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Prognostic Value of Neutrophil-to-Lymphocyte Ratio and Tumor-Infiltrating Lymphocytes (TILs), for Patients with Triple-Negative Breast Cancer

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ABSTRACT

During the process of carcinogenesis, inflammation originating in the tumor or its microenvironment promotes tumor growth. An elevated neutrophil-lymphocyte ratio (NLR) has been considered a biomarker associated with reduced survival in several types of cancer. A retrospective study was conducted and the results of 49 triple-negative breast cancer (TNBC) biopsies from 2012 to 2017 were reviewed. The patients were divided into two groups: those with nonbasal subtype TNBC and those with basal-like TNBC. To differentiate TNBC tumors by subtype, cytokeratin 5/6 (CK5/6) expression was assessed. The Chi-square test was used to analyze the association between the classical clinicopathological parameters and these two groups, revealing a statistically significant relationship with histological grade. For overall survival, the Kaplan–Meier method was used to analyze the data. The difference in survival was compared by univariate analysis. Patients with elevated tumor-infiltrating lymphocytes (TILs) and a NLR less than 3 had prolonged survival. NLR is a cost-effective and reliable tool that can be exploited in a wide number of scenarios during daily clinical practice.

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Introduction

Triple-negative breast cancer (TNBC) represents 10 to 17% of all breast tumors, which are characterized by the absence of estrogen and progesterone receptors and human epidermal growth factor receptor 2 (HER2) (1,2). TNBC is molecularly heterogeneous in gene expression analyses, is more common in women under 40 years of age, and the survival time of patients is shorter than that recorded for other types of breast cancer (3,4). Basal-like breast cancer is the most representative form of TNBC that receives its name from genes consistently expressed by basal/myoepithelial cells of the mammary gland (5,6). Some of the cell markers expressed by basal-like TNBC cells include high molecular weight cytokeratins (CK5/6, CK14, and CK17), vimentin, p-cadherin, aB crystallin, fascin, and caveolins 1 and 2. These cell markers are usually detected in breast tumor tissue sections by applying immunohistochemical procedures as an alternative to gene expression techniques.

Recent reports have indicated a potential role for the immune system in response to neoadjuvant chemotherapy (NAC) (7). Consequently, it has been observed that basal-like TNBC tissue is characterized by the presence of prominent tumor-infiltrating lymphocytes (TILs), areas of geographic necrosis, and central scarring, which are absent in other types of breast cancer (8). It has been postulated that TILs may provide prognostic and potentially predictive value, particularly for TNBC and HER2-enriched breast cancer (9). Some studies have demonstrated an association between TILs and the response to chemotherapy (10,11). In particular, TNBC and hormone receptors (HR)-negative/HER2-positive breast cancers exhibited elevated TILs scores in comparison to HR-positive breast cancers. Furthermore, high TILs scores were associated with an increased response rate to chemotherapy,

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as evidenced by studies (12,13). Leukocytes, including neutrophils, lymphocytes, and the neutrophil-to-lymphocyte ratio (NLR), have been proposed as potential prognostic factors for cancer (14). Additionally, evidence has described an association between the NLR and patient survival for different types of cancers, including those from the breast. A meta-analysis of 17,079 patients with breast cancer recently suggested that elevated NLRs are associated with poor overall survival (OS) and a high risk of recurrence for breast cancer patients. Therefore, there is a need to investigate further these prognostic survival factors associated with TNBC. In this study, we demonstrate that TNBC patients with a high number of tumor-infiltrating lymphocytes and a neutrophil-to-lymphocyte ratio lower than 3 exhibited prolonged survival.

Materials and methods

Selection of patients

A total of 597 biopsies from breast cancer patients were reviewed between 2012 and 2017. This number corresponds to the total number of biopsies diagnosed as breast cancer that were processed in the Anatomic Pathology laboratory of Hospital Base Valdivia (Chile). Of the cases, 49 were identified as TNBC, a subtype characterized by the absence of estrogen receptor (ER), progesterone receptor (PR), and HER2 expression. Furthermore, in cases where the HER2 status was uncertain or HER2-positive (2+), the HER2 status was confirmed by fluorescence in situ hybridization (FISH).

Detailed medical records were obtained and clinical examinations were conducted after the subjects had provided informed consent. The specimens were then processed and fixed in 10% formalin, after which they were grossly examined. The paraffin-embedded sections were stained with hematoxylin and eosin, and tumors were classified according to the Nottingham modification of the Scarff-Bloom-Richardson system. A summary of the histopathological variables evaluated in TNBC patients is presented in Table 1.

Patients with ductal carcinoma in situ with or without micro-invasion and patients lacking information on pathological or laboratory results were excluded. Additionally, patients with stage IV breast cancer were excluded.

This study was designed as an observational, retrospective registry and was approved by the institutional review board and Ethics Committee of the participating institution, in accordance with the Declaration of Helsinki and Good Clinical Practices.

Table 1. Clinicopathological variables.

Characteristics	N°	(%)
Age (year)		
Mean	57	
Median (range)	57 (28–85)	
Histological type		
Invasive carcinoma NST	44	89,8
Others	5	10,1
Medular	1	2
Metaplastic	2	4,1
Apocrine	1	2
Secretor	1	2
Histological grade		
Grade I	0	0
Grade II	12	24,5
Grade III	37	75,5
NLR		
>=3.0	8	22
<3.0	28	77,8
Ki 67		
>30%	34	97,1
<30%	1	2,9
CK 5/6		
Positive	32	78
Negative	9	22
TILs		
High	12	42,9
Intermediate	12	42,9
Low	4	14,3

Abbreviations: CK 5/6 Cytokeratin 5/6; Invasive carcinoma NST: No Special Type invasive carcinoma; NLR Neutrophil-to-Lymphocyte Ratio; TILs Tumor-Infiltrating Lymphocytes.

Analyses and scoring

Serial five-micrometer-thick histological sections of each breast tumor were mounted on silanized slides and oven-dried at 57 °C for 60 to 90 minutes. Subsequently, the samples were deparaffinized and subjected to antigen retrieval using citrate buffer (pH 6.0) in a steamer or Pascal pot for 20 to 30 minutes, in accordance with the manufacturer's instructions. Following this, the samples were washed with phosphate-buffered saline (pH 7.4). All staining procedures were conducted on the fully automated Roche immunohistochemical instrument (Roche Diagnostics, Shanghai, China) in accordance with the recommended standard protocols.

Estrogen and progesterone receptors were considered negative if less than 1% of tumor nuclei exhibited positive staining. The HER2 expression was scored in accordance with the ASCO/CAP 2007 guidelines. Immunohistochemical scores of 0 or 1+ were considered to indicate a negative result. In the event of equivocal 2+ cases, the HER2 status was confirmed by fluorescence in situ hybridization (FISH). For CK 5/6, any cell membrane staining was defined as positive. For the proliferation index obtained using an anti-Ki-67 antibody, 30% of the stained nuclei were considered to exhibit high proliferative activity.

The quantification of tumor-infiltrating lymphocytes (TILs) was conducted in accordance with the guidelines established by the International Immuno-Oncology Biomarker Working Group (9).

The neutrophil-to-lymphocyte ratio (NLR) was calculated from the full blood count, which was performed routinely after a breast cancer diagnosis (with a maximum of two months between the diagnostic biopsy and the NLR measurement) and before the initiation of any treatment modality, including surgery. Based on previous research, an NLR of 3.0 was selected as the cutoff value for distinguishing between a high-NLR ($>=3.0$) and a low-NLR (3.0).

Statistical analysis

The statistical significance of the results was evaluated using the SPSS Statistical Package, version 24 for Windows (SPSS Inc., Chicago, IL, USA). As required, the percentage of categorical variables was compared using either Pearson's Chi-square test or Fisher's exact test.

Overall survival (OS) was defined as the interval between histological diagnosis and death from breast cancer (as indicated on the death certificate).

Survival curves were estimated using the Kaplan–Meier product-limit method, and the significance of differences between survival curves was determined using the log-rank test. The results were considered statistically significant if the P value less than <0.05.

Results

Clinicopathological parameters

Of the total cases included in the study, 49 (8.21%) were identified as triple-negative breast cancer (TNBC), while 548 (91.79%) were non-TNBC. The mean age at presentation of TNBC was 46 years, although the median age at diagnosis was 57 years, with a range between 28 and 85 years (Table 1). The majority of TNBCs included in this study were invasive ductal carcinomas (Figure 1). A minority of cases were accounted for by medullary carcinoma, metaplastic carcinoma, and invasive carcinomas with apocrine features (Table 1). The histopathological analysis revealed that 37 tumors exhibited histologic grade III, 12 exhibited histologic grade II, and no grade-I tumors were identified. The architectural characteristics of TNBC included areas of geographic necrosis and central scarring accompanied by a prominent lymphocytic infiltrate. In this study, lymphocytic infiltration was observed in more than 80% of cases. In terms of histologic features, the majority of cases were identified as invasive ductal, non-special type (89.8%) and high grade (75.5%) (Figure 1, Table 1).

Table 2 presents the correlation between clinicopathological variables and CK5/6-positive (basal-like) and CK5/6-negative (non-basal subtype) TNBCs. All associated variables, including age, histologic type, lymphocyte infiltrate, NLR, and Ki-67, yielded a P-value greater than 0.05. Furthermore, a P value of 0.042 was obtained concerning histologic grade, indicating that histologic grade is associated with basal and non-basal triple-negative tumors.

TNBC phenotyping

CK5/6 immunoreactivity was employed to distinguish between the basal-like and non-basal subtypes of TNBC. Consequently, those biopsies that exhibited CK5/6 positivity and negativity for estrogen, progesterone, and HER2 receptors were classified as basal-like TNBC. Conversely, those tumors that were CK5/6 negative were designated as non-basal TNBC. According to this characterization, 78% of the TNBCs corresponded to the basal-like subtype, while 22% corresponded to

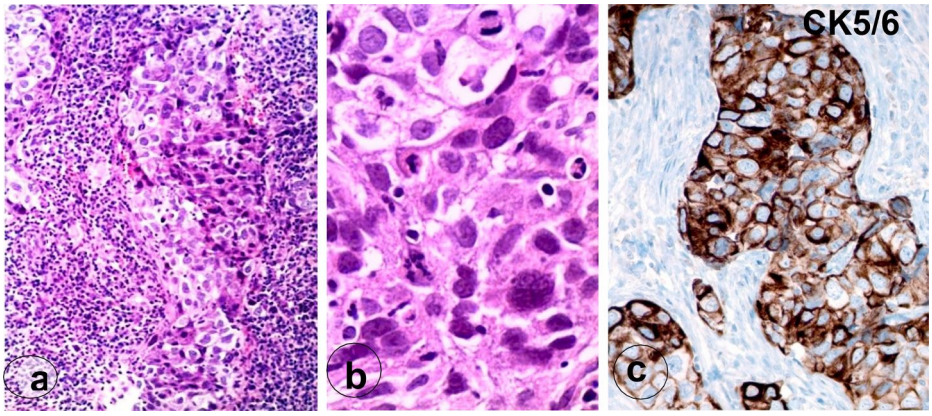


Figure 1. Triple-negative breast tumor. (a) Representative image of invasive ductal type with abundant lymphocytic infiltrate. Hematoxylin and eosin staining. (b) High histologic grade tumor. (c) Representative immunostaining for CK5/6.

Table 2. Correlation of CK5/6 with clinicopathological variables.

	CK5/6 Positive		CK5/6 Negative	*P- value*
		Age (years)		
< 50	13		3	1.000
>=50	19		6	
		Histopathological diagnosis		
IDC NOS	29		7	
Secretor carcinoma			1	
Metaplastic carcinoma	2		0	0.088
Medullary carcinoma	1		0	
apocrin carcinoma	0		1	
		Tumor grade		
Grade I	0		0	
Grade II	6		5	0.042[a]
Grade III	26		4	
		TILs		
High	10		1	
Intermediate	7		2	0.224
Low	2		2	
		Ki-67		
>=30	23		5	0.207
<30	0		1	
		NLR		
<3.0	18		7	0.293
>=3.0	6		1	

Abbreviations: IDC NOS, Non-special invasive ductal carcinoma; TILs, Tumor-infiltrating lymphocytes; NLR Neutrophil-to- Lymphocyte Ratio.
[a] statistically significant.

the non-basal subtype. Immunohistochemical detection of Ki-67 in TNBC tissues revealed that 97.1% of cases had a value greater than 30%, indicating the presence of highly proliferative tumor cells (Table 1). Additionally, amplification of the HER2 gene in TNBC CK5/6 positive cases was assessed using the FISH technique (Table 3).

Patient survival is related to the neutrophil-to-lymphocyte ratio and tumor-infiltrating lymphocytes. A total of 77.8% of cases exhibited an NLR of less than 3, indicating a low absolute neutrophil count compared to the total lymphocyte count in the peripheral blood. This observation was made based on the total number of blood counts with a maximum difference of two months at diagnosis.

A comparison of the NLR obtained for each TNBC case included in the study revealed that this index may serve as a valuable tool to anticipate patient survival. Consequently, patients with NLR values less than 3 exhibited prolonged survival, with an average of 51.4 months, in comparison to patients with an NLR greater than or equal to 3, who survived an average of 47.3 months. Furthermore, the median estimate indicated that in cases with an NLR less than 3, 50% of patients survived for more than 64 months, while in cases with an NLR greater than or equal to 3, 50% survived for less than 47 months (Figure 2).

Similarly, there was a notable increase in patient survival rates when tumor lymphocyte infiltration increased. Accordingly, patients with a low TIL had an estimated average survival of 27 months, whereas in those patients with an intermediate TIL, it was 43.7 months, and for patients with a high TIL, it was 48 months (Figure 3). Moreover, in patients with a high TIL, the median estimated survival for 50% was more than 31 months, whereas in patients with a low TIL, 50% survived less than 17 months.

Discussion

Breast cancer is a heterogeneous disease encompassing several distinct entities with markedly different biological characteristics and clinical behavior. The TNBC subtype is defined by the

Table 3. Immunohistochemical Characterization of Triple-Negative Breast Tumors.

	CK 5/6	positive	CK 5/6	Negative
	32/41	**78%**	**9/41**	**22%**
ER-, PR-,HER2 (0)	16/32	50 %	7/9	77,7%
ER-, PR-,HER2 (1+)	10/32	31,2 %	0/9	0%
ER-, PR-,HER2 (2+) unamplified	6/32	18,7 %	2/9	22,2%

Abbreviations: CK 5/6 Cytokeratin 5/6; ER estrogen receptor; HER2 human epidermal growth factor receptor-2; PR progesterone receptor.

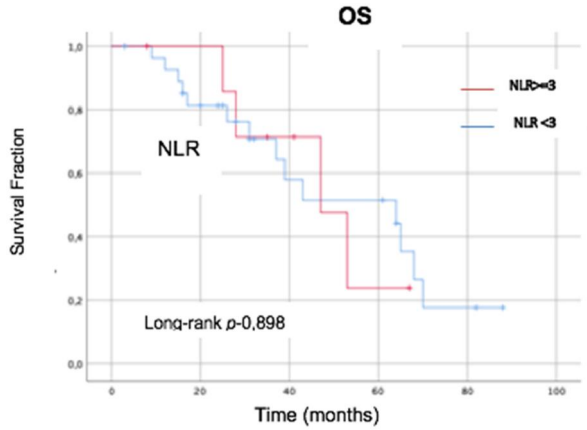


Figure 2. Overall survival graph comparing TNBC NLR> =3 vs NLR< 3. NLR: Neutrophil Lymphocyte Ratio.

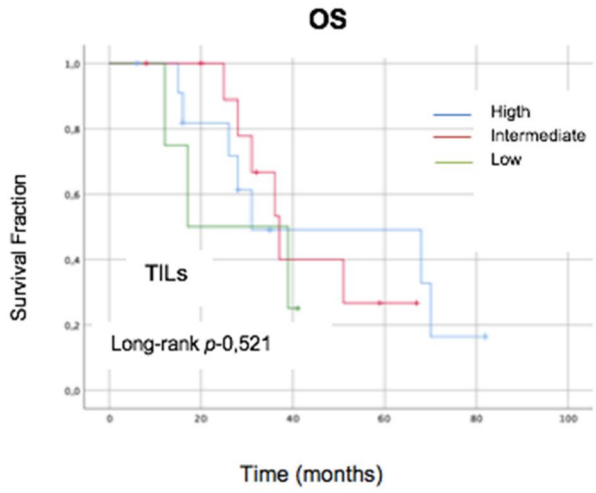


Figure 3. Overall survival graph comparing TNBC with low, intermediate, and high TILs. TNBC, Triple Negative Breast Cancer; TILs, Tumor-Infiltrating Lymphocytes.

absence of ER, PR, and HER2 gene amplification. It is characterized by overexpression of basal layer cytokeratins 5, 6, and 17 and expression of cell proliferation genes. This study estimated an incidence of TNBC of 8.2%. This percentage is considerably lower than that observed in other populations worldwide, which estimates the incidence of TNBC to be between 10 and 17% (2). A recent meta-analysis of the Indian population found a pooled prevalence of the 34 studies to be 27% (15).

The TNBC group was predominantly composed of women over 50 (65.3%), with only 14.5% being young women under 40. Another notable difference between our group and other TNBC populations is the average age at diagnosis, which was 57 years in our study. This is higher than the average age of 46 years reported for other TNBC populations (16,17). However, other authors have described an average age of 56 years at the time of diagnosis (18,19). The clinical and histopathological characteristics of the TNBC cases included in this study are consistent with those described for TNBC in other parts of the world (2,16,17). Based on the immunoreactivity to CK5/6, we distinguished between basal-like TNBC and non-basal TNBC and found that the proportion between both TNBC subtypes was similar to that reported by other authors (17,18). The differentiation between non-basal and basal triple-negative breast tumors is of significant importance, as basal-like TNBC is associated with a more aggressive phenotype and a shorter survival rate (16).

With regard to the association between variables, only a significant association was observed with histological grade. These results coincide with those of other authors who do not describe a significant association between clinical-pathological variables, except for histological grade. However, the information is diverse and not complementary since some authors find a relationship with histological grade III and other authors only with grade II (16,17).

When survival was associated with the NLR, it was observed that cases with values lower than 3 presented a greater survival, with an average of 51.4 months, compared with patients with an NLR higher than 3, who survived an average of 47.3 months. Azab et al. (20) described that a higher NLR resulted in a higher rate of advanced tumor stages, which corroborates the reciprocal relationship between tumor burden and peripheral neutrophil and lymphocyte counts. In this study, we did not perform the analysis by stage due to the limited sample size, which could have resulted in a lack of statistical power to detect

meaningful associations. However, we are currently developing a multicenter research proposal that would allow us to analyze the NLR as a prognostic factor for survival by stage.

We previously conducted a statistical power study for NLR and its association with survival, which indicated that the study had limited statistical power.

Pathological complete response (pCR) is a widely utilized surrogate endpoint in patients with breast cancer who have undergone neoadjuvant chemotherapy (NAC) with the objective of expediting the evaluation of novel treatment strategies. A number of studies have indicated that systemic inflammation markers, such as the NLR, are associated with pCR in breast cancer. Consequently, it would be of interest to include in the multicenter study an analysis of the predictive value of the NLR response to treatment, with a focus on patients treated with immunotherapy and those treated without immunotherapy.

In this study, TILs were observed in 84% of TNBC cases, with the majority exhibiting high or intermediate levels of infiltration. A comparison of groups according to the level of lymphocyte infiltration revealed that survival was higher as the level of lymphocyte infiltration increased. For instance, the mean estimated survival time for patients with a low lymphocyte infiltration level was 27 months, while that for patients with high infiltration levels was 48 months. However, no significant association was observed between survival and the level of lymphocyte infiltration. It is possible that the observed results may be influenced by the sample size, given that there is a historical association between increased Tils and increased survival in Her-2 and triple negative breast cancer. However, this association is not always accompanied by a complete pathological response following neoadjuvant therapy (21).

NLR is a cost-effective and reliable tool that can be exploited in a wide number of scenarios during daily clinical practice. High NLR values have been associated with features of tumor aggressiveness across many cancer types.

Consistently, a high NLR was associated with worse outcomes to chemotherapy and other treatment strategies in patients with advanced cancers, including colorectal, prostate, head and neck, and lung cancers (22,23). A number of other studies have investigated the potential correlation between the NLR and response to immunotherapy among patients with different malignancies, with a particular focus on lung cancer and melanoma (24–26).

It is important to note that the study is limited by its retrospective, single-center, and relatively small scale design. The NLR is influenced by a number of factors, including inflammatory diseases, autoimmune diseases, hematological diseases affecting white blood cell count, patients receiving steroid treatment or with infections. Ideally, patients with these conditions should be excluded from the analysis at the time the blood count is obtained.

Finally, it is noteworthy that the estimation of the median NLR and TIL in TNBC was highly encouraging. In cases with an NLR less than 3, 50% of patients survived more than 64 months, while in cases with an NLR equal to or greater than 3, 50% survived less than 47 months. Similarly, in patients with a high TIL, the median estimated survival for 50% was more than 31 months, whereas in patients with a low TIL, 50% survived less than 17 months.

It is of the utmost importance that large-scale multicenter studies be conducted in order to determine the impact of these biomarkers.

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Author contributions

Conception and design: Marcela Y. Soto and María T. Poblete

Manuscript writing: All authors

Final approval of manuscript: All authors

Accountable for all aspects of the work: All author

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