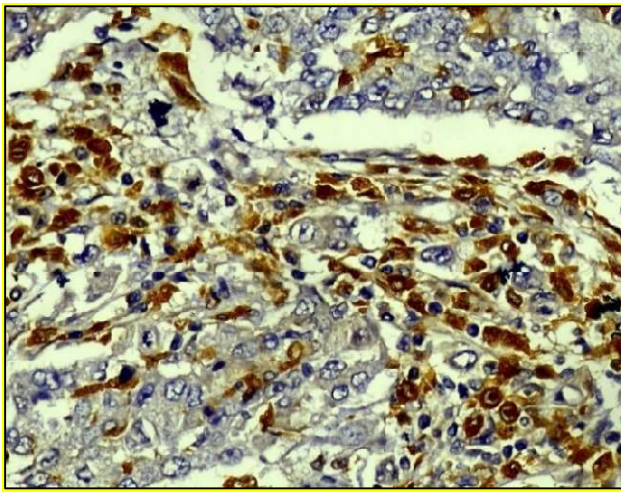

Figure 4b

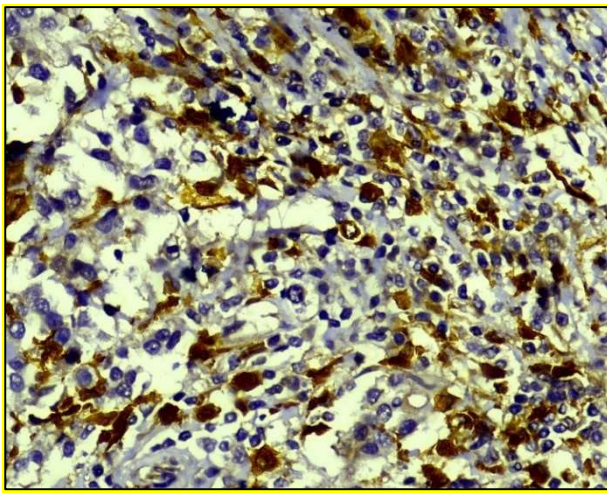

Figure 4c

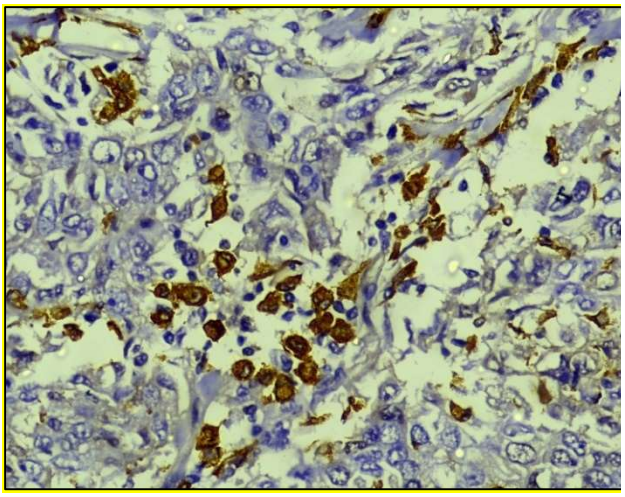

Figure 4d

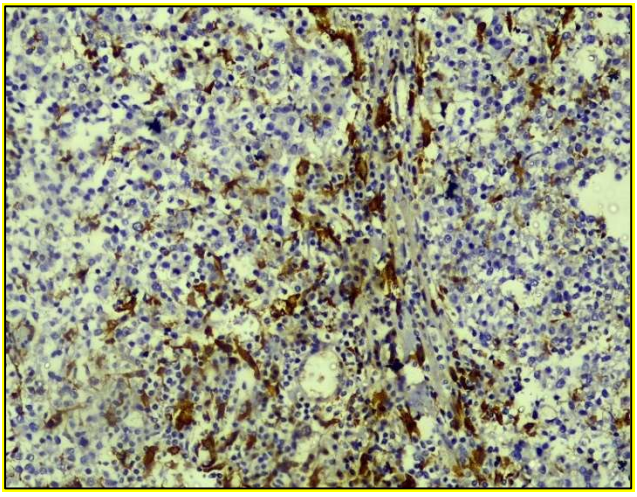

Figure 4e

Figure 4; COHORT B: a) Microphotograph shows poorly differentiated invasive duct carcinoma, ER, PR, Her2neu -ve (HPE, 400x), b) Positive CD68 expression in TN (IHC, 400x), c) Positive CD68 expression in TS (IHC, 400x), d) Positive CD163 expression in TN (IHC, 400x), e) Positive CD163 tumor expression in TS (IHC, 100x)

Collectively, these findings particularly those in triple-negative breast carcinoma (TNBC) suggest that CD68, a marker labeling both M1 and M2 macrophages, may lack specificity and limited prognostic value in evaluating TAM-related tumor biology in invasive breast cancer.In contrast, CD163 is a more selective marker of M2-polarized macrophages, which are typically involved in tumor progression and immune suppression. Medrek C et al. [8] found that $CD163^+$ TAMs in TS were associated with larger tumor size and showed an inverse relationship with hormone receptor expression and the luminal A subtype.

There were few limitations in our study. First, the background stain of CD68 and CD163 due to faulty antigen retrieval technique in few cases made the interpretation difficult. Secondly, though CD163 is a very specific marker for M2 macrophages, however it can be expressed by myeloid dendritic cells (MDCs). Macrophages are large cells with round to oval nucleus, vacuolated cytoplasm and having vesicular chromatin whereas MDCs are stellate shaped cells. Both of them belong to the mononuclear phagocyte system (MPS). Hence the presence of both cannot be differentiated in either TS or TN based on H&E and IHC with CD163 and need more markers to distinguish the two [8]. Thirdly, M1 macrophages express HLA-DR, CD11c, CD86, Inos, pSTAT1 and M2 macrophages express CD 206. Due to unavailability of markers in our institute, we could not include these markers in our IHC panel. We did not have immunofluorescence in our centre for further confirmation of TAMs by double labelling immunofluorescence methods.

In addition, the relatively small sample size (n = 72) limits the statistical power of the study and may affect the generalizability of the findings. The retrospective study design introduces the possibility of selection bias and limits control over confounding variables. Being a single-center study, institutional practices and patient demographics may have influenced the results, thereby restricting external validity. Furthermore, survival analysis was based on univariate Cox regression without multivariate adjustment for potential confounders such as tumor grade, nodal status, and age, which may independently influence prognosis. Finally, molecular subtyping was limited to ER, PR, and Her2/neu receptor status, and more detailed molecular classification (e.g., intrinsic subtypes or genomic profiling) was not performed, which could provide deeper insights into TAM-related tumor biology.

CONCLUSION

The present study highlights the critical role of TAMs in the progression and prognosis of breast cancers, especially in triple negative cases. It is among the first few studies on TAMs in India. The distinct patterns of TAMs infiltration in ER, PR positive and triple negative tumors suggest that TAMs actively shape the tumor microenvironment and influence clinical outcomes. Given the limitations of traditional hormone-based therapies, tumor-associated macrophages may represent a potential area for further research, and their modulation could be explored in future studies to better understand their role in treatment resistance and therapeutic response in breast cancer. Future research with larger cohorts is essential to further elucidate the mechanisms involved and to develop effective TAM targeted therapies for breast cancer patients.

Individual Author's Contribution: PM contributed to the study conception, data collection, and manuscript preparation. **PLP** contributed to the study conception, study design, data analysis and interpretation, and manuscript preparation. **PPD** contributed to data analysis and interpretation and manuscript preparation. **LM** contributed to the study design and manuscript preparation. **BHD** contributed to the study conception and manuscript preparation.

Availability of data: The data that support the findings of this study are available from the corresponding author on reasonable request.

Declaration of non-use of generative AI Tools: This article was prepared without the use of generative AI tools for content creation, analysis, or data generation. All findings and interpretations are based solely on the authors' independent work and expertise.

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