RESEARCH ARTICLE

Differential Effects of Anthracycline-based Neoadjuvant Chemotherapy on Stromal and Intratumoral FOXP3$^+$ Tumor-Infiltrating Lymphocytes in Invasive Breast Cancer of No Special Type

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Abstract

BACKGROUND: Neoadjuvant chemotherapy (NAC) plays a crucial role in the management of invasive breast cancer with no special type (IBC-NST), with the immune system's response to cancer heavily relying on the dynamics between tumor-infiltrating lymphocytes (TILs) and cancer cells. In this study, the differential effects of anthracycline-based NAC on stromal and intratumoral foxhead box P3 (FOXP3$^+$) TILs expressions were specifically examined.

METHODS: In this cross-sectional study, 32 IBC-NST samples were evaluated for pre- and post-NAC FOXP3$^+$ TIL expression as well as the changes of FOXP3$^+$ TIL expression. Comprehensive data collection regarding subjects' age, tumor size, grade, lymphovascular invasion, regional lymph node metastasis, and receptor status were conducted. Immunohistochemistry was utilized to quantify FOXP3$^+$ TILs. The stromal, intratumoral and total FOXP3$^+$ TILs expression were then analyzed.

RESULTS: Significant reductions in FOXP3$^+$ TIL expression post-NAC were observed, with stromal FOXP3$^+$ TILs showing a median decrease of 3.6 units in subjetcs aged ≥50 years (p=0.013) and a median decrease of 13.2 units in subjects with tumors ≥5 cm after NAC (p=0.006). In contrast, intratumoral FOXP3$^+$ TILs remained relatively stable, with minor changes. The total FOXP3$^+$ TIL expression, combining stromal and intratumoral components, was significantly decreased with a median of 13.0 units decreased to 5.3 units (p<0.001).

CONCLUSION: This study highlights the significant reduction in stromal FOXP3$^+$ TIL expression after NAC treatment in IBC-NST subjects, in contrast to the relatively stable intratumoral FOXP3$^+$ TILs. Understanding these differences may guide future therapeutic strategies and improve treatment outcomes for IBC-NST.

KEYWORDS: biomarkers, chemotherapy, FOXP3, prognostic, response, lymphocyte

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Introduction

Breast cancer accounted for roughly 30% of all new cancer diagnoses in women worldwide.(1-5) Invasive breast cancer (IBC) is a form of breast cancer that has the potential to disseminate to nearby tissues and organs.(6-8) Investigating the involvement of the immune system in IBC, namely in invasive breast cancer with no special type (IBC-NST), is an essential area of study. In this particular case, tumor-infiltrating lymphocytes (TILs), particularly those that express forkhead box P3 (FOXP3), are identified as important contributors.(9) FOXP3$^+$ TILs, which are mainly composed of regulatory T cells (Tregs), play a crucial

role in regulating immunological responses in the tumor microenvironment.(10)

Neoadjuvant chemotherapy (NAC) is a widely acknowledged treatment for patients diagnosed with IBC-NST. Treatment with NAC is aimed at shrinking tumors so that breast-conserving surgery may be performed, or at minimizing the need for mastectomy. Despite NAC's effectiveness in shrinking tumors, some patients do not respond to the treatment, and their tumors continue to develop.(11) The reasons for this dearth of response are not completely understood, and a greater understanding of the mechanisms involved is essential for optimizing breast cancer treatment outcomes.(12)

The immune system plays a pivotal role in tumor development and progression, and cancer interventions such as immunotherapy seek to stimulate immune responses against cancer cells.(13) TILs are immune cells that permeate tumor tissue and are associated with increased survival in patients with breast cancer.(14) TILs are composed of numerous subsets of immune cells, including $CD8^+$ cytotoxic T cells, $CD4^+$ helper T cells, and regulatory T cells (Tregs).(15) Tregs are a subpopulation of $CD4^+$ T cells that suppress immune responses and promote tolerance.(16) FOXP3 is the principal regulator of Tregs, and its expression is frequently used as a Treg marker.(16)

$FOXP3^+$ TILs are a subset of T cells, specifically Tregs, which maintain immune tolerance and modulate the immune response against tumor cells.(17) A growing body of evidence suggests that the presence of $FOXP3^+$ TILs in the tumor microenvironment is associated with a variety of clinical outcomes in various forms of cancer, with some studies reporting a favorable prognosis and others implying a worse clinical outcome.(18)

The differentiation between stromal and intratumoral $FOXP3^+$ TILs is essential in the complex field of tumor immunity. Stromal $FOXP3^+$ TILs, which are present in the connective tissue surrounding tumor cells, and intratumoral $FOXP3^+$ TILs, which are positioned inside the tumor parenchyma, may have distinct effects on tumor behavior and response to treatment.(19) The investigation of stromal versus intratumoral $FOXP3^+$ TILs in the setting of NAC in IBC-NST has not been thoroughly explored.(19) The examination of these lymphocytes has the ability to reveal and perhaps determine the tumor's reaction to chemotherapy, which could lead to new insights into the interactions between tumors and the immune system in IBC-NST.

Several studies have examined the association between TILs and NAC response in breast cancer.(20,21) However, the precise function of $FOXP3^+$ TILs in the NAC-induced IBC-NST response remains unknown.(20,22) In this study, we examine the correlation between changes in $FOXP3^+$ TILs expression and the NAC-induced IBC-NST response. We hypothesize that variations in $FOXP3^+$ TILs expression following administration of NAC will correlate with therapeutic efficacy. Therefore this study was conducted to characterize and to examine the association of the $FOXP3^+$ TILs expression in IBC-NST before and after NAC, the NAC-induced IBC-NST response.

Methods

Study Design and Data Collection

In this cross-sectional study, data from the Anatomical Pathology Database of the Faculty of Medicine, Universitas Indonesia, from September 2015 to February 2022 were gathered. The database provided access to detailed records of paraffin-embedded breast cancer samples. Thirty-two paraffin-embedded breast cancer samples were collected from women diagnosed with IBC-NST and treated with anthracycline-based NAC. Subjects with systemic diseases were not included in the study.

Information was gathered on the patient's age, tumor size, tumor grade, lymphovascular invasion (LVI), regional lymph node metastasis (RLNM), estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2), and Ki67 level. Meanwhile, breast cancer samples were examined quantitatively before and after the treatment with NAC. The protocol of this study was approved by The Institutional Review Board of Universitas Indonesia (No. KET-131/UN2.F1/ETIK/PPM.00.02/2022) in February 2022. The research protocol was made in accordance of on the Declaration of Helsinki.(23)

Slides Preparation and Immunohistochemistry (IHC)

The IHC staining procedures was developed in accordance with previous publications.(7,24-28) Breast cancer tissue samples were collected before and after NAC administration was analyzed. The biopsies yielded tissue samples that were prepared as paraffin slabs. From each paraffin block, a 4 μm slice was done using a microtome and then labeled and dehydrated. Tissue sections were deparaffinized, rehydrated in ethanol and running water, and then treated with hydrogen peroxide. The antigen was recovered with Tris EDTA at a pH of 9.0, and then the tissue sections were cleansed with phosphate-buffered saline. The tissue sections were treated with primary anti-FOXP3 antibody (Cat No. ab75763, Abcam, Cambridge, UK), biotinylated secondary

antibody (Cat No. ab99818, Abcam), and then cleansed and incubated with a diaminobenzidine tetrahydrochloride (Cat No. ab64238, Abcam). To better locate the target, Hematoxylin Mayer counterstaining was performed, followed by cleansing and drying with ethanol and xylol. Each sample contained both a negative and a positive control, and tonsil tissue served as the quality standard. The expression of FOXP3$^+$ TILs was measured quantitatively both before and after treatment with NAC.

FOXP3$^+$ TILs Expression Calculation

Two researchers independently assessed the FOXP3$^+$ TILs expression. Based on the findings of the IHC analysis of FOXP3$^+$ TILs expression, the tumor tissue slides were selected and evaluated. IHC analysis was used to evaluate intratumoral and stromal expression, and Image J software (National Institutes of Health, Bethesda, MD, USA) was used to analyze the results. Five fields of each slide were examined at 400x magnification using a 400x light microscope (Olympus Bx51, Olympus, Tokyo, Japan) for lymphocytes, and the total intratumoral and stromal FOXP3$^+$ TILs expression was calculated.

Statistical Analysis

Data was entered into a master table in Microsoft Excel (Microsoft Corporation, Redmond, WA, USA) before analysis. Statistical analysis was performed using RStudio (Posit, Boston, MA, USA), and the ggplot2 tool was used for data visualization. The Mann-Whitney U test or the Kruskal-Wallis test was used for univariate analysis of all variables. The Wilcoxon signed-rank test was used to compare baseline FOXP3$^+$ TIL expression levels to those obtained following NAC therapy.

Results

In current study, the overall reduction in FOXP3$^+$ TIL expression after therapy was depicted (Figure 1A, Figure 1B), emphasizing the efficacy of NAC in regulating the immunological environment. Figure 1C and Figure 1D showed a more detailed examination results, differentiating between stromal (highlighted by black arrows) and intratumoral (highlighted by red arrows) TILs. This visual evidence corroborates the following quantitative data.

Our analysis, segmented into distinct subsections based on the data obtained, revealed significant findings regarding FOXP3$^+$ TIL expressions pre- and post-NAC treatment in various clinicopathological features. The results were systematically presented in Table 1 for stromal FOXP3$^+$ TIL expression, Table 2 for intratumoral FOXP3$^+$ TIL expression, and Table 3 for total FOXP3$^+$ TIL expression, detailing medians before and after NAC, changes in expression, and associated *p*-values for each parameter.

Stromal FOXP3$^+$ TIL Expression

Significant reductions in stromal FOXP3$^+$ TIL expression were observed post-NAC across multiple clinicopathological features. For instance, subjects aged ≥50 years showed a median decrease of 3.6 units in stromal FOXP3$^+$ TIL expression, with this change being statistically significant (p=0.013). Similarly, tumor size after NAC treatment demonstrated a significant association with changes in stromal FOXP3$^+$ TIL expression, especially in tumors that remained ≥5 cm, indicating a median decrease of 13.2 units (p=0.006).

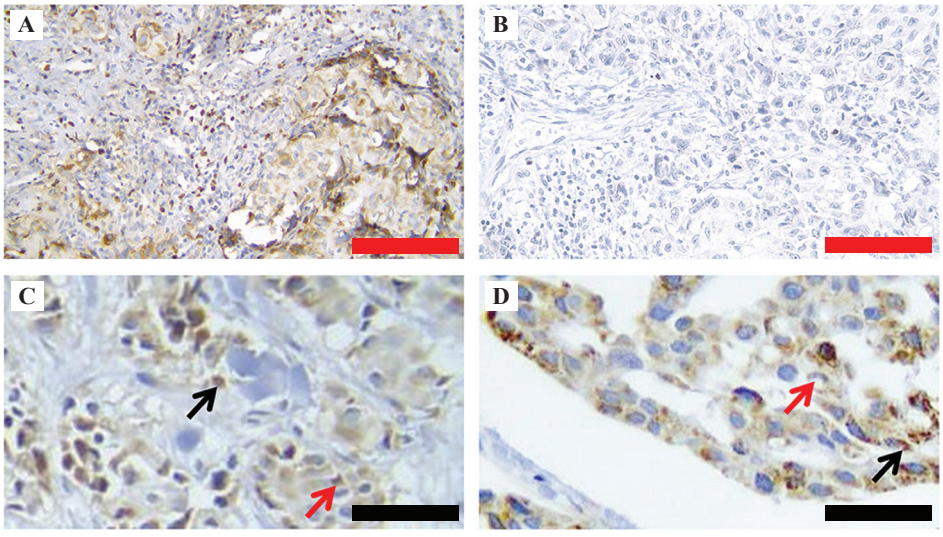

Figure 1. Expression of FOXP3$^+$ TILs before NAC and after NAC treatment. A: expression before NAC; B: expression after NAC; C: detailed stromal and intratumoral expression before NAC; D: detailed stromal and intratumoral expression after NAC. Black arrow: stromal expression; Red arrow: intratumoral expression. Red bar: 50 µm; Black bar: 10 µm.

Table 1. The subgroup analysis based on clinicopathological features in pre-NAC, post-NAC, and changes of stromal FOXP3⁺ TIL expression.

Variable	n	Pre–NAC		Post–NAC		Changes	
		Median (min–max)	*p*–value	Median (min–max)	*p*–value	Median (min–max)	*p*–value
Age							
≥50 years old	17	15.4 (1.8–72.8)	0.478	5.4 (2.2–26.4)	0.350	3.6 (-5.20–68.2)	0.013*
<50 years old	15	12.4 (3.0–19.2)		4.4 (0.6–12.4)		8.2 (-0.2–12.8)	
Tumor size before NAC							
≥5 cm	22	12.2 (1.8–34.8)	0.646	6.2 (0.6–26.4)	0.129	4.7 (-5.2–26.0)	0.260
<5 cm	10	14.4 (3.0–72.8)		4.4 (2.2–6.4)		9.6 (-0.20–68.2)	
Tumor size after NAC							
≥5 cm	7	22.6 (7.2–72.8)	0.030*	7.2 (2.2–26.4)	0.148	13.2 (-3.8–68.2)	0.006*
<5 cm	25	12.0 (1.8–34.8)		4.4 (0.6–12.4)		7.8 (-5.2–26.0)	
Tumor grade before NAC							
3	10	10.9 (2.0–34.8)		4.6 (2.6–12.0)		5.9 (-1.0–26.0)	
2	19	13.8 (1.8–72.8)	0.671	4.8 (0.6–26.4)	0.883	8.6 (-5.2–68.2)	0.669
1	3	13.0 (4.2–13.0)		4.4 (2.4–12.4)		0.6 (-0.2–10.6)	
Tumor grade after NAC							
3	12	16.9 (1.8–72.8)		7.1 (2.2–26.4)		5.9 (-5.2–68.2)	
2	15	12.4 (2.4–34.6)	0.542	4.4 (2.4–12.0)	0.157	8.6 (-1.0–23.6)	0.629
1	5	11.2 (3.0–19.6)		2.2 (0.6–12.4)		5.8 (0.6–15.2)	
LVI							
Yes	15	13.8 (1.8–72.8)	0.153	5.8 (0.6–9.2)	0.628	10.6 (-5.2–68.2)	0.180
No	17	11.6 (2.0–34.6)		3.4 (1.6–26.4)		3.6 (-3.8–22.6)	
RLNM							
Yes	16	12.7 (1.8–72.8)	0.867	4.7 (2.2–9.0)	0.564	9.6 (-5.2–68.2)	0.123
No	16	12.9 (2.0–34.6)		5.2 (0.6–25.8)		6.8 (-3.8–22.6)	
ER status							
Positive	31	12.8 (1.8–72.8)	-	4.8 (0.6–26.4)	-	7.8 (-5.2–68.2)	-
Negative	1	-		-		-	
PR status							
Positive	18	12.5 (3.2–34.6)	0.985	4.4 (0.6–26.4)	0.896	8.0 (-3.8–22.6)	0.077
Negative	14	14.1 (1.8–72.8)		5.3 (2.2–9.2)		7.2 (-5.2–68.2)	
HER2 status							
Positive	13	12.8 (2.0–34.8)	0.880	5.8 (2.2–26.4)	0.623	3.6 (-3.8 –26.0)	0.613
Negative	19	13.0 (1.8–72.8)		4.6 (0.6–12.4)		8.2 (-5.2–68.2)	
Ki67 status							
Positive	21	12.4 (2.0–72.8)	0.254	4.6 (1.6–26.4)	0.611	3.6 (-3.8–68.2)	0.984
Negative	11	15.4 (1.8–34.8)		5.8 (0.6–12.4)		12.6 (-5.2–26.0)	

The Mann-Whitney U test or the Kruskal-Wallis test was used for the univariate analysis. *p-value≤0.05 indicates statistical significance.

Intratumoral FOXP3⁺ TIL Expression

Changes in intratumoral FOXP3⁺ TIL expression post-NAC were less pronounced. The median change across all age groups and tumor sizes before NAC remained close to zero, reflecting minimal variation in intratumoral FOXP3⁺ expression following treatment. However, LVI status showed a significant median change in intratumoral FOXP3⁺ TIL expression, particularly in the absence of LVI, with a decrease of 0.0 units (p=0.034).

Total FOXP3⁺ TIL Expression

The total FOXP3⁺ TIL expression, combining both stromal and intratumoral components, exhibited substantial decreases post-NAC. For example, in the subgroup of subjects with tumors ≥5 cm after NAC, a significant median reduction of 13.2 units in total FOXP3⁺ TIL expression was recorded (p=0.015). This suggested a broad impact of NAC on diminishing the presence of FOXP3+ TILs within the tumor microenvironment.

Impact of NAC Treatment on Intratumoral, Stromal, and Total FOXP3⁺ TILs Expressions

In this study, the FOXP3⁺ TILs expressions were specifically separated into stromal, intratumoral, and total expressions, and then analyzed for both before and after NAC treatment, as shown in Table 4. There was a notable decrease in the expression of stromal FOXP3⁺ TIL, dropping from a median of 12.9 (1.8-72.8) before NAC to 4.7 (0.6-26.4) after NAC (p<0.001). The expression of FOXP3⁺ TIL within the tumor remained rather stable, with small changes observed before

Table 2. The association between clinicopathological features in pre-NAC, post-NAC, and changes of intratumoral FOXP3⁺ TIL expression.

Variable	n	Pre-NAC		Post-NAC		Changes	
		Median (min-max)	*p*-value	Median (min-max)	*p*-value	Median (min-max)	*p*-value
Age							
≥50 years old	17	0.2 (0.0–2.2)	0.433	0.2 (0.0–5.0)	0.576	0.0 (-0.4–5.0)	0.281
<50 years old	15	0.0 (0.0–1.0)		0.0 (0.0–15.0)		0.0 (-0.8–14.0)	
Tumor size before NAC							
≥5 cm	22	0.1 (0.0–1.0)	0.952	0.0 (0.0–15.0)	0.857	0.0 (-0.8–14.0)	0.134
<5 cm	10	0.0 (0.0–2.2)		0.2 (0.0–1.8)		0.0 (-0.6–1.2)	
Tumor size after NAC							
≥5 cm	7	0.0 (0.0–2.2)	0.503	0.0 (0.0–5.0)	0.964	0.0 (-0.4–5.0)	0.957
<5 cm	25	0.2 (0.0–1.0)		0.0 (0.0–15.0)		0.0 (-0.8–14.0)	
Tumor grade before NAC							
3	10	0.2 (0.0–1.0)		0.3 (0.0–15.0)		0.2 (-0.4–14.0)	
2	19	0.0 (0.0–2.2)	0.738	0.0 (0.0–1.8)	0.324	0.0 (-0.8–1.2)	0.086
1	3	0.0 (0.0–0.4)		0.0 (0.0–1.2)		0.0 (0.0–0.8)	
Tumor grade after NAC							
3	12	0.2 (0.0–2.2)		0.3 (0.0–15.0)		0.0 (-0.4–14.0)	
2	15	0.0 (0.0–0.6)	0.448	0.2 (0.0–5.0)	0.092	0.0 (-0.4–5.0)	0.426
1	5	0.0 (0.0–0.8)		0.0 (0.0–0.0)		0.0 (-0.8–0.0)	
LVI							
Yes	15	0.2 (0.0–2.2)	0.882	0.2 (0.0–2.4)	0.576	0.0 (-0.4–1.4)	0.034*
No	17	0.0 (0.0–1.0)		0.0 (0.0–15.0)		0.0 (-0.8–14.0)	
RLNM							
Yes	16	0.3 (0.0–2.2)	0.402	0.1 (0.0–3.0)	0.752	0.0 (-0.4–2.4)	0.047*
No	16	0.0 (0.0–1.0)		0.0 (0.0–15.0)		0.0 (-0.8–14.0)	
ER status							
Positive	31	0.0 (0.0–2.2)	-	0.0 (0.0–15.0)	-	0.0 (-0.8–14.0)	-
Negative	1	-		-		-	
PR status							
Positive	18	0.0 (0.0–1.0)	0.837	0.1 (0.0–15.0)	0.536	0.0 (-0.8–14.0)	0.038*
Negative	14	0.1 (0.0–2.2)		0.0 (0.0–2.4)		0.0 (-0.6–1.4)	
HER2 status							
Positive	13	0.0 (0.0–1.0)	0.821	0.2 (0.0–5.0)	0.821	0.0 (-0.6–5.0)	0.428
Negative	19	0.0 (0.0–2.2)		0.0 (0.0–15.0)		0.0 (-0.8–14.0)	
Ki67 status							
Positive	21	0.2 (0.0–2.2)	0.144	0.2 (0.0–15.0)	0.289	0.0 (-0.8–14.0)	0.571
Negative	11	0.0 (0.0–1.0)		0.0 (0.0–5.0)		0.0 (-0.2–5.0)	

The Mann-Whitney U test or the Kruskal-Wallis test was used for the univariate analysis. *p-value≤0.05 indicates statistical significance.

and after NAC treatment (p=0.106). The overall expression of FOXP3⁺ TILs also significantly decreased after NAC, with a median value of 13.0 (2.0-75.0) decreasing to 5.3 (0.6-26.4). This indicates a major reduction in FOXP3⁺ TILs after treatment, which was statistically significant (p<0.001).

Figure 2 displayed a time series (before and after) that illustrates the overall impact of NAC on the changes in intratumoral, stromal, and total FOXP3⁺ TILs expressions. This visual depiction facilitated comprehension of the fluctuating characteristics of immune response alteration during the duration of NAC therapy. The trends illustrated in Figure 2 supported the findings of the data analysis, indicating a substantial reduction in FOXP3⁺ TIL expression in various tumor areas.

Discussion

This study demonstrates that patients with IBC-NST benefit from NAC therapy, with a notable decrease in the number of FOXP3⁺ TILs observed. Using FOXP3⁺ TILs as a potential biomarker for NAC therapy response in breast cancer is supported by the response of FOXP3⁺ TILs to NAC treatment and the relationship between this response and clinical parameters.

The significant reduction in stromal FOXP3⁺ TIL expression post-NAC, as opposed to the unchanged intratumoral FOXP3⁺ expression, underscores a pivotal aspect of tumor microenvironment dynamics in IBC-NST. This phenomenon may reflect the differential susceptibilities

Table 3. The association between clinicopathological features in pre-NAC, post-NAC, and changes of total FOXP3⁺ TIL expression.

Variable	n	Pre-NAC		Post-NAC		Changes	
		Median (min-max)	*p*-value	Median (min-max)	*p*-value	Median (min-max)	*p*-value
Age							
≥50 years old	17	15.8 (2.0-75.0)	0.478	6.2 (2.6-26.4)	0.390	3.6 (-5.0 – 68.6)	0.029*
<50 years old	15	12.4 (3.6-20.2)		4.4 (0.6-26.0)		6.6 (-5.8 – 12.8)	
Tumor size before NAC							
≥5 cm	22	12.2 (2.0-35.8)	0.675	6.6 (0.6-26.4)	0.219	3.5 (-5.8 – 23.6)	0.243*
<5 cm	10	14.7 (3.6-75.0)		4.4 (2.2-7.2)		9.3 (-0.2 – 68.6)	
Tumor size after NAC							
≥5 cm	7	22.6 (7.2-75.0)	0.030*	7.2 (2.6-26.4)	0.148	13.2 (-3.8 – 68.6)	0.015*
<5 cm	25	12.0 (2.0-35.8)		4.4 (0.6-26.0)		6.6 (-5.8 – 23.6)	
Tumor grade before NAC							
3	10	11.2 (2.0-35.8)	0.655	4.7 (3.0-26.0)	0.830	1.8 (-5.8 – 23.6)	0.512
2	19	13.8 (2.0-75.0)		6.2 (0.6-26.4)		8.4 (-5.0 – 68.6)	
1	3	13.0 (4.2-13.4)		4.4 (3.6-12.4)		0.6 (-0.2 – 9.8)	
Tumor grade after NAC							
3	12	17.1 (2.0-75.0)	0.488	7.1 (2.6-26.4)	0.124	3.4 (-5.8 – 68.6)	0.786
2	15	12.4 (2.8-34.6)		4.4 (3.0-17.0)		8.4 (-0.8 – 17.6)	
1	5	11.2 (3.6-19.6)		2.2 (0.6-12.4)		6.6 (0.6 – 15.2)	
LVI							
Yes	15	13.8 (2.0-75.0)	0.132	6.4 (0.6-12.2)	0.433	9.8 (-5.0 – 68.6)	0.204
No	17	11.6 (2.0-34.6)		3.6 (1.6-26.4)		1.4 (-5.8 – 17.6)	
RLNM							
Yes	16	12.9 (2.0-75.0)	0.867	5.3 (2.6-12.2)	0.616	9.2 (-5.0 – 68.6)	0.131
No	16	13.0 (2.0-34.6)		5.6 (0.6-26.4)		5.1 (-5.8 – 17.6)	
ER status							
Positive	31	13.0 (2.0-75.0)	-	5.8 (0.6-26.4)	-	6.6 (-5.8 – 68.6)	-
Negative	1	-		-		-	
PR status							
Positive	18	12.5 (3.6-34.6)	0.955	4.4 (0.6-26.4)	0.955	6.6 (-5.8 – 17.6)	0.104
Negative	14	14.4 (2.0-75.0)		6.1 (2.2-12.2)		6.7 (-5.0 – 68.6)	
HER2 status							
Positive	13	13.0 (2.0-35.8)	0.880	5.8 (2.2-26.4)	0.762	3.6 (-3.8 – 23.6)	0.376
Negative	19	13.0 (2.0-75.0)		4.8 (0.6-26.0)		6.6 (-5.8 – 68.6)	
Ki67 status							
Positive	21	12.4 (2.0-75.0)	0.271	4.8 (1.6-26.4)	0.696	3.4 (-5.8 – 68.6)	0.788
Negative	11	15.8 (2.0-35.8)		5.8 (0.6-17.0)		12.6 (-5.0 – 23.6)	

The Mann-Whitney U test or the Kruskal-Wallis test was used for the univariate analysis. *p-value≤0.05 indicates statistical significance.

of stromal and intratumoral compartments to chemotherapy. The observed disparity in the response of stromal and intratumoral FOXP3⁺ TILs to NAC might be further explained by considering the molecular pathways involved in the regulation of these cells within the tumor microenvironment. The stroma, being more accessible to the systemic circulation, is likely exposed to higher concentrations of chemotherapeutic agents, which could directly impact the viability and functionality of FOXP3⁺ TILs located in this region.(29) This exposure could lead to a significant reduction in stromal FOXP3⁺ TILs through mechanisms such as increased apoptosis or altered trafficking of these cells out of the tumor microenvironment.(29,30)

In contrast, intratumoral FOXP3⁺ TILs, which are embedded within the tumor mass and in direct contact with cancer cells, might be influenced by a unique set of microenvironmental factors, including cytokines and growth factors secreted by the tumor cells themselves.(31) These factors could promote the survival and suppressive function of FOXP3⁺ TILs, thus rendering them more resistant to the effects of chemotherapy. Moreover, the tumor microenvironment can induce a state of hypoxia, which has been shown to support the maintenance and function of Tregs through pathways involving hypoxia-inducible factor 1-alpha (HIF-1α).(32)

Furthermore, the differential impact of NAC on stromal versus intratumoral FOXP3⁺ TILs might also reflect the role of chemokine gradients in mediating the localization and retention of these cells. Chemokines such as CCL22, secreted by tumor cells and other cells in the tumor microenvironment, are known to attract Tregs via

Table 4. Fluctuations in the expression of FOXP3$^+$ TILs both before and after NAC treatment.

FOXP3$^+$ TIL	Category	n	Median (min–max)	*p* –value
Stromal	Before	32	12.9 (1.8–72.8)	<0.001*
	After	32	4.7 (0.6–26.4)	
Intratumoral	Before	32	0.0 (0.0–2.2)	0.106
	After	32	0.0 (0.0–15.0)	
Total	Before	32	13.0 (2.0–75.0)	<0.001*

Wilcoxon signed rank test was utilized for univariate analysis. *p-value≤0.05 indicates statistical significance.

their CCR4 receptor (33,34), potentially contributing to the relatively stable levels of intratumoral FOXP3$^+$ TILs post-NAC treatment.

The unchanged expression of intratumoral FOXP3$^+$ TILs despite NAC treatment raises questions regarding their direct interaction with tumor cells and the correlation to clinical outcomes. Investigating the expression of CD8$^+$ TILs and the CD8$^+$/FOXP3$^+$ TIL ratio could illuminate the changes in cytotoxic and regulatory T cell dynamics within the tumor stroma and intratumoral regions, offering insights into the immune evasion mechanisms employed by tumor cells and the differential modulation of immune responses by NAC.(35) The necessity of evaluating the expression of CD8$^+$ TILs and the CD8$^+$/FOXP3$^+$ TIL ratio is underscored by these considerations, as changes in these parameters could provide further insight into the balance between cytotoxic and regulatory immune responses following NAC. An increase in the CD8$^+$/FOXP3$^+$ ratio, for example, could indicate a shift towards a more cytotoxic environment conducive to tumor clearance, especially in the stroma, where FOXP3$^+$ TILs are significantly reduced post-treatment.(35)

It is also critical to delineate whether the observed associations between stromal FOXP3$^+$ TILs and NAC response are specific to IBC-NST. This specificity could be influenced by the unique tumor biology of IBC-NST, including its molecular and cellular composition, which may differentially modulate the immune landscape in response to chemotherapy.(36) Further, the specific association of stromal FOXP3$^+$ TIL reduction with NAC response in IBC-NST highlights the potential of targeting stromal components to enhance chemotherapy efficacy. This specificity suggests that modulating the stromal tumor, possibly through combined immunotherapeutic approaches, could be a strategic direction for improving treatment outcomes in IBC-NST. Recent studies corroborate our findings, demonstrating a variable impact of chemotherapy on TIL populations across different tumor types. For instance, a similar reduction in stromal FOXP3$^+$ TILs in rectal cancer following chemotherapy was observed, suggesting a common mechanism of immune modulation by NAC across cancers.(37) Conversely, an increase in intratumoral CD8$^+$ TILs post-NAC in gastric cancer was reported, highlighting the diverse effects of NAC on the

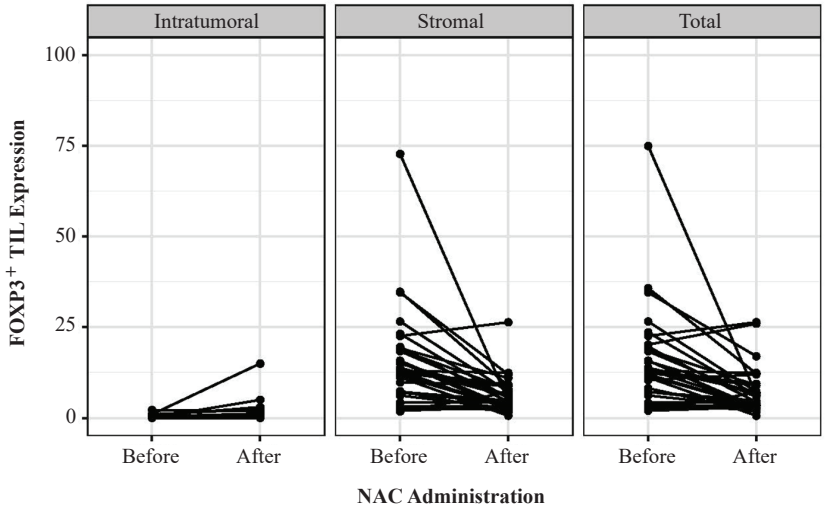

Figure 2. A time series depicting the cumulative effect of NAC on intratumoral, stromal, and total FOXP3$^+$ TIL expressions change.

tumor microenvironment and the need for cancer-specific studies.(38)

The present study has some limitations that must be acknowledged. Only 32 samples comprised the sample size, which was relatively small. This could potentially limit the generalizability of the findings and the ability to detect smaller effect sizes. Future research with larger sample sizes is required to corroborate and expand upon these findings. Second, other immune cell populations within the tumor microenvironment were not evaluated for their presence or function. A deeper exploration incorporating $CD8^+$ TILs and the $CD8^+/FOXP3^+$ ratio is essential for a full understanding of the immune dynamics at play and their impact on NAC response, underlining the importance of future research in this area.

Conclusion

In this study, we demonstrated that NAC treatment in IBC-NST patients significantly reduces the number of $FOXP3^+$ TILs, suggesting a potential mechanism through which NAC enhances clinical outcomes and prognosis. The specific reduction in stromal $FOXP3^+$ TILs post-treatment, compared to the unchanged intratumoral $FOXP3^+$ expression, highlights the complex interplay between chemotherapy and the tumor microenvironment. Future research should focus on delineating the direct correlation between the decrease in $FOXP3^+$ TIL numbers and the clinical outcomes in IBC-NST, further refining the prognostic value of TIL analysis in this context.

Authors Contribution

PR, MP, and SCM were involved in the conceptualization of the study. PR, EW, and SCM were involved in the preparation of methodology and conducted formal analysis. EW performed the analysis using software. PR performed the validation test. PR, EW, MP, and SCM performed the investigation. PR and MP prepared the study resources. EW and SCM performed the data curation. EW and SCM performed the visualization of the data. PR and EW prepared the original manuscript draft. All authors are involved in the review and editing of the manuscript. PR supervised the study and involved in the funding acquisition, while EW managed the project administration. All authors have read and agreed with the final version of the manuscript.

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