High immune infiltrate is a prognostic and predictive factor in triple negative breast cancer patients from Colombia.

Carlos Alexander Huertas-Caro[1], Mayra Alejandra Ramirez[1], Juan Carlos Mejía[2], Diego-Felipe Ballen[3], Marcela Nuñez Lemus[4], Luz F. Sua[2], Alicia Cock-Rada[5], Laura Fejerman[6,7], María Carolina Sanabria-Salas[1], Silvia J. Serrano-Gomez[4]

[1]Grupo de investigación en biología del cáncer, Instituto Nacional de Cancerología, Bogotá, Colombia.
[2]Servicio de patología de patología, Fundación Valle del Lili, Cali, Colombia.
[3]Grupo de oncología, Instituto Nacional de Cancerología, Bogotá, Colombia.
[4]Grupo de apoyo y seguimiento para la investigación, Instituto Nacional de Cancerología, Bogotá, Colombia
[5]Grupo de oncología, Instituto de Cancerología Las Americas, Medellín, Colombia.
[6]Department of public health sciences, University of California Davis, Davis, CA
[7]UC Davis Comprehensive Cancer Center, University of California Davis, Davis, CA.

Correspondence to: Silvia J. Serrano-Gómez, Grupo de apoyo y seguimiento para la investigación, Instituto Nacional de Cancerología, Calle 1 No. 9 -85, Bogotá DC, Colombia, (phone: (+57) (601) 3905012 ext. 4907, e-mail sserrano@cancer.gov.co

ABSTRACT

Background: Triple negative breast cancer (TNBC) is highly immunogenic and high levels of tumor infiltrating lymphocytes (TILs) have been associated with a better overall survival and higher probability to achieve pathological complete response (pCR). This study aims to explore stromal TILs (sTILs) level and composition as a prognostic and predictive biomarker in TNBC Colombian women.
Methods: 195 TNBC tumor biospecimens was analyzed from stromal TILs (sTILs), positive cells for CD4 and CD8 in agreement the clinical pathological features, pathological complete response, and survival analysis.
Results: Tumors with high sTILs levels were more likely to be early stage (64.4% stage I/II) compared to tumors with low sTILs levels (35.5%). Additionally, when compared patients with high sTILs vs. low sTILs, a higher percentage of patients with high sTILs didn't receive neoadjuvant chemotherapy (NAC) (high:50% vs. low:32.7%, p=0.025) and received more conservative surgeries (high:60% vs. low:37.9%, p<0.05). Similar results were observed for patients with high CD4/8 infiltration. Longer overall survival times were observed in patients with high sTILs (84.9 mo vs. 41.4 mo, p<0.05), as well as in patients with high CD4+ infiltration (p<0.05) and CD8+ (p<0.015). In the multivariate analysis, low levels of sTILs was found to be independent prognostic factor associated with a higher risk of death (HR:

1.59, 95% CI 1.01-2.48). Regarding immune infiltrate as a predictive biomarker, a statistically significant association between sTILs and pCR was observed (OR: High sTILs 1.486, 95% CI 1.14 - 2.013). Similar results were observed for high CD4 and CD8 infiltration (OR: 1.262, 95% CI 1.061 - 1.536, OR: 1.337, 1.085 - 1.694, respectively).
Conclusions: Our results suggest that sTILs levels are a prognostic marker for overall survival and a predictive marker for pCR in TNBC patients from Colombia as has been reported in previous studies including biospecimens from mostly European ancestry patients.

Keywords: Triple negative breast cancer, Tumor infiltrating lymphocytes, Prognosis, Predictive.

INTRODUCTION

Triple negative breast cancer (TNBC) is characterized by the lack of expression of estrogen receptor (ER), progesterone receptor (RP), and the epidermal growth factor receptor 2 (HER2) (1). It represents 10% - 20% of all breast cancers and occurs more frequently in young (under 50 years old) Non- Hispanic Black (NHB) and Latina women (2–4). It is considered the most aggressive subtype of breast cancer as it groups tumors with larger size and higher histological grade at diagnosis thus it has short survival times and high recurrence rates compared to other breast cancer subtypes (5–7). TNBC patients benefit mainly of cytotoxic chemotherapy, achieving higher pathological complete response (pCR) rates compared to hormone receptor (HR) positive tumors. Furthermore, in TNBC, to achieve a pCR has been associated with longer times of disease-free survival (DFS) and overall survival (OS) (8,9).

Among all breast cancer subtypes, TNBC is the most immunogenic due to the relatively high levels of tumor infiltrating lymphocytes (TILs) (10), the most abundant immune cells in the breast tissue microenvironment (11). Previous studies have reported that high levels of TILs in TNBC tumors have been associated with long term survival as well as a risk reduction of death and recurrence (12–15). Additionally, high levels of TILs have been shown to be an independent predictive factor for pCR after neoadjuvant chemotherapy (10,16,17), and high TILs levels showed a higher rate of pathological complete response (pCR) (18,19).

TILs consist of T and B lymphocytes that can be differentiated into subpopulations by specific markers using for example, immunohistochemical techniques (20,21). It is noteworthy that various subpopulations of TILs can show inhibitory or stimulatory effects thus differentially impacts on the prognosis of the disease (22). PD-L1 is an immune checkpoint involved in the maintenance of T cell. It is expressed on several types of cells, such as B cell, dendritic cells, macrophages, and on the surface of cancer cell (23). In TNBC, PD-L1 expression has been correlated to TILs expression and its expression has been

associated with a good prognosis (24–26), however, the prognosis value of PD-L1 expression in TNBC remain controversial (5,27,28).

Little is known about TILs and its prognostic value in TNBCs from Latinas such as Colombian women. The goal of this study was to evaluate the differences in the clinic-pathological variables, clinical outcomes and pCR according to TILs levels. Also, to evaluate if TILs are an independent prognostic factor in TNBC from Colombian patients.

METHODS

Patient selection

This is a retrospective study that included 195 TNBC patients diagnosed between 2008 and 2016 at the Instituto Nacional de Cancerología (INC), Colombia, Fundación Valle de Lili, Colombia, and Clínica las Américas, Colombia. TNBC was defined by a lack of immunoreactivity (<1%) of ER and PR and a HER2 score of 0+, 1+ or 2+ (The cases with expression of HER2 2+, it discards the overexpression for HER2 through FISH or DISC) by immunohistochemistry (IHC) according to the current recommendations of the American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP) guidelines (1). All the cases were reviewed by a single pathologist for ER, PR, HER2 to confirm the histological diagnosis.

The inclusion criteria included histologically confirmed diagnosis of primary TNBC, availability of formalin-fixed paraffin-embedded (FFPE) tissues from biopsy and surgery that were suitable for immunohistochemical tests and that contained at least 10% of the invasive tumor and the availability of relevant clinical data in the medical records.

Pathology reports were reviewed to obtain information regarding histopathological diagnosis, nodal status, lymphovascular and perineural invasion, surgical margins, and histological grade. Demographic information including place of birth, age at diagnosis, Body mass index (BMI) and region of origin as well as information on tumor size, clinical stage, treatments (neoadjuvant and/or adjuvant), recurrence and death was extracted from clinical records. The pathological response was evaluated according to recommendation of Chevallier et al (29).

This study was approved by the Institutional Review Board of the Colombian National Cancer Institute. Informed consent was required to use the biopsies. If patient did not receive neoadjuvant treatment and has available surgery material, no inform consent was required.

TILs assessment

Analysis of TILs was performed on a single full-face hematoxylin and eosin (H&E) stained pre-treatment sections following criteria described by the International TILs Working Group 2014 Guideline. The score was estimated as a proportion of presence of mononuclear cells

in tumoral stromal (sTILs), sTILs were defined as the percentage of tumor stroma containing infiltrating lymphocytes located dispersed in the stroma between the carcinoma cells and do not directly contact (30). Histopathologic evaluation of TILs was performed by a single pathologist that was blinded to clinicopathological and survival information.

Immunohistochemistry

The immune markers CD4 (clone SP35, Ventana Medical System) and CD8 (clone SP57, Ventana Medical System) and immunocheckpoint marker PD-L1 (clone SP142, Ventana Medical System) expressions were examined by immunohistochemistry on tissue. Four μm section were cut from pre-treatment FFPE block and transferred to slides which were processed and stained using a Ventana Benchmark XT staining system (Ventana Medical Systems, Tucson, AZ, USA) with the Optiview DAB Detection Kit (Ventana Medical Systems) following the manufacturer's protocols. All solutions were from Ventana Medical Systems unless otherwise specified.

Tonsil tissue was used as positive control for each biomarker, whereas the negative control was prepared by replacing the primary antibody with a nonimmune immunoglobulin of the same isotype. Positive and negative controls were included in each staining run.

Quantification of CD4+, CD8+ and Immunocheckpoint marker PD-L1

The quantification of the expression of CD4 and CD8 was perform from slide immunohistochemistry, the pathologist selects three tumoral areas (higher infiltration of cells immune) of each sample, these areas was digitalized by a digital camera for microscopy (Olympus EP50) in high definition of each area select at ×400 magnification (×40 objective), the score of positive cells in two areas (stromal and intratumoral, in agreement was mentioned earlier) was performed using the program ImageJ through plugin cell counter, the mean of three readings for each tumor sample, was calculated and took on what the representative number of cells positive of each tumor. In the case that one area was uninformative (contained no tumor tissue or infiltrate), the mean score was calculated of the remaining areas. Furthermore, the cases in which all three areas were uninformative, this sample were excluded from the analyses. To define cut-off of immune markers, we categorized according to the median of expression of overall samples, for each marker. The median values for: CD4 (>101.33), CD8 (>105). The expression of PD-L1 was assayed in immune cells, and it was reported according to the expression as positive (≥1%) or negative (0%). All cases were evaluated by a pathologist.

Statistical analysis

Statistical analysis was performed using R version 4.2.1 statistical software package. The frequencies' differences of clinical-pathological characteristics in agreement to immune

infiltrate were examined using chi-squared test or Fisher's exact test. Differences in the immune infiltration were analyzed between the groups of patients who achieved pCR and those who did not, using t-student test or the Wilcoxon signed-rank test, in agreement to distribution of the data, according to the Kolmogórov-Smirnov test, Multivariable analysis of pCR was carried out using logistic regression model. The Kaplan-Meier method was used to estimate OS and DFS according to the groups and compare through log-rank test. The Cox proportional hazard analysis was performed in a model univariate and multivariate adjusting for clinicopathological variables of clinical importance (tumor size and lymph node involvement). The results are presented as Hazard ratios (HR) and 95% confidence intervals. P values < 0.05 were considered statistically significant.

Results

Clinicopathological characteristics

The clinicopathological characteristics of 195 tumors TNBC showed in table 1. All patients were female and 124 (64.2%) belonged at paid health, and 112 (57.7%) birth in Andean region. In clinical characteristics, the most of patients presented BMI of <29.9 (78.9%), diagnosis age >50 years (67.2%), no family history of breast cancer (86.1%), node involvement at diagnosis (56%), no bilateral breast cancer (97.4%), advanced clinic state (III, 49%), tumor sizes greater than 2cm (81.7%), no genetic test (76.2%), no receive chemotherapy neoadjuvant (60%), of the patients that receive chemotherapy neoadjuvant only 15 (15.8%) had clinical complete response, 104 (54.2%) patients underwent how surgery the modified radical mastectomy (MRM), in agreement of pathological characteristics 184 (94.4%) cases were diagnoses as invasive ductal carcinoma of no special type (IDC), 168 (86.6%) poorly differentiated, the most of patients presented in the surgery, no invasion of breast cancer in product of surgery (51.5%), no involvement of tumor margins (90.6%), but received axillary lymph node dissection (69.1%), and only 21 (23.3%) cases gain pCR, of the 195 patients 77 (40.3%) presented recurrence and 108 (55.4%) had died at date of this study.

Table 1. Population description

Characteristics	Number (%)
n	195
Sociodemographic characteristics	
Health regimen	
Contributory	124 (64.2)
Subsidized	69 (35.8)
No data	2
Birth's region	
Amazon-Orinoquia	13 (6.7)
Andean	112 (57.7)
Pacific-Caribbean	69 (35.6)
No data	1
Clinical characteristic	

BMI	
<29.9	153 (78.9)
>30	41 (21.1)
No data	1
Diagnostic's age	
<50 years	64 (32.8)
>50 years	131 (67.2)
Family history of breast cancer	
No	167 (86.1)
Yes	27 (13.9)
No data	1
Node involvement at diagnosis	
No	85 (44.0)
Yes	108 (56.0)
No data	2
Bilateral breast cancer	
No	190 (97.4)
Yes	5 (2.6)
Clinical stage	
I-II	91 (46.9)
III	95 (49.0)
IV	8 (4.1)
No data	1
Size tumor at diagnosis	
≤2cm	33 (18.3)
>2cm	147 (81.7)
No data	15
Genetic test	
No	147 (76.2)
Yes	46 (23.8)
No data	2
Neoadjuvant treatment	
No	78 (40.0)
Yes	117 (60.0)
Clinical response (CR)	
cCR	15 (15.8)
nCR	80 (84.2)
Not apply	78
No data	22
Surgery	
Quadrantectomy	88 (45.8)
MRM	104 (54.2)
No data	3
Pathological characteristics	
Histology diagnosis	
IDC	184 (94.4)
Other	11 (5.6)
Tumor differentiated	
Good differentiated	1 (0.5)

Moderate differentiated	25 (12.9)
Poorly differentiated	168 (86.6)
No data	1
Present invasion	
No	84 (51.5)
Yes	79 (48.5)
No data	32
Involvement of tumor margins	
No	173 (90.6)
Yes	18 (9.4)
No data	4
Axillary lymph node dissection	
No	60 (30.9)
Yes	134 (69.1)
No data	1
Pathological response	
pCR	21 (23.3)
pNR	18 (20.0)
pPR	51 (56.7)
No apply	78
No data	27
Prognosis characteristics	
Recurrence	
No	114 (59.7)
Yes	77 (40.3)
No data	4
Died	
No	87 (44.6)
Yes	108 (55.4)

Abbreviatures: *BMI* Body mass index, *cm* centimeters, *cCR* clinical complete response, *nCR* nonclinical response, *MRM* Modified radical mastectomy, *IDC* invasive ductal carcinoma, *pCR* pathological complete response, *pNR* pathological no response, *pPR* pathological partial response.

Frequency of clinicopathological characteristics

In the patients with clinical stage I-II and III, high sTILs was observed in 82 (44.1%) tumors and was more frequency in absence of lymph node involvement at diagnosis (58% vs 36.9%, p= 0.007), early clinical stage (I-II, 64.6% vs 36.9%, p= <0.001) and smaller tumor sizes (≤ 2cm, 29.5% vs 10.5%, p=0.003), compared to tumors with low sTILs. Additionally, the patients whit high sTILs were more likely to don't receive chemotherapy neoadjuvant (50% vs 32.7%, p= 0.025), more conservative surgeries like quadrantectomy (60% vs 37.9%, p=0.005) and don't receive axillary lymph node dissection, in the patients that receive neoadjuvant treatment was more frequently gain the cCR (28.6% vs 9.3%, p=0.022) and pCR (42.9% vs 15.8%, p= 0.023) in comparison whit the patients that presents low sTILs. In

disease outcome was observe high sTILs had more probability of don't die (61% vs 34.6%, *p*= 0.001), data show in table 2.

In concordance whit observed in the frequency of characteristics of patients with TNBC and levels sTILs, the number of cells positive for CD4 and CD8 show similar results. High CD4 and CD8 was more frequently in patient whit characteristics of better prognosis in comparison of low CD4 and CD8, like no node involvement at diagnosis (CD4: 59.6% vs 34.5%, *p*= 0.001, CD8: 59.8% vs 34.8%, *p*= 0.002), early clinical stage (CD4: 70% vs 31.8%, *p*= <0.001, CD8: 62.9% vs 39.3%, *p*= 0.003) and smaller tumor sizes (CD4: 31.6% vs 7%, *p*= <0.001, CD8: 26.6% vs 11.6%, *p*= 0.024), in the same way of the therapeutic option to don't receive chemotherapy neoadjuvant (CD4: 57.8% vs 25%, *p*= <0.001, CD8: 52.8% vs 30.3%, *p*= 0.004), more conservative surgeries like quadrantectomy (CD4: 67.8% vs 31.8%, *p*= <0.001, CD8: 64% vs 36%, *p*= <0.001) and don't receive axillary lymph node dissection (CD4: 46.7% vs 20.5%, *p*= <0.001, CD8: 43.8% vs 23.6%, *p*= 0.007). In the response to treatment the high CD4 and CD8 was more frequent in patients that gain the cCR (CD4: 29% vs 9.4%, *p*= 0.032, CD8: 28.6% vs 8.2%, *p*= 0.018) and pCR only for high CD4 (43.3% vs 16.3%, *p*= 0.006) in comparison whit the patients that presents low CD4 and CD8.

In the case of PD-L1 the positivity was observed whit better frecuency in patients that birth in region Andean (75.8% vs 53.3%, *p*=0.012), this region have predominance of major ancestry European, too we were observed that the positive are present in patients with obesity (BMI >30, 36.4% vs 17.4%, *p*=0.029).

Table 2. Frequency of clinicopathological characteristics according to level of immune infiltrating in TNBC.

Characteristics	sTILs			CD4			CD8			PD-L1		
	High (>10%)	Low (≤10%)	*p* valor	High (>101.33)	Low (≤101.33)	*p* valor	High (>105)	Low (≤105)	*p* valor	Negative	Positive	*p* valor
	82	104		90	88		89	89		150	33	
Sociodemographic characteristics												
Birth's region												
Amazon-Orinoquia	6 (7.3)	6 (5.8)	0.555[a]	5 (5.6)	7 (8.0)	0.815[b]	6 (6.7)	6 (6.7)	0.729[a]	9 (6.0)	3 (9.1)	0.012[b*]
Andean	44 (53.7)	64 (61.5)		51 (56.7)	50 (56.8)		53 (59.6)	48 (53.9)		80 (53.3)	25 (75.8)	
Pacific-Caribbean	32 (39.0)	34 (32.7)		34 (37.8)	31 (35.2)		30 (33.7)	35 (39.3)		61 (40.7)	5 (15.2)	
Clinical characteristic												
BMI												
<29.9	67 (81.7)	80 (77.7)	0.623[a]	68 (76.4)	71 (80.7)	0.610[a]	65 (73.9)	74 (83.1)	0.187[a]	123 (82.6)	21 (63.6)	0.029[a*]
>30	15 (18.3)	23 (22.3)		21 (23.6)	17 (19.3)		23 (26.1)	15 (16.9)		26 (17.4)	12 (36.4)	
No data	0	1		1	0		1	0		1	0	
Node involvement at diagnosis												
No	47 (58.0)	38 (36.9)	0.007[a]	53 (59.6)	30 (34.5)	0.001[a*]	52 (59.8)	31 (34.8)	0.002[a*]	70 (47.0)	13 (40.6)	0.646[a]
Yes	34 (42.0)	65 (63.1)		36 (40.4)	57 (65.5)		35 (40.2)	58 (65.2)		79 (53.0)	19 (59.4)	
No data	1	1		1	1		2	0		1	1	
Clinical stage												
I-II	53 (64.6)	38 (36.5)	<0.001[a*]	63 (70.0)	28 (31.8)	<0.001[a*]	56 (62.9)	35 (39.3)	0.003[a*]	73 (48.7)	18 (54.5)	0.675[a]
III	29 (35.4)	66 (63.5)		27 (30.0)	60 (68.2)		33 (37.1)	54 (60.7)		77 (51.3)	15 (45.5)	
Size tumor at diagnosis												
<=2cm	23 (29.5)	10 (10.5)	0.003[a*]	25 (31.6)	6 (7.0)	<0.001[a*]	21 (26.6)	10 (11.6)	0.024[a*]	26 (18.4)	6 (20.7)	0.983[a]
>2cm	55 (70.5)	85 (89.5)		54 (68.4)	80 (93.0)		58 (73.4)	76 (88.4)		115 (81.6)	23 (79.3)	
No data	4	9		11	2		10	3		9	1	
Neoadjuvant treatment												
No	41 (50.0)	34 (32.7)	0.025[a*]	52 (57.8)	22 (25.0)	<0.001[a*]	47 (52.8)	27 (30.3)	0.004[a*]	53 (35.3)	21 (63.6)	0.005[a*]
Yes	41 (50.0)	70 (67.3)		38 (42.2)	66 (75.0)		42 (47.2)	62 (69.7)		97 (64.7)	12 (36.4)	
Clinical response (CR)												
cCR	10 (28.6)	5 (9.3)	0.022[b*]	9 (29.0)	5 (9.4)	0.032[b*]	10 (28.6)	4 (8.2)	0.018[b*]	14 (17.9)	1 (10.0)	1.000[b]
nCR	25 (71.4)	49 (90.7)		22 (71.0)	48 (90.6)		25 (71.4)	45 (91.8)		64 (82.1)	9 (90.0)	
No apply	41	34		52	22		47	27		53	21	
No data	6	16		7	13		7	13		19	2	
Surgery												
Quadrantectomy	48 (60.0)	39 (37.9)	0.005[a*]	59 (67.8)	28 (31.8)	<0.001[a*]	55 (64.0)	32 (36.0)	<0.001[a*]	69 (46.3)	18 (54.5)	0.506[a]
MRM	32 (40.0)	64 (62.1)		28 (32.2)	60 (68.2)		31 (36.0)	57 (64.0)		80 (53.7)	15 (45.5)	
No data	2	1		3	0		3	0		1	0	
Pathological characteristics												
Axillary lymph node dissection												
No	36 (43.9)	24 (23.1)	0.004[a*]	42 (46.7)	18 (20.5)	<0.001[a*]	39 (43.8)	21 (23.6)	0.007[a*]	49 (32.7)	11 (33.3)	1.000[a]

Yes	46 (56.1)	80 (76.9)		48 (53.3)	70 (79.5)		50 (56.2)	68 (76.4)		101 (67.3)	22 (66.7)	
Pathological response												
pCR	12 (42.9)	9 (15.8)	0.023[b*]	13 (43.3)	8 (16.3)	0.006[a*]	13 (38.2)	8 (17.8)	0.106[a]	20 (27.0)	1 (10.0)	0.059[b]
pNR	3 (10.7)	14 (24.6)		7 (23.3)	7 (14.3)		6 (17.6)	8 (17.8)		12 (16.2)	5 (50.0)	
pPR	13 (46.4)	34 (59.6)		10 (33.3)	34 (69.4)		15 (44.1)	29 (64.4)		42 (56.8)	4 (40.0)	
No apply	41	34		52	22		47	27		53	21	
No data	13	13		8	17		8	17		23	2	
Prognosis characteristics												
Died												
No	50 (61.0)	36 (34.6)	0.001[a*]	55 (61.1)	29 (33.0)	<0.001[a*]	52 (58.4)	32 (36.0)	0.004[a*]	69 (46.0)	16 (48.5)	0.947[a]
Yes	32 (39.0)	68 (65.4)		35 (38.9)	59 (67.0)		37 (41.6)	57 (64.0)		81 (54.0)	17 (51.5)	

Abbreviatures: *TNBC* triple negative breast cancer, *sTILs* stromal tumoral infiltrating lymphocytes, *PD-L1* Programmed Death-ligand 1, *BMI* body mass index, *cm* centimeters, *cCR* clinical complete response, *nCR* nonclinical response, *MRM* Modified radical mastectomy, *pCR* pathological complete response, *pNR* pathological no response, *pPR* pathological partial response.

[a] Chi-square test

[b] Fisher's exact test

[*] *p* value is significant

229

230

Correlation infiltrating immune and pCR

The correlations between pathological response and immune infiltrating were analyzed in Fig 1. sTILs were scored as continuous variable, Mann-Whitney test showed that tumors whit better percentage of sTILs were present in patients that gain pCR (p= 0.0076, Figure 1A). T-test show similar results for CD4 (p=0.012, Figure 1B) and CD8 (p=0.019, Figure 1C), it was associated whit pCR.

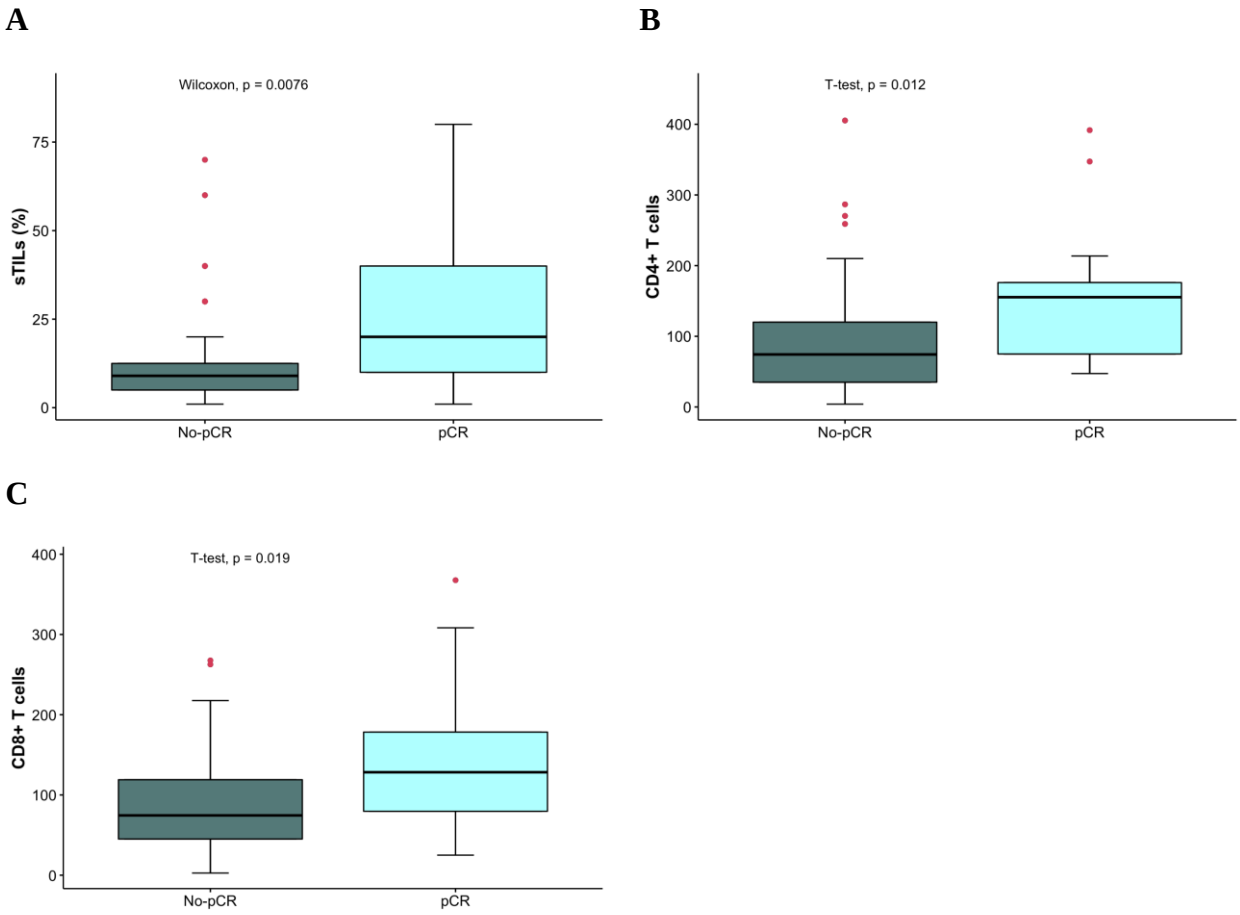

Fig 1. The correlation of infiltrating immune with pathological response in TNBC. Boxplot represented the scores of immune infiltrating in agreement pathological response (No-pCR or pCR). **A**, **B** and **C:** sTILs, CD4, CD8 were positively associated with pCR (sTILs: p= 0.0076, CD4: p= 0.012, CD8: p= 0.019). p value was calculated for sTILs by Mann-Whitney test, CD4 and CD8 were calculate for t-test. *sTILs* stromal tumoral infiltrating lymphocytes, *TNBC* triple negative breast cancer.

Immune infiltrating as factor predictor of pCR

The relationship between sTILs, specific population of immune infiltrating and pCR was analyzed by logistic regression analysis (Table 3). sTILs were scored as continuous variable per 10% increment. Univariate analysis shows that increments per 10% of sTILs was significative correlated with pCR (OR: 1.486, 95% CI 1.14 – 2.013, *p*= 0.00531), higher sTILs score indicate better probability of gain pCR after neoadjuvant administration. However, in multivariate analysis adjusted by node involvement at diagnosis, sTILs (OR: 1.310, 95% CI 0.984 - 1.807, *p*= 0.0763) didn't show as a factor independent predictor for pCR. For specific population CD4 and CD8 were score as continuous variable per 30 cell increment. Univariate analysis showed that for both CD4 (OR: 1.262, 95% CI 1.061 - 1.536, *p*= 0.012) and CD8 (OR: 1.337, 95% CI 1.085 - 1.694, *p*= 0.00948) were significantly associated whit pCR. Multivariate analyses on the contrary for sTILs, CD4 (OR: 1.206, 95% CI 1.006 - 1.468, *p*= 0.0470) and CD8 (OR: 1.285, 95% CI 1.022 - 1.658, *p*= 0.0396) demonstrated that were independent predictor for pCR, irrespective of other clinical pathological characteristics.

Table 3. univariate and multivariate analysis of immune infiltrating by pCR in neoadjuvant treated TNBC.

Marker	**Univariate analysis**			**Multivariate analysis**		
	OR	95% CI	p	OR	95% CI	p
sTILs (per 10 %)	1.486	1.14 - 2.013	0.00531	1.310	0.984 - 1.807	0.0763
CD4 (per 30 cells)	1.262	1.061 - 1.536	0.012	1.206	1.006 - 1.468	0.0470
CD8 (per 30 cells)	1.337	1.085 - 1.694	0.00948	1.285	1.022 - 1.658	0.0396

Abbreviatures: *TNBC* triple negative breast cancer, *sTILs* stromal tumoral infiltrating lymphocytes, *pCR* pathological complete response.

* *p* value is significant

Prognostic value of immune infiltrating in the TNBC

In agreement explore the prognostic value of immune infiltrating in the patients with TNBC were analyzed by Kaplan Meier, among 186 tumors, high sTILs was associated with prolonged OS (median of survival: 84.9, 95% CI 62.9 – 100, vs. 41.4, 95% CI 35.0 – 58.2, *p*= 0.00043) and DFS (*p*= 0.022), similarly in 178 tumors of TNBC show that high CD4 (OS: *p*= 0.0074, DFS: *p*= 0.021) and high CD8+ (OS: *p*= 0.008, DFS: *p*= 0.017) were as also associated with good outcome at present long times of OS and DFS (Figure 2 and 3).

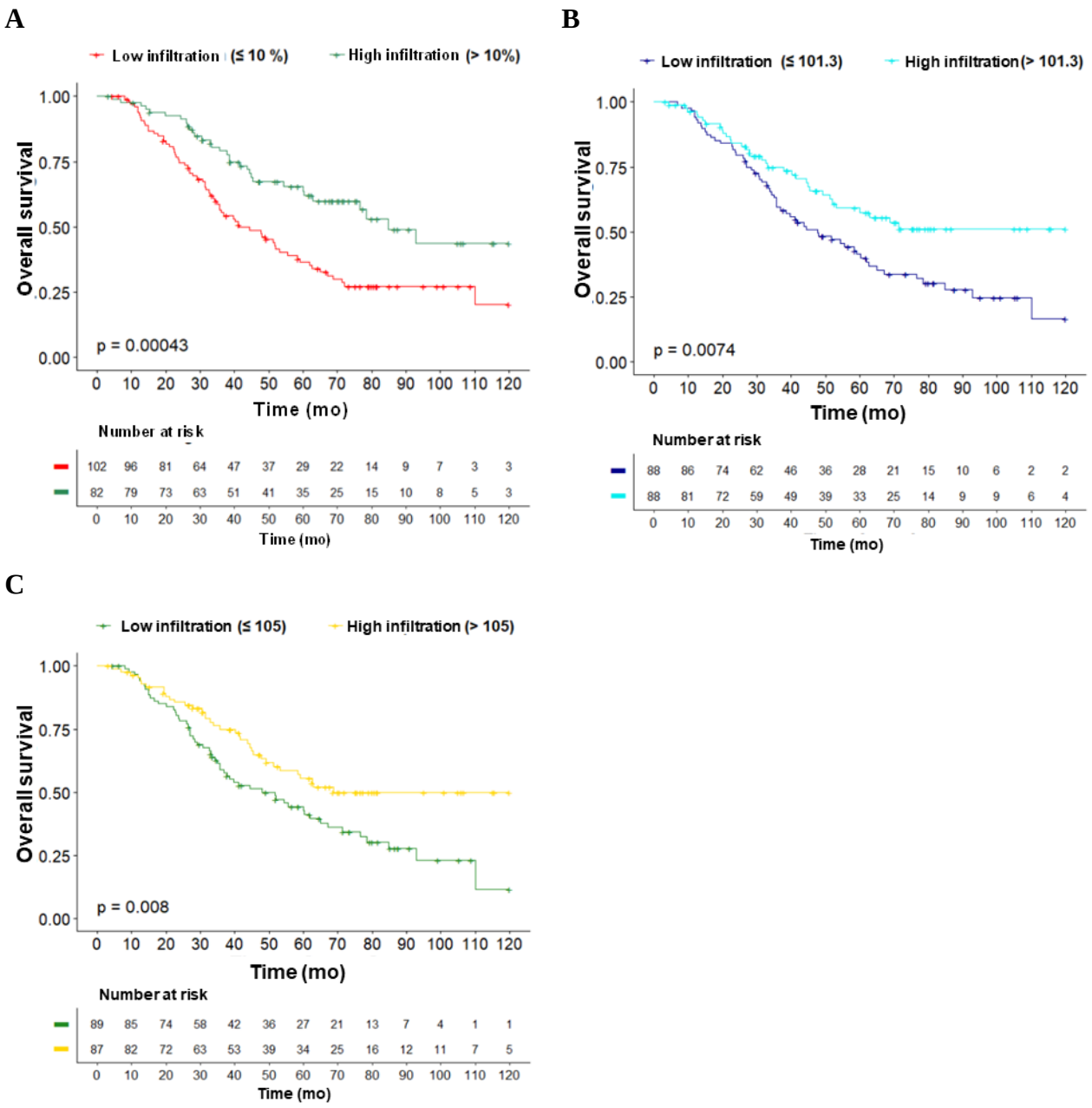

Fig 2. Prognostic value of immune infiltrating in the TNBC. Overall survival was analyzed in the TNBC for 186 tumors by **A** sTILs and 178 tumors by **B** CD4 and **C** CD8. Scores of sTILs were categorized in two groups of low and high infiltration (cut off 10%), scores of CD4 and CD8 were categorized by median of all cases. *sTILs* stromal tumoral infiltrating lymphocytes, *TNBC* triple negative breast cancer.

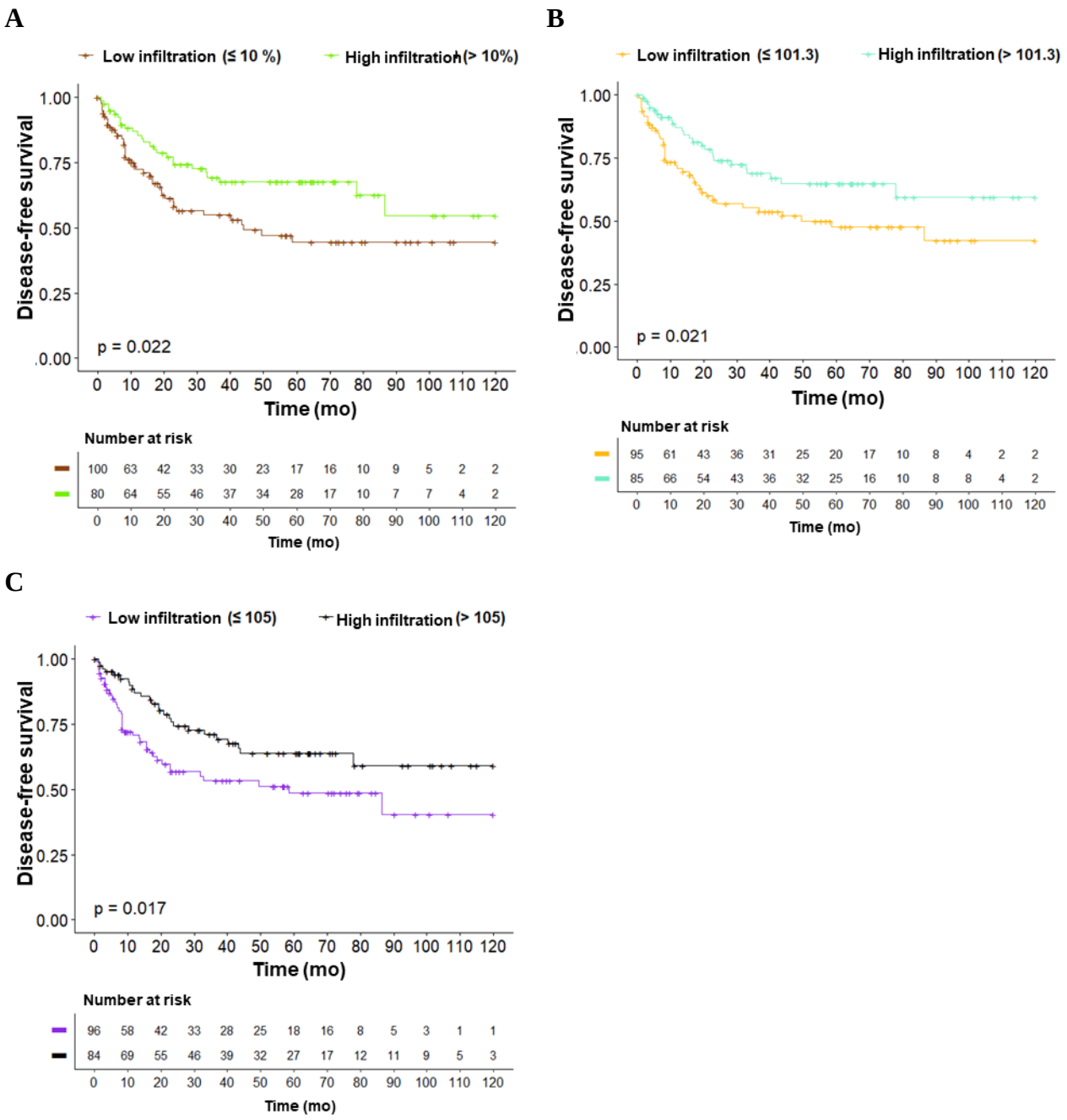

Fig 3. Prognostic value of immune infiltrating in the TNBC. Disease free survival was analyzed in the TNBC for 186 tumors by **A** sTILs and 178 tumors by **B** CD4 and **C** CD8. Scores of sTILs were categorized in two groups of low and high infiltration (cut off 10%), scores of CD4 and CD8 were categorized by median of all cases. *sTILs* stromal tumoral infiltrating lymphocytes, *TNBC* triple negative breast cancer.

Low infiltration of sTILs, CD4 and CD8 was associated with increased risk of death and recurrence in univariate analysis, clinical – pathological characteristics associated with outcomes evaluated were lymph node involvement and tumoral size (Table 4). In the multivariate analysis of sTILs adjusted by tumor size and lymph node involvement in a Cox

regression proportional hazard model show that low sTILs was an independent prognostic factor associated whit higher risk of death (HR: 1.59, 95% CI 1.01-2.48, p= 0.043), this didn't observe in CD4 and CD8, neither didn't for risk of recurrence (Table 4).

Table 4. Association of sTILs with OS in a Cox proportional hazards model

Factor		Overall survival		Disease-free survival	
		HR (CI 95%)	***p***	**HR (CI 95%)**	***p***
sTILs	High	Ref.		Ref.	
	Low	2.08 (1.35 – 3.20)	<0.001	1.78 (1.08 – 2.92)	0.023
CD4	High	Ref.		Ref.	
	Low	1.85 (1.19 – 2.89)	0.007	1.73 (1.05 – 2.87)	0.033
CD8	High	Ref.		Ref.	
	Low	1.79 (1.16 – 2.77)	0.008	1.77 (1.07 – 2.92)	0.027
Lymph node involvement	No	Ref.		Ref.	
	Yes	2.50 (1.59 – 3.95)	<0.001	4.38 (2.42 – 7.92)	<0.001
Tumoral size	≤2cm	Ref.		Ref.	
	>2cm	3.79 (1.75 – 8.82)	<0.001	1.80 (0.98 – 3.31)	0.057

Abbreviatures: *HR* Hazard Ratio, *CI* Confidence interval, *sTILs* stromal tumoral infiltrating lymphocytes.

* *p* value is significant

Discussion

Although breast cancer has not been described as an immunogenic cancer with the capacity to generate strong immune response, different studies have describe that mainly ER-negative subtypes such as HER2-enriched and TN have a greater immune infiltration, and this has been associated as a prognostic biomarker and as a target for immune-therapeutic treatments (31,32).

The results of this study showed the relation between clinical-pathological features and the prognostic and predictive value of the TILs in the TNBC, in accordance with other studies (22,33–35). However, it has not been established a standard methodology for evaluating immune infiltrate in TNBC. It had been presented in differences in the evaluation between studies, change the cutoff or the management how categorical variable or how continuous variable, the group international of TILs analyzed different studies for to generate recommendations on how evaluate this TILs in tissue starting of different studies and its results (20).

In accordance with reported in the literature, a greater immune infiltrate in TNBC has been related to clinical-pathological characteristics of a good prognosis (34,35). Consistently in our study, it was observed that patients with high levels of sTILs, CD4+ and CD8+ cells more

frequently presented earlier clinical stages, tumor sizes less than 2 cm and absence of lymph node involvement, which is related to also with the therapeutic management that these patients receive, that is, they receive less management with neoadjuvant chemotherapy and a greater number of conservative surgeries such as quadrantectomy. Additionally, in our study, we found sTILs as an independent prognostic marker for overall survival. In contrast, CD4+ and CD8+ cells were found to be associated with risk of death in the univariate model, but lost statistical significance in the multivariate model.

The results in other studies regarding the prognostic value of TILs and of specific populations in TNBC are controversial and require more studies to have a better understanding of it. The differences in the prognostic impact of the TILs between the studies could also be related to the clinical stage of the patients included in each of the studies. Presumably, there are lower amounts of tumor antigens among patients in earlier stages (36,37), which could give rise to erroneous interpretations regarding the relationship of the TILs and the outcomes of interest. To date, few studies have evaluated the prognostic impact of TILs in patients with early-stage TNBC.

The specific immune populations CD4 and CD8 representing a very general fraction of the immune infiltrate within TNBC and can't be informative to explore its association with the prognosis of the disease. Since the role of the different subpopulations of immune cells in the development of TNBC has been described, for example, Th2 (CD4+) cells promote the proliferation of B lymphocytes and humoral responses and have been associated with a poor prognosis for breast cancer (38,39). However, CD4+/FOXP3+/CD25+ cells participate in immune escape by suppressing the antitumor activity and have been reported associated with longer OS and DFS times in TNBC (40–43). The immune infiltrate must continue being explored to learn more about the interactions between the immune cell populations specific and thus have a better understanding of its functioning and its role in TNBC.

Different studies have shown an association between the percentage of sTILs and pCR, so it has been suggested that high levels of sTILs could be an independent predictor of pCR. (18–20,44). We found the association for the CD4 and CD8 markers, and suggestive for the sTILs with pCR. The relationship observed between a greater infiltration of sTILs and higher rates of pCR could be explained by the degree of the antitumor immune response of sTILs against cancer cells, which acts synergistically with natural immunity, restoring the antitumor immune response (12,45). In addition, it has been shown that chemotherapy treatment can promote the antitumor immune response due to the production of DAMP (Danger Associated Molecular Patterns) danger signals during cell death; In addition, the expression of calreticulin (CALR) and the high mobility group release box 1 (HMGB1), enhance this antitumor immune response (46).

This study presents some limitations, among them the type of sample used stands out, being tissues in FFPE blocks taken from the pathology archives of the different institutions, we found the wear of the FFPE block from the biopsy, which limited the assembly of IHC markers for the evaluation of the immune infiltrate.

This investigation is the first study in the Colombian women in exploring the immune infiltrate as a prognostic and predictive biomarker in triple negative breast cancer. The interdisciplinary work between pathologists, oncologists, tumor molecular biology experts and others enriches the work favoring the advancement of science in Colombia.

Acknowledgments

We would like to acknowledge all the patients who have given so generously to help this study, to the participating institutions and all those who contributed to the development of this project.

Funding

Funding for this work was provided by the Instituto Nacional de Cancerología and Minciencias, Bogotá, Colombia.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

References

1. Wolff AC, Hammond MEH, Hicks DG, Dowsett M, McShane LM, Allison KH, et al. Recommendations for human epidermal growth factor receptor 2 testing in breast. J Clin Oncol. 2013 Nov 1;31(31):3997–4013.
2. Dietze EC, Sistrunk C, Miranda-Carboni G, O'Regan R, Seewaldt VL. Triple-negative breast cancer in African-American women: Disparities versus biology. Nature Reviews Cancer. 2015.
3. Abramson VG, Lehmann BD, Ballinger TJ, Pietenpol JA. Subtyping of triple-negative breast cancer: Implications for therapy. Cancer [Internet]. 2015 Jan 1 [cited 2022 May 18];121(1):8–16. Available from: https://onlinelibrary.wiley.com/doi/full/10.1002/cncr.28914
4. Scott LC, Mobley LR, Kuo TM, Il'yasova D. Update on triple-negative breast cancer disparities for the United States: A population-based study from the United States Cancer Statistics database, 2010 through 2014. Cancer [Internet]. 2019 Oct 1 [cited 2022 May 18];125(19):3412–7. Available from: https://onlinelibrary.wiley.com/doi/full/10.1002/cncr.32207
5. Li X, Yang J, Peng L, Sahin AA, Huo L, Ward KC, et al. Triple-negative breast

cancer has worse overall survival and cause-specific survival than non-triple-negative breast cancer. Breast Cancer Res Treat 2016 1612 [Internet]. 2016 Nov 25 [cited 2022 May 18];161(2):279–87. Available from: https://link.springer.com/article/10.1007/s10549-016-4059-6

6. Lin NU, Vanderplas A, Hughes ME, Theriault RL, Edge SB, Wong YN, et al. Clinicopathologic features, patterns of recurrence, and survival among women with triple-negative breast cancer in the National Comprehensive Cancer Network. Cancer [Internet]. 2012 Nov 15 [cited 2022 May 18];118(22):5463–72. Available from: https://onlinelibrary.wiley.com/doi/full/10.1002/cncr.27581

7. Ignatov A, Eggemann H, Burger E, Ignatov T. Patterns of breast cancer relapse in accordance to biological subtype. J Cancer Res Clin Oncol 2018 1447 [Internet]. 2018 Apr 19 [cited 2022 May 18];144(7):1347–55. Available from: https://link.springer.com/article/10.1007/s00432-018-2644-2

8. von Minckwitz G, Martin M. Neoadjuvant treatments for triple-negative breast cancer (TNBC). Ann Oncol. 2012 Aug 1;23(SUPPL. 6):vi35–9.

9. Spring L, Greenup R, Niemierko A, Schapira L, Haddad S, Jimenez R, et al. Pathologic Complete Response After Neoadjuvant Chemotherapy and Long-Term Outcomes Among Young Women With Breast Cancer. J Natl Compr Cancer Netw [Internet]. 2017 Oct 1 [cited 2022 May 18];15(10):1216–23. Available from: https://jnccn.org/view/journals/jnccn/15/10/article-p1216.xml

10. He TF, Yost SE, Frankel PH, Dagis A, Cao Y, Wang R, et al. Multi-panel immunofluorescence analysis of tumor infiltrating lymphocytes in triple negative breast cancer: Evolution of tumor immune profiles and patient prognosis. PLoS One [Internet]. 2020 [cited 2022 May 18];15(3):e0229955. Available from: https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0229955

11. Kim G, Pastoriza JM, Condeelis JS, Sparano JA, Filippou PS, Karagiannis GS, et al. The Contribution of Race to Breast Tumor Microenvironment Composition and Disease Progression. Front Oncol. 2020 Jun 30;10:1022.

12. Loi S, Sirtaine N, Piette F, Salgado R, Viale G, Van Eenoo F, et al. Prognostic and predictive value of tumor-infiltrating lymphocytes in a phase III randomized adjuvant breast cancer trial in node-positive breast cancer comparing the addition of docetaxel to doxorubicin with doxorubicin-based chemotherapy: BIG 02-98. J Clin Oncol. 2013;

13. Loi S, Michiels S, Salgado R, Sirtaine N, Jose V, Fumagalli D, et al. Tumor infiltrating lymphocytes are prognostic in triple negative breast cancer and predictive for trastuzumab benefit in early breast cancer: Results from the FinHER trial. Ann Oncol. 2014;25(8):1544–50.

14. Ibrahim EM, Al-Foheidi ME, Al-Mansour MM, Kazkaz GA. The prognostic value of tumor-infiltrating lymphocytes in triple-negative breast cancer: a meta-analysis. Breast Cancer Research and Treatment. 2014.

15. Pruneri G, Gray KP, Vingiani A, Viale G, Curigliano G, Criscitiello C, et al. Tumor-infiltrating lymphocytes (TILs) are a powerful prognostic marker in patients with triple-negative breast cancer enrolled in the IBCSG phase III randomized clinical trial 22-00. Breast Cancer Res Treat. 2016;

16. Denkert C, Von Minckwitz G, Brase JC, Sinn B V., Gade S, Kronenwett R, et al. Tumor-infiltrating lymphocytes and response to neoadjuvant chemotherapy with or without carboplatin in human epidermal growth factor receptor 2-positive and triple-

negative primary breast cancers. J Clin Oncol. 2015;
17. Ono M, Tsuda H, Shimizu C, Yamamoto S, Shibata T, Yamamoto H, et al. Tumor-infiltrating lymphocytes are correlated with response to neoadjuvant chemotherapy in triple-negative breast cancer. Breast Cancer Res Treat [Internet]. 2012 Apr [cited 2022 Feb 11];132(3):793–805. Available from: https://pubmed.ncbi.nlm.nih.gov/21562709/
18. Asano Y, Kashiwagi S, Goto W, Takada K, Takahashi K, Hatano T, et al. Prediction of treatment response to neoadjuvant chemotherapy in breast cancer by subtype using tumor-infiltrating lymphocytes. Anticancer Res. 2018;
19. Ruan M, Tian T, Rao J, Xu X, Yu B, Yang W, et al. Predictive value of tumor-infiltrating lymphocytes to pathological complete response in neoadjuvant treated triple-negative breast cancers. Diagn Pathol. 2018;
20. Salgado R, Denkert C, Demaria S, Sirtaine N, Klauschen F, Pruneri G, et al. The evaluation of tumor-infiltrating lymphocytes (TILS) in breast cancer: Recommendations by an International TILS Working Group 2014. Annals of Oncology. 2015.
21. Swisher SK, Wu Y, Castaneda CA, Lyons GR, Yang F, Tapia C, et al. Interobserver Agreement Between Pathologists Assessing Tumor-Infiltrating Lymphocytes (TILs) in Breast Cancer Using Methodology Proposed by the International TILs Working Group. Ann Surg Oncol. 2016;
22. Ibrahim EM, Al-Foheidi ME, Al-Mansour MM, Kazkaz GA. The prognostic value of tumor-infiltrating lymphocytes in triple-negative breast cancer: a meta-analysis. Breast Cancer Res Treat 2014 1483 [Internet]. 2014 Nov 1 [cited 2022 May 18];148(3):467–76. Available from: https://link.springer.com/article/10.1007/s10549-014-3185-2
23. Francisco LM, Sage PT, Sharpe AH. The PD-1 pathway in tolerance and autoimmunity. Immunol Rev [Internet]. 2010 Jul 1 [cited 2022 May 18];236(1):219–42. Available from: https://onlinelibrary.wiley.com/doi/full/10.1111/j.1600-065X.2010.00923.x
24. Mittendorf EA, Philips A V., Meric-Bernstam F, Qiao N, Wu Y, Harrington S, et al. PD-L1 expression in triple-negative breast cancer. Cancer Immunol Res [Internet]. 2014 Apr 1 [cited 2022 May 18];2(4):361–70. Available from: https://aacrjournals.org/cancerimmunolres/article/2/4/361/467294/PD-L1-Expression-in-Triple-Negative-Breast
25. Wimberly H, Brown JR, Schalper K, Haack H, Silver MR, Nixon C, et al. PD-L1 expression correlates with tumor-infiltrating lymphocytes and response to neoadjuvant chemotherapy in breast cancer. Cancer Immunol Res [Internet]. 2015 Apr 1 [cited 2022 May 18];3(4):326–32. Available from: https://aacrjournals.org/cancerimmunolres/article/3/4/326/467790/PD-L1-Expression-Correlates-with-Tumor
26. Ali HR, Glont SE, Blows FM, Provenzano E, Dawson SJ, Liu B, et al. PD-L1 protein expression in breast cancer is rare, enriched in basal-like tumours and associated with infiltrating lymphocytes. Ann Oncol. 2015 Jul 1;26(7):1488–93.
27. Muenst S, Schaerli AR, Gao F, Däster S, Trella E, Droeser RA, et al. Expression of programmed death ligand 1 (PD-L1) is associated with poor prognosis in human breast cancer. Breast Cancer Res Treat 2014 1461 [Internet]. 2014 May 20 [cited 2022 May 18];146(1):15–24. Available from:

https://link.springer.com/article/10.1007/s10549-014-2988-5
28. Beckers RK, Selinger CI, Vilain R, Madore J, Wilmott JS, Harvey K, et al. Programmed death ligand 1 expression in triple-negative breast cancer is associated with tumour-infiltrating lymphocytes and improved outcome. Histopathology [Internet]. 2016 Jul 1 [cited 2022 May 18];69(1):25–34. Available from: https://onlinelibrary.wiley.com/doi/full/10.1111/his.12904
29. Chevallier B, Roche H, Olivier JP, Chollet P, Hurteloup P. Inflammatory breast cancer. Pilot study of intensive induction chemotherapy (FEC-HD) results in a high histologic response rate. Am J Clin Oncol [Internet]. 1993;16(3):223–8. Available from: http://europepmc.org/abstract/MED/8338056
30. Adams S, Gray RJ, Demaria S, Goldstein L, Perez EA, Shulman LN, et al. Prognostic value of tumor-infiltrating lymphocytes in triple-negative breast cancers from two phase III randomized adjuvant breast cancer trials: ECOG 2197 and ECOG 1199. J Clin Oncol [Internet]. 2014 Sep 20 [cited 2022 May 18];32(27):2959–66. Available from: https://pubmed.ncbi.nlm.nih.gov/25071121/
31. Becht E, Giraldo NA, Dieu-Nosjean MC, Sautès-Fridman C, Fridman WH. Cancer immune contexture and immunotherapy. Curr Opin Immunol [Internet]. 2016 Apr 1 [cited 2022 Jun 27];39:7–13. Available from: https://pubmed.ncbi.nlm.nih.gov/26708937/
32. Kroemer G, Senovilla L, Galluzzi L, André F, Zitvogel L. Natural and therapy-induced immunosurveillance in breast cancer. Nat Med [Internet]. 2015 Oct 7 [cited 2022 Jun 27];21(10):1128–38. Available from: https://pubmed.ncbi.nlm.nih.gov/26444637/
33. Castaneda CA, Mittendorf E, Casavilca S, Wu Y, Castillo M, Arboleda P, et al. Tumor infiltrating lymphocytes in triple negative breast cancer receiving neoadjuvant chemotherapy. World J Clin Oncol. 2016;
34. Gu-Trantien C, Loi S, Garaud S, Equeter C, Libin M, De Wind A, et al. CD4+ follicular helper T cell infiltration predicts breast cancer survival. J Clin Invest [Internet]. 2013 Jul 7 [cited 2022 Jun 27];123(7):2873. Available from: /pmc/articles/PMC3696556/
35. Jang N, Kwon HJ, Park MH, Kang SH, Bae YK. Prognostic Value of Tumor-Infiltrating Lymphocyte Density Assessed Using a Standardized Method Based on Molecular Subtypes and Adjuvant Chemotherapy in Invasive Breast Cancer. Ann Surg Oncol. 2018;
36. Park HS, Heo I, Kim JY, Kim S, Nam S, Park S, et al. No effect of tumor-infiltrating lymphocytes (TILs) on prognosis in patients with early triple-negative breast cancer: Validation of recommendations by the International TILs Working Group 2014. J Surg Oncol. 2016 Jul 1;114(1):17–21.
37. Savas P, Salgado R, Denkert C, Sotiriou C, Darcy PK, Smyth MJ, et al. Clinical relevance of host immunity in breast cancer: From TILs to the clinic. Nature Reviews Clinical Oncology. 2016.
38. Stanton SE, Adams S, Disis ML. Variation in the Incidence and Magnitude of Tumor-Infiltrating Lymphocytes in Breast Cancer Subtypes: A Systematic Review. Vol. 2, JAMA oncology. 2016. p. 1354–60.
39. Teschendorff AE, Gomez S, Arenas A, El-Ashry D, Schmidt M, Gehrmann M, et al. Improved prognostic classification of breast cancer defined by antagonistic activation patterns of immune response pathway modules. BMC Cancer. 2010;

40. Yeong J, Thike AA, Lim JCT, Lee B, Li H, Wong SC, et al. Higher densities of Foxp3+ regulatory T cells are associated with better prognosis in triple-negative breast cancer. Breast Cancer Res Treat. 2017;
41. Lee S, Cho EY, Park YH, Ahn JS, Im YH. Prognostic impact of FOXP3 expression in triple-negative breast cancer. Acta Oncol (Madr). 2013;
42. Matsumoto H, Koo SL, Dent R, Tan PH, Iqbal J. Role of inflammatory infiltrates in triple negative breast cancer. J Clin Pathol. 2015;
43. West N, Kost S, Martin S, Milne K, Deleeuw R, Nelson B. Tumour-infiltrating FOXP3(+) lymphocytes are associated with cytotoxic immune responses and good clinical outcome in oestrogen receptor-negative breast cancer. Br J Cancer. 2013;108(1):155–62.
44. Denkert C, von Minckwitz G, Darb-Esfahani S, Lederer B, Heppner BI, Weber KE, et al. Tumour-infiltrating lymphocytes and prognosis in different subtypes of breast cancer: a pooled analysis of 3771 patients treated with neoadjuvant therapy. Lancet Oncol. 2018;
45. Adams S, Gray RJ, Demaria S, Goldstein L, Perez EA, Shulman LN, et al. Prognostic value of tumor-infiltrating lymphocytes in triple-negative breast cancers from two phase III randomized adjuvant breast cancer trials: ECOG 2197 and ECOG 1199. J Clin Oncol. 2014;
46. Galluzzi L, Buqué A, Kepp O, Zitvogel L, Kroemer G. Immunogenic cell death in cancer and infectious disease. Nat Rev Immunol 2016 172 [Internet]. 2016 Oct 17 [cited 2022 Feb 11];17(2):97–111. Available from: https://www.nature.com/articles/nri.2016.107