Breast Cancer: Targets and Therapy

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ORIGINAL RESEARCH

Tumor Microenvironment Modulation by Tumor-Associated Macrophages: Implications for Neoadjuvant Chemotherapy Response in Breast Cancer

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Background: Tumor-associated macrophages (TAMs) constitute an important part of the tumor microenvironment of breast cancer (BC), and they play an essential role in modulating tumor growth and invasion. However, the role of TAMs in neoadjuvant chemotherapy (NAC) has not been fully elucidated. Therefore, the aim of this study was to assess the function of TAM subtypes and investigate their role in the response to NAC in BC.

Methods: Presence of TAMs was examined immunohistochemically (IHC) in pre- and post- NAC treatment tumor tissue in a cohort of 138 BC patients. IHC staining with monoclonal antibodies for CD68 and CD163 were performed. Positivity was defined as staining > 1% TAMs in stroma and tumor cell nests. Response to NAC was evaluated according to tumor size change and Residual Cancer Burden (RCB) index.

Results: CD68+ and CD163+ TAMs decreased significantly in both the stroma and tumor nests (TN) after NAC. The median CD68+ TAMs in the stroma decreased significantly from 5% to 1% ($p < 0.005$), while CD163+ TAMs showed a marked reduction from 20% to 5% ($p < 0.001$). Post-NAC, the persistence of CD68+ and CD163+ TAMs in the stroma was strongly correlated with larger residual tumor size ($p < 0.005$ and $p < 0.001$, respectively). Changes in CD163+ TAM levels in the stroma were significantly associated with RCB classes ($p < 0.005$). Pre-NAC, CD163+ TAMs in the stroma and TN showed a significant association with TILs; however, no correlations with TILs were observed post-NAC.

Conclusion: This study highlights the critical role of TAMs dynamics in shaping NAC response in BC. Notably, CD163+ TAMs may emerge as pivotal players in mechanisms of chemotherapy resistance and response, underscoring their potential as biomarkers and therapeutic targets in breast cancer treatment.

Keywords: tumor-associated macrophages, CD68, CD163, breast cancer, neoadjuvant chemotherapy

Introduction

Breast cancer represents a complex and heterogeneous malignancy, characterized by various molecular subtypes and clinical presentations. Despite early diagnosis and improved treatment modalities, breast cancer (BC) accounts for approximately 15% of cancer-related deaths.[1] Accumulating evidence indicates that the evolving interplay between tumor cells, stromal cells, immune cells in the tumor microenvironment (TME) and fibroblasts throughout the progression of the cancer significantly influences patients' survival and their response to therapies.[2–4] This highlights the importance of considering the dynamic nature of cancer biology in clinical management and treatment strategies.

Received: 11 September 2024
Accepted: 15 January 2025
Published: 21 February 2025

The TME plays a pivotal role in modulating tumor growth, invasion, metastasis, and response to therapy.[5] Among the myriad components of the TME, tumor-associated macrophages (TAMs) have emerged as one of the key regulators of BC progression, BC metastasis and treatment resistance.[6–8] TAMs originate from peripheral blood monocytes and differentiate into macrophages following recruitment to tumor sites.[9] TAMs are divided into subgroups by participating in certain immunological processes according to the environment and growth factors secreted by them.[10,11] Although, M1 macrophage is proinflammatory and tumoricidal, M2 macrophages play a role in the release of anti-inflammatory cytokines, tissue repair, wound healing, angiogenesis, and tumor progression.[11,12] CD68 and CD163 are two prominent markers used to identify and characterize TAMs in BC, as well as in various types of cancer.[12,13] CD68+ TAMs in BC can exhibit a spectrum of phenotypes, ranging from M1-like to M2-like, depending on the local microenvironmental interaction. In contrast, CD163, which is predominantly expressed on M2-like macrophages, plays an immunosuppressive and tumor-promoting role.[12–14] In BC, high levels of TAMs have been associated with higher proliferation rates, lower tumor cell differentiation, and a lack of hormone receptor (HR) expression.[15] In addition, high infiltration of macrophages in BC were associated with an impaired disease-free survival (DFS) and overall survival (OS) in triple negative breast cancer (TNBC).[15–17] However, the role and dynamic changes of TAMs in response to chemotherapy have not yet been thoroughly investigated in clinical studies. Furthermore, the functions of macrophages within the TME across BC subtypes remain elusive.

It is crucial to better understand TAMs to ensure the effectiveness of treatment modalities in BC and reduce cancer-related mortality. Understanding the dynamic interplay between TAMs and the TME offers insights into novel therapeutic strategies and personalized approaches for BC management. Therefore, this study attempts to shed lights on the role of TAMs in response to neoadjuvant therapy in different BC types.

Materials and Methods

Patients and Clinical Data Selection

In this study, we analyzed 138 patients with locally advanced breast cancer who had neoadjuvant chemotherapy (NAC) and underwent either mastectomy or breast-conserving surgery (BCS) at the Multidisciplinary Breast Clinic of Antwerp University Hospital between 2014 and 2018. This retrospective clinical study was conducted following approval by the Institutional Ethics Review Board (File number: 20/26/349, Edge number: 001251). Additionally, all patients had pre- or post-operative slides available in the pathology archive. Patients with carcinoma in situ, stage IV breast cancer, bilateral BC, inflammatory BC, as well as those who received any form of therapy (chemotherapy, endocrine therapy, or radiotherapy) before NAC, were excluded from this study. Initial staging was determined by physical examination, ultrasonography, magnetic resonance imaging (MRI), and positron emission tomography-computed tomography (PET-CT), which helped exclude distant metastasis.

Oestrogen receptor (ER) and progesterone receptor (PR) were stained by using monoclonal antibodies respectively clone EP1 (Dako) and clone PR1294 (Dako) and scored according to the Allred method. ER and PR were considered positive in case of a population score of at least 2/5 (>1% tumour cells staining) in conformity with the ASCO/CAP guidelines. Ki-67 was stained using clone MIB-1 (Dako). HER-2 expression (DG44Dako Omnis) was also scored according to ASCO/CAP guidelines and tumor samples were considered HER2-positive when a fluorescence in situ hybridisation (FISH) test documented amplification.

Clinicopathological and follow-up data of all patients were collected from hospital medical records. The absence of residual invasive carcinoma in the resected breast specimen and in all sampled regional lymph nodes after NAC was defined as pCR.

Immunohistochemistry and Macrophage Quantification

Four-micron consecutive sections were cut from representative formalin-fixed, paraffin-embedded (FFPE) diagnostic tissue blocks, mounted on adhesive glass slides and stained for CD68 (Clone KP1, Dako) on the Dako Omnis platform, according to the manufacturer's protocol. And for CD163 (Clone MRQ-26, Ventana) on the VENTANA BenchMark ULTRA platform, according to the manufacturer's protocol (Ventana Medical Systems, Tucson, AZ, USA). The CD68+

and CD163+ TAMs were quantified in three randomized high-power fields (40 X) with the pathologists who were blinded to the clinicopathological features and prognosis of these patients. The CD68+ and CD163+ TAMs were counted in the stroma and tumor nest (TN) separately (Figure 1). TAMs in TN were defined as intraepithelial tumor infiltrating

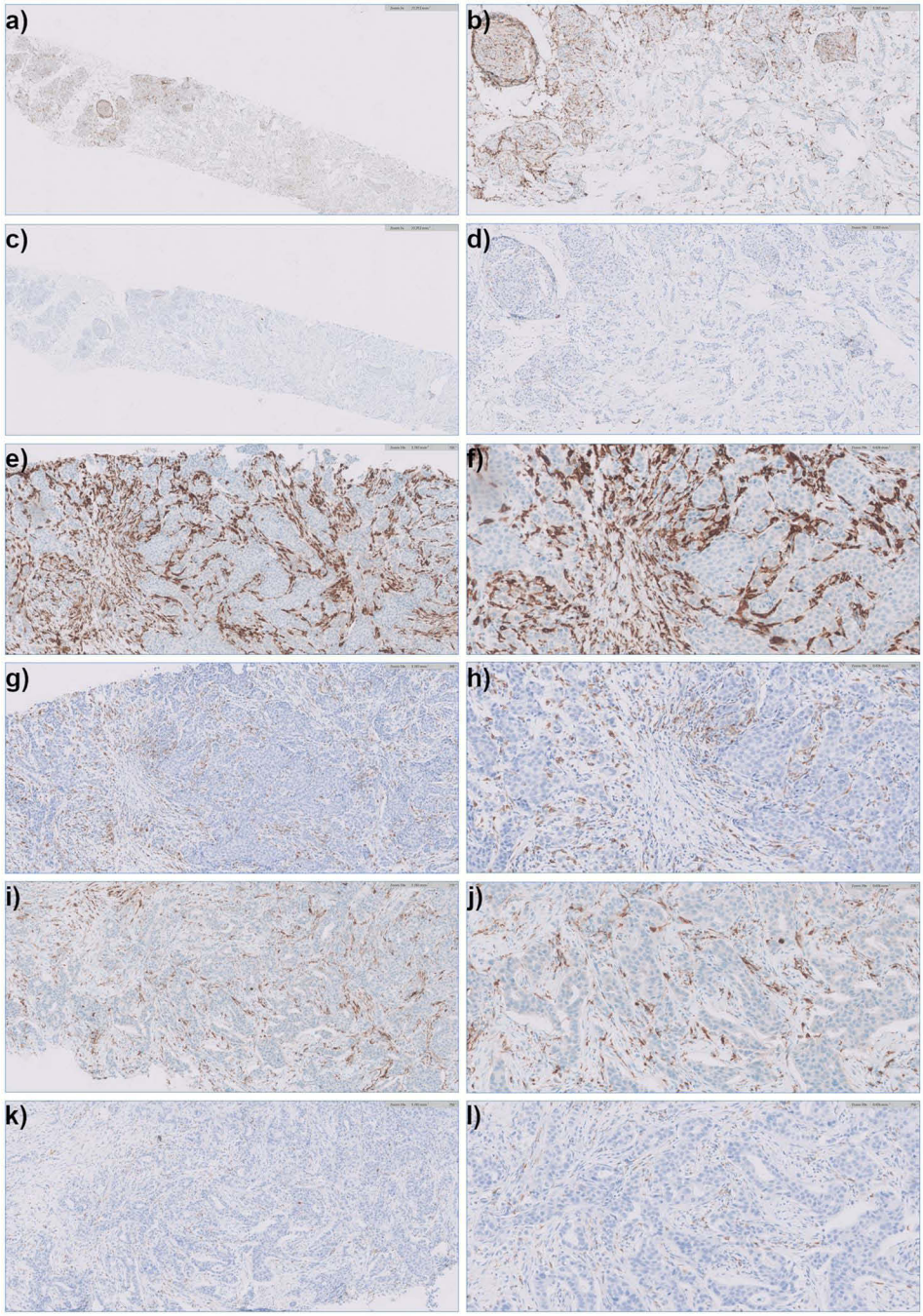

Figure 1 Histological images of CD68+TAMs and CD163+TAMs by immunohistochemistry. Scoring for markers was done by measuring the percentage of cells stained in stroma and TN. Positive staining was evaluated quantitatively and, TAMs were categorized into high and low infiltration groups based on the median level of infiltration. (**a–d**) The difference in CD163 and CD68 staining on the consecutive FFPE sections: (**a**) CD163+ TAMs 20% in stroma and 20% in TN (2x, scale bar = 400 μm), (**b**) CD163+ TAMs 20% in stroma and 20% in TN (10x, scale bar = 100 μm), (**c**) CD68+ TAMs 5% in stroma and 5% in TN (2x, scale bar = 400 μm), (**d**) CD68+ TAMs 5% in stroma and 5% in TN (10x, scale bar = 100 μm). (**e–h**) The difference in CD163 and CD68 staining on consecutive FFPE sections: (**e**) CD163+ TAMs 30% in stroma and 20% in TN (10x, scale bar = 100 μm), (**f**) CD163+ TAMs 30% in stroma and 20% in TN (20x, scale bar = 40 μm), (**g**) CD68+ TAMs 10% in stroma and 10% in TN (10x, scale bar = 100 μm), (**h**) CD68+ TAMs 10% in stroma and 10% in TN (20x, scale bar = 40 μm).(**i–l**) The difference in CD163 and CD68 staining on consecutive FFPE sections: (**i**) CD163+ TAMs 10% in stroma and 10% in TN (10x, scale bar = 100 μm), (**j**) CD163+ TAMs 10% in stroma and 10% in TN (20x, scale bar = 40 μm), (**k**) CD68+ TAMs 5% in stroma and 10% in TN (10x, scale bar = 100 μm), (**l**) CD68+ TAMs 5% in stroma and 10% in TN (20x, scale bar = 40 μm).

Abbreviations: TAMs, tumor-associated macrophages; TN, tumor nest; FFPE, Formalin-fixed paraffin-embedded.

https://doi.org/10.2147/BCTT.S493085

macrophages. The quantification was performed by pathologists who were blinded to the clinicopathological features and prognosis of the patients to ensure objectivity. TAMs were analyzed both categorical and as a continuous variable. TAMs were categorized into high and low infiltration groups based on the median level of infiltration. Percentages were calculated as the number of positively stained TAMs in the stroma or TN divided by the total number of cells in the respective compartment. When pCR was achieved after NAC, TAMs were evaluated only in the stroma.

Treatment and Chemotherapy Response

Among the patients who received NAC, all underwent anthracycline- and taxane-based regimens, including docetaxel, epirubicin, and cyclophosphamide (TEC); epirubicin and cyclophosphamide followed by docetaxel (EC-T); and paclitaxel and epirubicin (PE). Following NAC, operations (mastectomy or BCS) were performed to remove the primary tumor and axillary sentinel lymph node biopsy or axillary lymph node dissection were conducted to excise the lymph nodes.

Stromal Tumor-Infiltrating Lymphocytes (sTIL)

Morphological evaluation of TILs and TILs scoring was performed on haematoxylin and eosin (H&E) stained 4-µm sections of FFPE pre-treatment tumor tissue and post-treatment tumor tissue by different researchers according to the international consensus recommendations of the International TILs Working Group. All evaluations were performed avoiding areas with necrosis, technical artefacts and suboptimal tissue preservations. TILs were reported for the stromal compartment (% stromal TILs, sTIL) in all areas containing invasive tumor cells on the H&E slide. TILs were considered both as continuous variable and dichotomized in <10% (category 1), ≥10–40% (category 2), and ≥40% (category 3).

Residual Cancer Burden Index

"MD Anderson Cancer Center Residual Cancer Burden Index" was used to measure NAC response. The following parameters are required in order to calculate Residual Cancer Burden (RCB) index after NAC treatment: a) The two largest dimensions of the residual tumor bed (the largest tumor bed in multicentric cases is included in the calculation), b) The histologic assessment of the percentage of the tumor bed area that contains carcinoma, c) The histologic estimate of the percentage of the carcinoma in the tumor bed that is in-situ, d) The number of metastatic lymph nodes e) The diameter of the largest lymph node metastasis. RCB was determined using the official online RCB index calculator (http://www3.mdanderson.org/app/medcalc/index.cfm?pagename=jsconvert3) and the RCB classification was based on this scoring. In this classification, the lowest category is considered as pCR (RCB-pCR, like category RCB-0), whereas the highest category (RCB-III) is considered as neo-adjuvant therapy resistant.

Peripheral Blood Parameters

Peripheral blood cell count results were extracted from medical records. Blood tests, which were part of the routine management of patients prior to any therapeutic intervention, were considered pre-NAC blood results. As a post-NAC blood sample, blood result at the earliest one month after receiving the latest NAC and before surgery were included in this study.

Statistical Analysis

Data were analysed using R project in R studio (Version 2024.04.0+735). Cases with missing data were maintained in the database but excluded from the statistical analyses on a per test basis. Categorical variables were compared using Fisher's exact test or Chi-square test. Pearson chi2 test (categorical variables) and ANOVA (continuous variables) were used to assess the relationship between the different parameters. Changes in quantitative biomarkers from before to after NAC were made using Wilcoxon signed rank test. Significant parameters were included in a multivariate regression model. Survival data were last updated on March 1, 2023. All p values considered statistically significant when < 0.05 and were calculated two-sided.

Results

Clinicopathological Characteristics

A total of 138 BC patients [median age 53.7 years (27–82)] were enrolled this retrospective study. Patient and tumor characteristics are presented in Table 1. All these patients received NAC and majority of the patients underwent breast-conserving surgery (77/138, 56%). With a mean follow-up of 53 months (9–105), twelve patients experienced a breast cancer related event. Among these, two patients had local recurrence, ten patients had metastasis and there were five cancer related deaths during follow-up. Tumor tissues from all 138 patients were evaluated immunohistochemically before and after NAC.

CD68+ TAMs Change in the Immune Microenvironment Before and After NAC

Before NAC, CD68+ TAMs were present in the stroma in 93% (128) of cases, while CD68+ TAMs were present in 77% (106) of patients within TN. After NAC, there was a decrease in CD68 + TAMs in both the stroma (80%, 106) and TN (40%, 55). Before NAC, the median percentage of CD68+ TAMs in the stroma was 5% (0–30) and in the TN was 1% (0–30), respectively. After NAC, the median percentage of CD68+ TAMs in the stroma was 1% (1–40) and in the TN was 1% (1–40). The decrease of CD68+ TAMs expression in the stroma and TN is statistically significant ($p < 0.001$) (Table 2 and Figure 2).

Table 1 Patient and Tumor Characteristics of the Study Population

Patients Characteristics (N=138)		**BEFORE - NACn (%)**	**AFTER- NACn (%)**
Median age	53.7 years (27–82 years)		
Menopausal status	*Premenopausal*	51 (37)	
	Postmenopausal	87 (63)	
Tumor size (TNM – cT- ypT)	T0	-	59 (43)
	T1	26 (19)	50 (36)
	T2	88 (64)	25 (18)
	T3	19 (14)	3 (2)
	T4	4 (3)	1 (1)
Nodal status (TNM – cN- ypN)	N0	66 (48)	96 (69)
	N1	53 (38)	30 (22)
	N2	11 (8)	8 (6)
	N3	8 (6)	4 (3)
Intrinsic subtype	*HR +*	90 (65)	
	HER-2 +	50 (36)	
	TNBC	37 (27)	
Histology	*Ductal*	134 (97)	
	Lobular	4 (3)	

(*Continued*)

https://doi.org/10.2147/BCTT.S493085

Table 1 (Continued).

Patients Characteristics (N=138)		BEFORE - NACn (%)	AFTER- NACn (%)
Nuclear Grade	*G1*	13 (9)	
	G2	49 (36)	
	G3	42 (30)	
	Unknown	34 (25)	
Tumor size (median)(mm)		26 (range 1–85)	5.55 (range 0–90)
Ki-67 (median)		40 (range 1–99)	20 (range 1–85)
TILs	<10% (category 1)	40 (71)	44 (80)
	≥10–40% (category 2)	13 (24)	9 (16)
	≥ 40% (category 3)	3 (5)	2 (4)
Residual Cancer Burden Category	RCB-pCR		59 (43)
	RCB-I		21 (15)
	RCB-II		41 (30)
	RCB-III		17 (12)

Abbreviations: TNM, tumor node metastasis classification; sTIL, stromal tumor-infiltrating lymphocytes; RCB, Residual Cancer Burden; n, number of patients; %, percentage; mm, millimetre.

Table 2 Comparison of Continuous Parameters Before and After NAC

	Continuous Parameters (Median (Min-max))		
	Before NAC	**After NAC**	**p value**
Tumor Size (mm)	26 (2–70)	5.55 (0–90)	**<0.001**
CD68+ TAMs in tumor nest	1 (0–30)	1 (0–40)	**<0.005**
CD68 + TAMs in stroma	5 (0–30)	1 (0–40)	**<0.005**
CD163+ TAMs in tumor nest	10 (0–60)	5 (0–60)	**0.008**
CD163+ TAMs in stroma	20 (0–60)	5 (0–60)	**<0.001**
Monocytes (10e9/L)	0.39 (0.03–4.49)	0.54 (0.14–1.47)	**<0.005**
TILs	9 (1–85)	5 (1–60)	**<0.005**

Notes: Comparison of the continuous parameters was done using Wilcoxon signed rank test. Bold values denote statistical significance at the $p < 0.05$ level.

CD 163+ TAMs Change in the Immune Microenvironment Before and After NAC

Before NAC, CD163+ TAMs were observed in the stroma of 99% (136) of patients, while CD163+ TAMs were detected in the TN of 92% (127) of patients. On the other hand, following NAC, CD163+ TAMs were detected in the stroma of 91% (125) of patients, whereas it was observed in the TN of 49% (68) of patients. Before NAC, the median percentage of CD163+ TAMs in the stroma was 20% (0–60), while in TN it was 10% (range: 0–60). After NAC, there was a statistically significant decrease ($p < 0.001$) in the median percentage of CD163+ TAMs in the stroma to 5% (1–40).

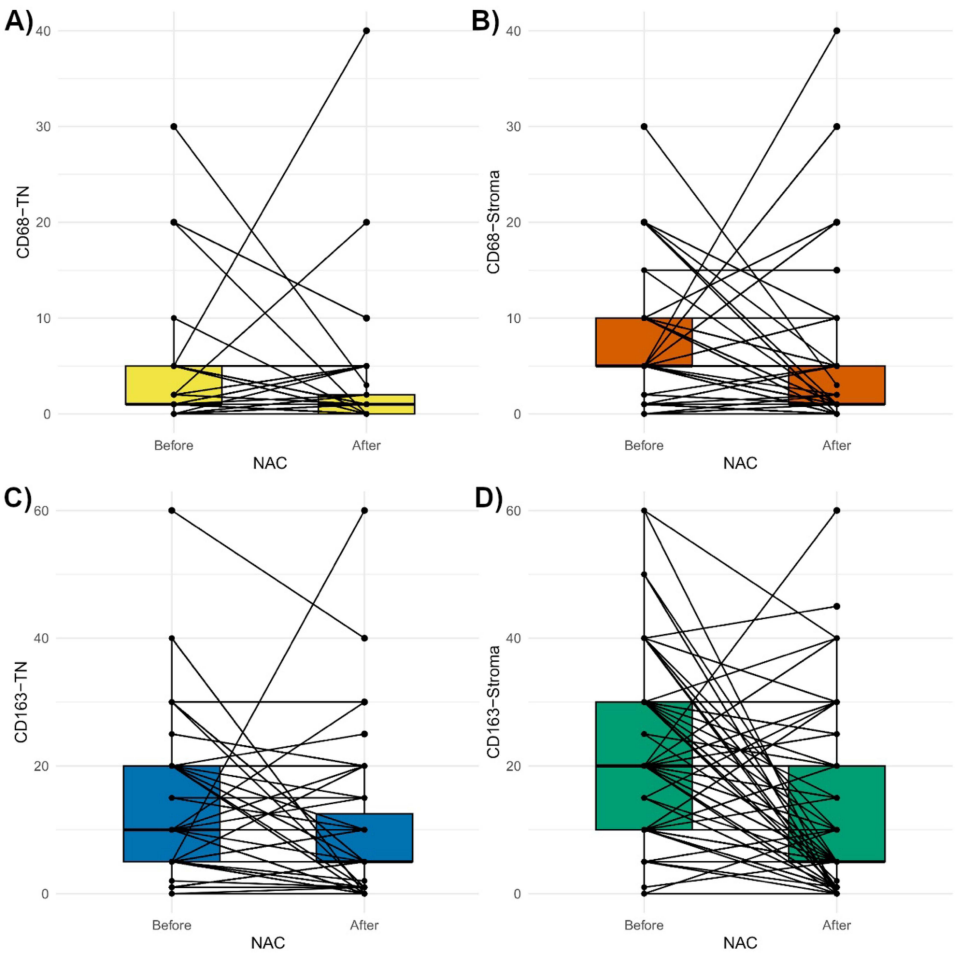

Figure 2 Boxplot graphs of the change of CD68 + and CD 163+ TAMs in the stroma and TN before and after neoadjuvant chemotherapy (NAC). Comparison of CD68 + and CD 163+ TAMs before and after NAC was done using Wilcoxon signed-rank test. (**A**) Boxplot showing the evolution of CD68+ TAMs in TN during NAC ($p<0.005$), (**B**) Boxplot showing the evolution of CD68 + TAMs in the stroma during NAC ($p<0.005$), (**C**) Boxplot showing the evolution of CD 163+ TAMs in the TN during NAC ($p<0.008$), (**D**) Boxplot showing the evolution of CD68+ TAMs in the stroma during NAC ($p<0.001$). Each boxplot represents the 25th to 75th percentile with the median indicated as the central line and whiskers indicating 1.5 × interquartile range.

In the TN, the median percentage of CD163+ TAMs also decreased to 5% (1–60) and this decrease was also statistically significant ($p<0.008$) (Table 2 and Figure 2).

Continuous Variable Analysis of TAM Correlation With NAC Response

The analysis showed a significant correlation between primary tumor size and the level of CD68+ stromal TAMs before NAC, as indicated by a coefficient of 1.032 (95% CI: 1.0037–1.0629, $p <0.05$). This suggests that larger primary tumors are accompanied by a higher infiltration of CD68+ TAMs within the stromal compartment. In addition, CD68 expression in the TN before NAC exhibited a statistically significant positive association with primary tumor size (coefficient = 1.03, 95% CI: 1.005 to 1.055, $p <0.05$). However, CD163 + stromal TAMs did not show a significant correlation with primary tumor size (coefficient = 0.99, 95% CI: 0.97 to 1.022, $p = 0.8$).

The presence of CD68+ TAMs in the stroma after NAC is an indicative of a less favorable response to chemotherapy as evidenced by the significant positive correlation with residual tumor size (coefficient = 1.05, 95% CI: 1.02 to 1.08, $p <0.005$). The presence of CD163+ TAMs in the stroma after NAC demonstrated a significant positive correlation with residual tumor size (coefficient = 1.109, 95% CI: 1.065 to 1.16, $p <0.001$). In addition, there was a significant positive correlation between CD68+ TAMs in the TN (coefficient = 1.05, 95% CI: 1.01 to 1.11, $p=0.01$) and residual tumor size.

https://doi.org/10.2147/BCTT.S493085

Similarly, CD163+ TAMs in the TN (coefficient = 1.07, 95% CI: 1.02 to 1.12, p=0.005) also showed a significant positive correlation with residual tumor size.

The change in CD 68+ and CD163+ TAMs expression from pre- to post-treatment was found to significantly influence tumor differences before and after NAC (coefficient = 1.008 95% CI: 1.003–1.012 p < 0.001 and coefficient = 1.01 95% CI: 1.005–1.014, p<0.001, respectively).

Correlation of TAMs With Various Clinicopathological Features

The differences between the density of CD68+ or CD163+ TAMs (low and high expression), before and after NAC, and various clinicopathological features is presented in Table 3. Before NAC, CD163+ TAMs in the stroma showed a significant association with TILs (OR = 1.79, 95% CI: 1.14–2.86, p = 0.013), and CD163+ TAMs in the TN revealed an even stronger association with TILs (OR = 2.28, 95% CI: 1.39–3.89, p = 0.002). Additionally, CD68+ TAMs in the TN and stroma were significantly associated with TILs (OR = 2.1, 95% CI: 1.32–3.42, p = 0.002, and OR = 2.5, 95% CI: 1.52–4.25, p = 0.0004, respectively) (Figure 3). However, no correlation was found between the presence of TAMs and TILs after NAC. We also did not find any correlation between monocytes count in peripheral blood analysis and TAMs before and after NAC.

Before NAC, the proportion of CD163+ TAMs in the stroma and TN showed a correlation with the RCB categories (OR=0.28, 95% CI: 0.09–0.84, p = 0.02, and OR=0.16 (95% CI: 0.04–0.54, p = 0.005, respectively). Following NAC, the presence of CD163+ TAMs in both the stroma and the TN demonstrated significantly elevated odds ratios of 6.09 (95% CI: 1.94–20.8, p = 0.002) and 5.84 (95% CI: 1.77–23.4, p = 0.006), respectively. Further analysis revealed significant differences in the CD163 difference, reflecting the variance in CD163+ TAMs expression before and after NAC in the stroma, across the RCB categories. Specifically, when comparing RCB class I to II, a statistically significant difference was observed with a p-value of 0.01. Similarly, comparing RCB class I to III resulted in a highly significant difference with a p-value <0.005. Additionally, a significant difference was found when comparing RCB class II to III, with a p-value <0.005. Furthermore, when compared with the pCR group, significant differences were evident across RCB II and RCB III (Figure 4). There was no statistically significant correlation observed between the presence of CD68+ TAMs in both the stroma and the TN and the RCB categories. On the other hand, the variance in CD68+ TAMs expression before and after NAC in the stroma exhibited significant distinctions across the Residual RCB categories and the pCR group (p = 0.01 for RCB class I, 0.05 for RCB class II, and 0.04 for RCB class III, compared to the pCR group) (Figure 4).

Discussion

TAMs, as an important component of the TME, play a critical role in both the response and resistance mechanisms of BC to chemotherapy.[5–8] A more comprehensive understanding of the characterization of TAMs before and after NAC could offer valuable insights into how TAMs may alter in response to treatment, potentially influencing drug resistance, metastasis and prognosis. However, the correlation between TAMs and response to NAC has not been thoroughly explored in the literature. Clinically, TAMs were associated with poor patient survival.[18–25] Ye et al retrospectively analysed the association between TAMs and the pCR rate of TNBC to NAC.[18] Patients were categorized into high and low infiltration groups based on the median of CD163+ macrophage infiltration. However, the specific numerical value of this cut-off was not provided in the article. A significantly higher pCR rate was obtained in patients with low CD163+ macrophage infiltration. In addition, survival analysis showed that OS and recurrence-free survival (RFS) rates were significantly lower in patients with high TAMs infiltration than in those with low infiltration (P=0.023 and P=0.013, respectively).[18] Furthermore, a high infiltration of CD68+ and CD163+ TAMs was correlated with worse DFS, OS and breast cancer specific survival (BCSS).[18] Zhao et al reported that CD68+ TAMs were a more sensitive prognostic indicator than CD163 in predicting OS while Ni et al reported the opposite result.[24,25] We did not perform a survival analysis in this study because, with a mean follow-up of 53 months (range: 9–105 months), there were limited breast cancer-related events (twelve in total). Specifically, two patients experienced local recurrence, ten patients developed metastasis, and five patients had cancer-related deaths during follow-up. On the other hand, our research revealed several significant associations between TAMs and tumor size before and after NAC. Specifically, CD68+ TAMs in the stroma and TN showed a positive correlation with primary tumor size before NAC, while CD163+ stromal TAMs did not show a positive correlation. Furthermore, post-NAC presence of both CD68+ and CD163+ TAMs correlated positively with residual tumor size. These findings underscore the potential of TAMs as indicators of response to treatment. In addition, subsequent analysis

Table 3 The Differences Between the Density of CD68+ or CD163+ TAMs (Low and High Expression), Before and After NAC, and Various Clinicopathological Features

Clinicopathological features (N=*138*)	CD68+ TAMs Before NAC						CD163+ TAMs Before NAC						CD68+ TAMs After NAC						CD163+ TAMs After NAC					
	Tumor			*Stroma*			*Tumor*			*Stroma*			*Tumor*			*Stroma*			*Tumor*			*Stroma*		
	nest						*nest*						*nest*						*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*	Low	High	p-*value*	Low	High	p-*value*
Age (years)			**<0.005**			*0.62*			*0.21*			*0.74*			*0.43*			*0.68*			*0.11*			*0.39*
<50	*20*	*34*		*38*	*16*		*18*	*36*		*33*	*21*		14	8		31	21		9	14		28	23	
≥ 50	52	32		62	22		37	47		49	35		40	15		46	36		23	33		39	42	
Menopausal status			0.35			0.98			0.63			0.54			0.18			0.92			0.27			0.48
Premenopausal	24	27		37	14		19	32		32	19		13	9		29	21		2	5		26	22	
Postmenopausal	48	39		63	24		36	51		50	37		41	14		48	36		40	32		41	43	
Tumor size			0.38			0.65			0.22			0.67			0.99			**<0.05**			0.99			**<0.05**
≤2cm	15	11		20	6		7	19		15	11		34	16		44	14		32	18		45	11	
>2cm	56	54		79	32		47	64		67	44		11	17		33	43		12	16		22	54	
Lymph node status			0.22			0.78			0.45			0.23			0.09			0.74			**<0.05**			**<0.005**
Absent	30	36		48	18		24	42		36	30		24	15		52	40		27	14		55	35	
Present	38	30		48	20		29	39		44	24		30	8		25	17		15	23		12	30	
Nuclear grade			0.05			0.09			0.07			0.11			**<0.005**			0.32			0.79			0.24
I	37	25		49	13		30	32		42	20		34	9		36	24		22	21		25	35	
II–III	17	25		27	15		13	29		22	20		8	12		20	20		12	10		21	18	
HR			0.97			0.62			0.73			0.18			0.059			0.60			0.055			**<0.05**
Positive	47	43		67	23		36	54		58	32		43	15		49	39		27	31		36	49	
Negative	25	23		33	15		19	29		24	24		11	8		28	18		15	6		31	16	
HER-2 status			0.07			0.62			0.29			0.79			0.34			0.23			0.81			0.11
Positive	21	29		35	15		17	33		29	21		11	7		32	18		10	8		28	19	
Negative	51	37		65	23		38	50		53	35		43	16		45	39		32	29		39	46	

(*Continued*)

https://doi.org/10.2147/BCTT.S493085

https://doi.org/10.2147/BCTT.S493085

Table 3 (Continued).

Clinicopathological features (N=*138*)	CD68+ TAMs Before NAC						CD163+ TAMs Before NAC						CD68+ TAMs After NAC						CD163+ TAMs After NAC					
	Tumor nest			*Stroma*			*Tumor nest*			*Stroma*			*Tumor nest*			*Stroma*			*Tumor nest*			*Stroma*		
	Low	High	p-*value*	Low	High	p-*value*	Low	High	p-*value*	Low	High	p-*value*	Low	High	p-*value*	Low	High	p-*value*	Low	High	p-*value*	Low	High	p-*value*
TNBC status			*0.9*			*0.93*			*0.76*			*0.43*			*0.34*			*0.96*			***<0.05***			***<0.05***
Positive	*19*	*18*		*27*	*10*		*14*	*23*		*20*	*17*		*9*	*6*		*20*	*15*		*14*	*3*		*23*	*12*	
Negative	*53*	*48*		*73*	*28*		*41*	*60*		*62*	*39*		*45*	*17*		*57*	*42*		*28*	*34*		*44*	*53*	
TILs			***<0.005***			***<0.005***			***<0.005***			***<0.05***			*0.66*			*0.77*			*0.59*			*0.81*
Category 1	*44*	*23*		*58*	*9*		*35*	*32*		*45*	*22*		*41*	*19*		*27*	*34*		*31*	*29*		*19*	*42*	
Category 2	*20*	*26*		*29*	*17*		*16*	*30*		*28*	*18*		*11*	*1*		*6*	*4*		*7*	*5*		*3*	*10*	
Category 3	*8*	*17*		*13*	*12*		*4*	*21*		*9*	*16*		*1*	*3*		*0*	*4*		*1*	*3*		*2*	*2*	
RCB.class			*0.93*			*0.63*			***0.005***			***<0.05***			*0.30*			*0.44*			***<0.005***			***<0.05***
pCR	*24*	*35*		*38*	*21*		*19*	*40*		*32*	*27*		-	-		*42*	*16*		-	-		*44*	*12*	
I	*12*	*9*		*17*	*4*		*4*	*17*		*8*	*13*		*16*	*5*		*11*	*10*		*17*	*4*		*13*	*8*	
II	*23*	*18*		*31*	*10*		*24*	*17*		*28*	*13*		*24*	*14*		*16*	*22*		*16*	*22*		*8*	*30*	
III	*13*	*4*		*14*	*3*		*8*	*9*		*14*	*3*		*13*	*4*		*8*	*9*		*6*	*11*		*2*	*15*	
Ki-67			**0.05**			0.39			0.71			0.21			0.20			0.53			0.94			**<0.05**
Low	25	13		29	9		17	21		26	12		38	6		32	22		21	23		18	34	
High	46	49		68	27		37	47		52	43		11	15		15	20		14	12		18	17	

Notes: Categorically divides TAMs into "low" and "high" groups based on their median levels. Bold values denote statistical significance at the $p < 0.05$ level. Pre-NAC "Tumor size", "Lymph node status", "TILs", and "Ki-67" were compared with pre-NAC CD68+ and CD163+ TAMs, while post-NAC "Tumor size", "Lymph node status", "TILs", and "Ki-67" were compared with post-NAC CD68+ and CD163+ TAMs.

Abbreviations: NAC, neoadjuvant chemotherapy; TILs, stromal tumor infiltrating lymphocytes; HR, hormone receptor; TNBC, triple-negative breast cancer; RCB, Residual Cancer Burden.

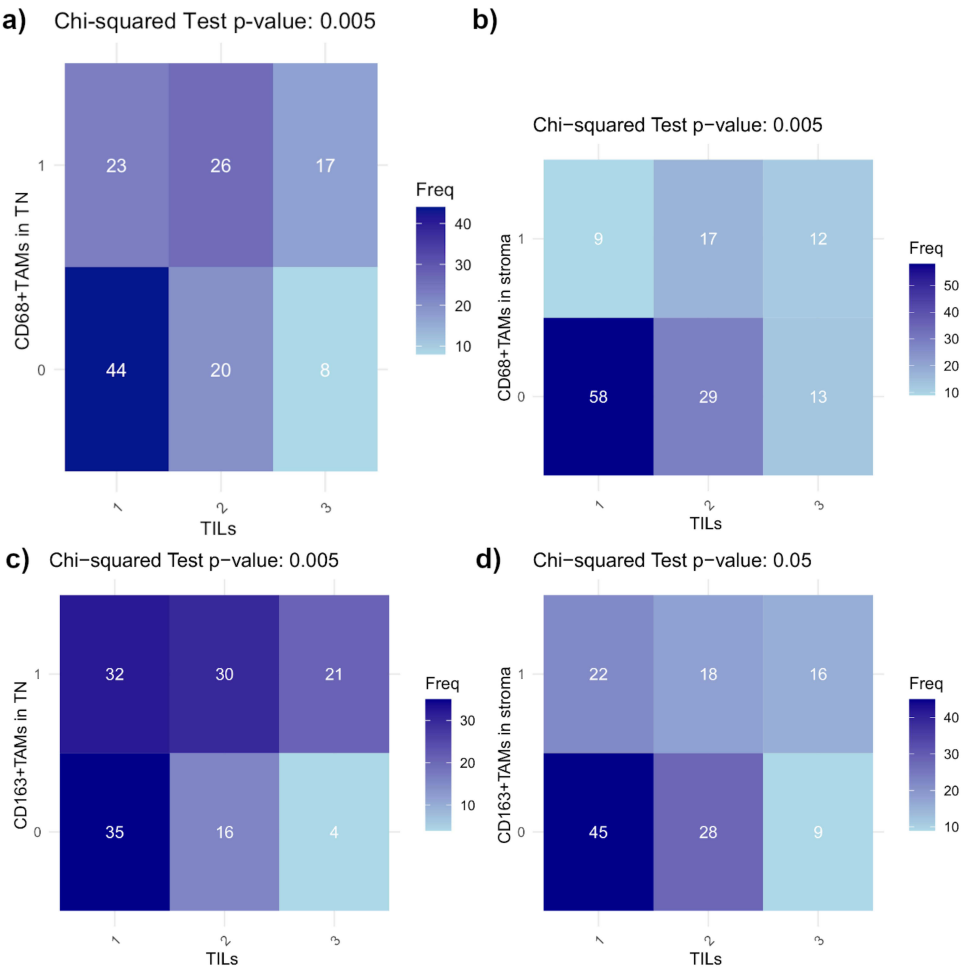

Figure 3 Heatmap illustrations showing the association between tumor-associated macrophages (TAMs) and tumor-infiltrating lymphocytes (TILs) in the tumor microenvironment before neoadjuvant chemotherapy (NAC). Chi-squared test p-values indicate statistical significance. The color intensity represents the frequency of occurrences, with darker shades indicating higher frequencies. In this analysis, 0 represents low expression of tumor-associated macrophages (TAMs), including CD68+ or CD163+ TAMs, while 1 represents higher expression. The TIL categories are based on the defined percentage ranges: Category 1 (<10%), Category 2 (≥10–40%), and Category 3 (≥40%). (**a**) CD68+ TAMs in the tumor nest (TN) versus TILs. (**b**) CD68+ TAMs in the stroma versus TILs. (**c**) CD163+ TAMs in the tumor nest (TN) versus TILs. (**d**) CD163+ TAMs in the stroma versus TILs. Chi-squared test results show p-values indicating statistical significance for all panels, with (**a**), (**b**), and (**c**) having p-values of 0.005, and (**d**) having a p-value of 0.05.

highlighted significant differences in the changes of CD163+ TAMs before and after NAC across RCB categories. This suggests that there may be potential benefit in observing changes in CD163+ TAMs expression to assess treatment response in the TME.

High density of CD163+ and CD68+ TAMs in primary BC have shown a strong association with adverse clinicopathological characteristics.[20–29] The meta-analysis result revealed that high CD68+ macrophage infiltration indicated advanced histological grade, high Ki67 expression, negative HR expression and high TNBC proportion.[24,25] In addition, high CD163+ TAM infiltration correlated with advanced histological grade, high Ki67 expression, T category and negative HR expression.[30–32] Zwager et al have found positive associations between high CD68+ and CD163+ TAMs numbers and higher tumor grade in the Luminal-B group.[33] In our study, no correlation was found between the presence of TAMs and receptor status before NAC. On the other hand, after NAC, the analysis showed a statistical correlation between HR+ and CD163+ TAMs in the stroma. In vitro studies have showed that the functions of TAMs may differ depending on the type of BC and therefore TAMs should be evaluated differently according to BC subgroups.[34,35] Compared with luminal-like BC, basal-like BC are more likely to express a broader range of receptors for macrophage-derived cytokines, which could recruit macrophages into the TME and promote monocyte differentiation into M2-like macrophages.[34–37] Levano et al have demonstrated that there are differences in the cytokine receptor profile according to breast cancer types. Basal-like cells express preferentially granulocyte monocyte colony stimulating factor (GM-CSF), hepatocyte growth factor receptor (HGFR, also known as c-MET), CD44, epithelial growth factor receptor (EGFR), transforming growth factor receptor 2 (TGFR2) and oncostatin M receptor (OSMR). Luminal-type breast cancer cells express RET (a proto-oncogene

https://doi.org/10.2147/BCTT.S493085

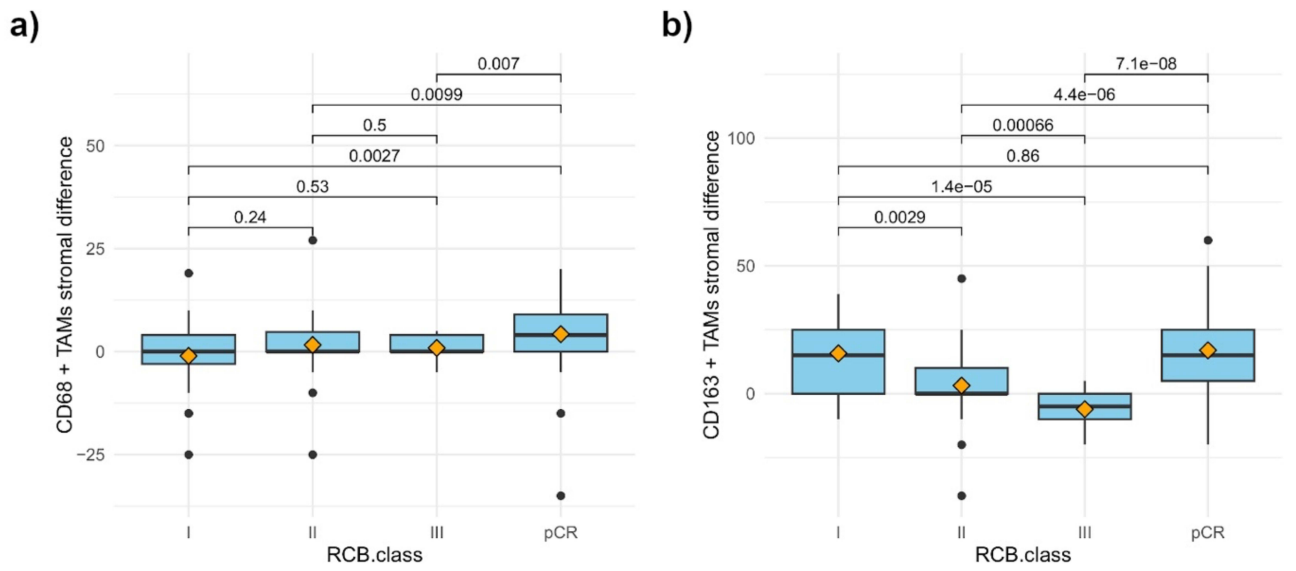

Figure 4 Boxplots that illustrate the differences in stromal tumor-associated macrophages (TAMs) expression before and after neoadjuvant chemotherapy (NAC) across different Residual Cancer Burden (RCB) classes and the pathological complete response (pCR) group. **Boxplot a)** shows the differences in CD68+ TAMs in the stroma. The p-values indicate statistically significant differences between various RCB classes and the pCR group. **Boxplot b)** displays the differences in CD163+ TAMs in the stroma. The p-values highlight significant differences between different RCB classes and the pCR group.

which encodes for a receptor tyrosine kinase for members of the glial cell line-derived neurotrophic factor).[17] These results allow us to conclude that TAMs have a different influence depending on the BC subtype. The results of our study showed a positive correlation between the presence of CD163+ TAMs in both TN and stroma, and lymph node positivity after NAC ($p<0.05$). Additionally, a correlation was noted between CD163+ TAM expression in the stroma post-NAC and Ki-67 expression post-NAC. Our results also showed an association between TAMs and TILs before NAC and this suggest significant interactions between TAMs and TILs within the TME.

Although the importance of TAMs in the TME of BC has been highlighted by extensive research, some controversies exist. Firstly, it is unclear which macrophage biomarkers can be used for prognosis prediction of TAMs and the relevance of these biomarkers to various breast cancer subtypes. CD68 has been widely used as a human pan-macrophage marker. However, CD68 as a marker for TAMs has some limitations. CD68 is expressed by a wide variety of cells, including fibroblasts, granulocytes, dendritic cells, endothelial cells, and some lymphoid subsets and, as a pan-macrophage marker, CD68 is unable to distinguish TAM subpopulations.[13,28,38] While many markers such as CD163, CD204, and CD206 were used for M2 macrophages, markers such as CD11c, CD80, and CD86 were used for M1 macrophages.[31,39] A study using CD68 and CD163 to detect TAMs showed a high density of CD163+ TAMs rather than CD68+ TAMs in TME.[40] Our research revealed similar results. CD68+ TAMs in the stroma before NAC showed a median value of 5, with values ranging from 0 to 30. In contrast, CD163+ TAMs in the stroma before NAC demonstrated a higher median value of 20, with values spanning from 0 to 60. After NAC, the median of CD163+ TAMs in the stroma was also higher than CD68+ TAMs. In vitro study with Basal-like BC cell line suggested that since cancer line cells produce high amounts of colony stimulating factor-1 (CSF-1), CSF-1 induces M2 polarization and therefore CD163+ macrophage expression increases in TME.[12] Secondly, variations exist among studies regarding the classification of macrophages as stromal, TN or total.[15,24,41] Finally, the cut-off value also varies between publications. The majority of these studies utilized the median number of macrophages as the cut-off value to categorize TAMs into high and low TAM groups.[15,24] As a result, the findings of the meta-analysis strikingly highlight the disparities in the literature concerning the evaluation of TAMs.

This study has both strengths and limitations. One of the strengths is the exploration of TAMs in the TME before and after NAC. Additionally, we used different macrophage markers to understand the functional heterogeneity, which is reflected by the heterogeneous expression of TAM markers. However, there are limitations to our exploratory study that need to be acknowledged. Most notably, it is a retrospective study. In addition, the sample group was heterogeneous, and the sample size small.

Conclusion

In conclusion, our study confirms the important role of TAMs in the TME of BC. TAMs, especially CD163+ TAMs, are strongly linked to worse clinical features and poorer treatment outcomes. The distinct behavior of TAMs across different BC subtypes highlights the need for subtype-specific evaluation and treatment strategies. Despite these findings, inconsistencies in macrophage classification and biomarker cut-off values across studies underscore the necessity for standardized approaches in future research to accurately evaluate TAMs' impact across various BC subtypes. Future research should focus on standardizing TAM assessment methods and further investigating the interactions between TAMs and TILs to better understand their combined influence on BC progression and treatment outcomes.

Ethics Approval

This retrospective study was conducted in accordance with the ethical principles outlined in the Declaration of Helsinki and was approved by the University Hospital Antwerp (UZA), File number: 20/26/349, Edge number: 001251.

All data used in the study were anonymized prior to analysis to ensure patient confidentiality and privacy. No direct patient contact occurred, and no identifiable personal information was used. The study adhered to all relevant ethical and legal standards for the use of retrospective data in medical research.

Funding

This study is funded by UZA Foundation grant and Kocaeli University, Department of Scientific Research Projects with the following grant numbers "TSA-2019-1611".

Disclosure

The authors declare that they have no competing interest.

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