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A Standardized Evaluation Method for FOXP3+ Tregs and CD8+ T-cells in Breast Carcinoma: Association With Breast Carcinoma Subtypes, Stage and Prognosis

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Abstract. *Background/Aim: The role of FOXP3+ Tregs and CD8+ T-cells in different stages and subtypes of breast carcinoma (BC) is yet to be fully defined, mainly because of methodological variations between studies. The aim of this study was to assess FOXP3+ and CD8+ intratumoral stromal TILs (sTILs) by a standardized method, in order to discern differences between the histological subtypes and BC stage and evaluate the applicability of the method. Patients and Methods: FOXP3+ and CD8+ sTILs were studied immunohistochemically in 207 BCs and counted on digital images, amounting to a standard stromal area of a 10×10 grid on ×40 magnification. The results were correlated with clinicopathological features and outcomes. Results: Tregs and CD8+ TILs were more abundant in HER2+ BCs (p=0.02, p=0.007, respectively), estrogen receptor (ER)- BCs (p<0.001, for both cell types), and triple-negative BCs (TNBCs) (p=0.01, p=0.006, respectively). Tregs and CD8+ TILs were associated with high grade (p<0.001 and p=0.002, respectively) and high Ki67 index (p<0.001, for both cell types). Lower CD8/FOXP3 ratio was associated with node metastases (p=0.007). Node metastases and advanced stage paralleled with decreased CD8+ sTILs (p=0.023, p=0.019, respectively). In the entire group and in ER– BCs, CD8+ TILs were associated with favorable distant metastasis-free survival (p=0.021, p<0.001, respectively), disease-free survival (p=0.022, p<0.001, respectively) and breast cancer specific survival (BCSS) (p=0.022, p=0.005). In ER- BCs, Tregs were associated with favorable BCSS (p=0.02). Conclusion: Tregs and CD8+ TILs are higher in early-stage TNBCs and HER2+ BCs and diminish with progression to advanced stages. The findings provide support for immunotherapeutic manipulation of TILs, particularly in early stages of these BC subtypes. The evaluation methodology can be easily implemented for standardization of immunohistochemically-detected TILs.*

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Key Words: Breast carcinoma, regulatory T cells (Tregs), FOXP3, Cytotoxic T lymphocytes (CTLs), CD8+ T cells, TILs, immunosurveillance.

Tumor-infiltrating lymphocytes (TILs), a vital component of the tumor microenvironment (TME), represent the local immune response against cancer (1). Most TILs in solid tumors are T lymphocytes, including CD4+ helper cells, CD4+FOXP3+ regulatory T-cells (Tregs) and CD8+ cytotoxic T-cells (CTLs), distributed within the tumor, infiltrating the stromal compartment or the epithelial cell islands, and around the invasive tumor margin (2, 3). Disease progression and clinical outcome are influenced by the subtype of TILs and their biological and functional characteristics, rather than their density (4). Recent clinical research focused on TILs as a biomarker of the immunological response and clinical outcome in various types of cancers, such as malignant melanoma (5, 6), colorectal (7, 8), oral squamous cell (9-11), ovarian (12, 13) and gastrointestinal carcinomas (14, 15).

In breast carcinoma (BC), TILs observed in hematoxylin-eosin (H-E) sections, have been assessed in several large studies, reviews and metaanalyses, and high numbers of TILs have been associated with triple-negative BC (TNBC) and human epidermal growth factor receptor 2 (HER2) + BC (16-25). The prognostic significance of H-E TILs has been shown in lymphocyte-predominant BC (LPBC) (26), TNBC (16-18, 27), and HER2+ BC (26, 28), in contrast to the lack of prognostic value in estrogen receptor (ER)+ BC (16). Reflecting the emerging importance of H-E TILs in BC, the International TILs Working Group has published consensus guidelines for the evaluation methodology of H-E TILs in invasive BC (28),

adopted by the International Immuno-Oncology Biomarkers Working Group (29). Furthermore, guidelines have been proposed for the evaluation of H-E TILs in the post-neoadjuvant setting and in ductal carcinoma in situ of the breast (30).

The various subtypes of TILs have been investigated in BC by immunohistochemistry, flow cytometry or gene expression analysis (20, 22, 31-35). CTLs, through a Th1 immune response, exert a direct lytic effect on tumor cells by triggering apoptosis and are considered the frontline defense against cancer. Several studies, reviews and metaanalyses have linked notable amounts of effector T-cells with a favorable clinical outcome (20, 22-24, 31, 32). In contrast, during Th2 responses, Tregs suppress cytokine production, CD8+ T-cell proliferation and release of cytotoxic granules and induce apoptotic death of CTLs and other cell types, resulting in suppression of the antineoplastic immune response (36, 37). Association of Tregs with prognosis, clinicopathological parameters and BC subtypes (20, 22, 32-34, 38-40) results in often contradictory findings, possibly attributed to methodological differences between the studies that blur the underlying associations. To the best of our knowledge no standardized methodology has been advocated for immunohistochemically outlined TILs subpopulations in BC.

The present study aimed to analyze the major, clinically significant, TIL subtypes of the TME in early and locally advanced BC with a standardized method and discern differences in TME composition during disease progression. The average presence of FOXP3+ Tregs and CD8+ TILs identified by immunohistochemistry within a standard surface area of the intratumoral stroma was quantified and their possible association to BC subtypes and clinicopathological parameters, and their contribution to progression and outcome of BC was explored.

Patients and Methods

The present retrospective study included 207 consecutive patients operated for invasive BC, in the University Hospital of Patras, from January 2000 to December 2011, with available histopathological slides, tissue blocks as well as regular follow-up clinical information until death or their last appointment. Patients who received preoperative radiation or neoadjuvant chemotherapy or had metastatic disease at the time of diagnosis were excluded. For each patient, the following clinicopathological parameters were recorded: age, tumor size, histologic grade, ER, progesterone receptor (PR), HER2, Ki67 index, lymphatic/blood vessel invasion, regional lymph node metastasis, and follow-up data. This protocol was approved by the Ethics Research Committee of University Hospital of Patras, Patras, Greece.

All available H-E tumor slides were reviewed for each case, in order to re-assess the above described histopathological parameters, to evaluate stromal TILs (sTILs), and select a representative tumor block depicting the average immune infiltration of the tumor, as TILs density varied within each tumor section and between different sections of the same tumor. The median age of the enrolled patients was 60 years [interquartile range (IQR)=48.75-70]. The age at diagnosis was over 50 years in 149 patients (71.98%). The tumor size was ≤5 cm (T1 and T2) in 179 patients (86.47%), and lymph node metastases were observed in 100 patients (48.31%). The Nottingham histologic grade (Elston–Ellis modification of Scarff–Bloom–Richardson grading system) (41) was used for the assessment of the histologic grade. Using this classification system, 18 cases met criteria for Nottingham grade 1, 93 cases were characterized as Nottingham grade 2, and 98 cases were characterized as Nottingham grade 3. BCs were classified in immunohistochemically defined surrogate molecular subtypes, according to the recommendations of the St. Gallen International Breast Cancer Conference recommendations (42). They included 46 luminal A (ER+ and/or PR+, and Ki-67 <20%) (22.22%), 58 HER2- luminal B (ER+ and/or PR+ and Ki-67≥20%) (28.02%), 16 HER2+ luminal B (ER+ and/or PR+ and HER2+) (7.73%), 31 hormone receptor (HR)-/HER2+ (ER–, PR–, HER2+) BCs (14.98%), and 56 TNBC (ER–, PR–, HER2–) (27.05%). Tumor stage was recorded according to the American Joint Committee on Cancer (AJCC) classification (7th edition). The median follow-up time after surgery was 70 months (IQR=55-88.5).

Immunohistochemical analysis. Immunohistochemistry was performed on 4-μm-thick formalin-fixed, paraffin-embedded (FFPE) full sections of BCs, mounted on positively charged glass slides. Briefly, paraffin-embedded tissue sections were dewaxed and rehydrated in xylene and graded alcohol solutions. Antigen retrieval was performed in an electric microwave oven, using antigen retrieval solution (EDTA, pH 9.0) for 30 min. Subsequently the slides were incubated with the primary antibodies anti-FOXP3 (mouse monoclonal antibody, clone 236/E7, Abcam, Cambridge, UK, 1:100 dilution) and anti-CD8 (mouse monoclonal antibody, clone C8/144B, Dako, CA, USA, ready to use) for 30 min at room temperature. After appropriate washes, the slides were incubated with the detection system (Dako EnVision Labelled Polymer, Dako) for 30 min. Diaminobenzidine (Dako) was used as chromogen. Nuclei were counterstained with hematoxylin. Sections from human tonsil were used as positive controls for FOXP3+ and CD8+ lymphocytes.

Assessment of immunohistochemical staining. Immunohistochemical stains for CD8+ and Foxp3+ stromal TILs (sTILs) were considered positive when evident membranous/cytoplasmic or nuclear immunoreactions, respectively, were noted. The evaluation included sTILs present within the confines of the tumor. TILs present around the tumor border (peritumoral TILs) or infiltrating the epithelial cell nests were not assessed. Immunostained sections were scanned at low magnification, in order to select areas depicting intermediate lymphocytic infiltration. Hot spots, sTIL-depleted, necrotic or desmoplastic areas were excluded. Subsequently, at high magnification (×40) 4-6 non-overlapping tumor foci were captured as digital images (Amscope MU1000, Irvine, CA, USA). The digital images were processed by digital processing software (ImageJ, version 1.48 Wayne Rasband, National Institutes of Health, Bethesda, MD, USA), in order to acquire the regions of interest (ROI), corresponding to the intratumoral stromal compartment. Tumor nests within the selected area were erased. The absolute score was obtained by observer counting and manual clicking on the cells of interest within the images of stroma. These were chosen to add up to a standard stromal area of 0.0625 mm^2. This value corresponds to the surface of a 10×10 square grid at 40× magnification (Figure 1). Upon completion of counting, the software provided the absolute number of the cells counted, which

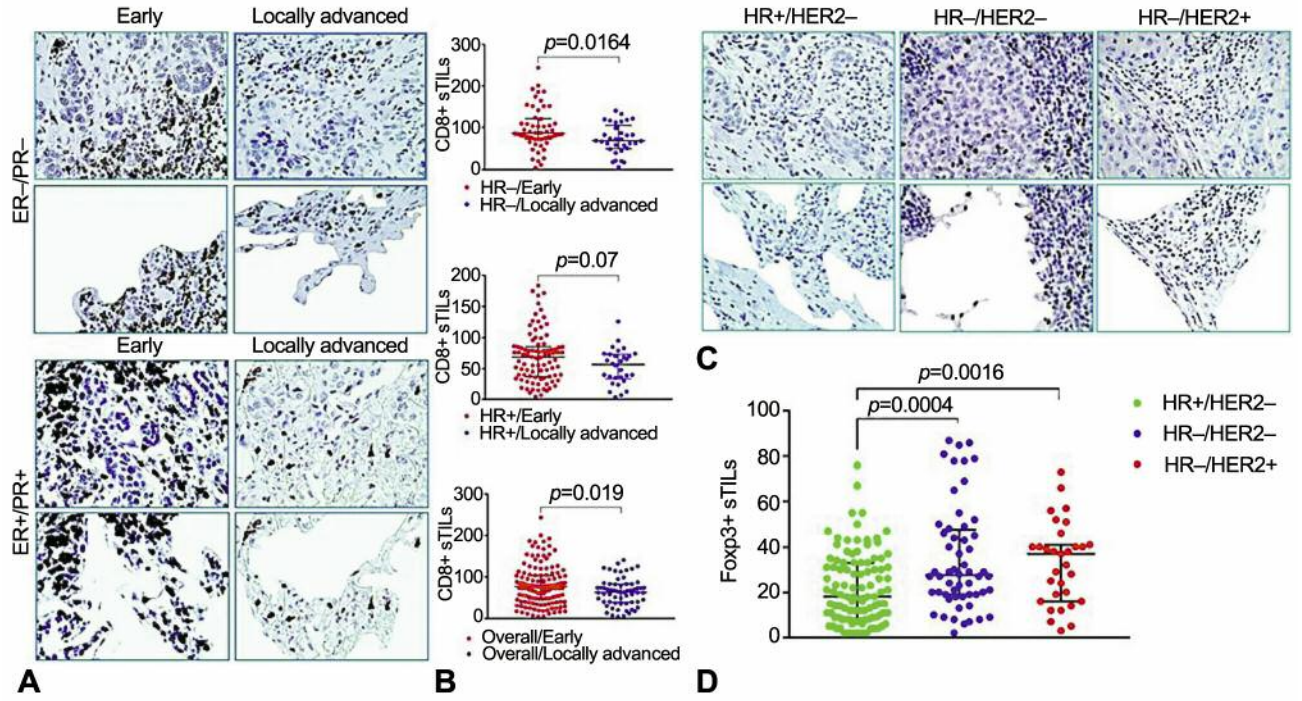

Figure 1. *CD8+ and FOXP3+ sTILs in breast carcinoma (BC). (A) Representative images CD8+ sTILs in sections of early and locally advanced BCs, according to estrogen receptor (ER) status. Diminished numbers of CD8+ T cells were observed in locally advanced BCs in both the hormone receptor (HR)– and HR+ subgroups, while high numbers of CD8+ cells were detected in early BCs. (B) Scatter dot plots display the significantly lower numbers of CD8+ T cells both in the overall (HR– and HR+) and HR– BC subtypes, among early and locally advanced BCs (p=0.0187; p=0.0164). (C) Representative images of FOXP3+ sTILs in sections of BCs, according to the HR/HER2 status. Higher numbers of FOXP3+ T cells were detected in the HR– HER2+ subtypes as compared to the HR+HER2- and the triple-negative BCs (TNBCs). (D) Scatter dot plot of FOXP3+ sTILs with respect to HR+HER2–, HR-HER2– and HR-HER2+ subtypes. The median number of FOXP3+ sTILs was found significantly higher in the HR–HER2+ group and TNBCs compared to the HR+HER2– BC group. p-Values were calculated using the Mann–Whitney test. The black colored vertical line represents the median value and the interquartile range. Scattered dots were deployed using GraphPad Prism 7.04.*

was exported and used for statistical analysis. The median of CD8+ and FOXP3+ TILs per 0.0625 mm^2 stromal area defined the cut-off for low and high values. Density of sTIL subtypes was analyzed in relation to the clinicopathologic parameters and clinical outcome.

Survival endpoints. Events such as locoregional recurrence, distant metachronous metastases, death, and loss to follow-up were recorded. Different survival endpoints, including distant metastasis-free survival (DMFS), disease-free survival (DFS) and breast cancer-specific survival (BCSS) were calculated. DMFS was defined as the time period from the date of surgery until an event of metastasis occurred. DFS was defined as the time period from the date of surgery until the occurrence of metastasis and/or local recurrence, or until last follow-up visit, in the absence of disease recurrence. BCSS was defined as the time period from the date of surgery to the date of death attributed to BC or to the last follow-up visit. Dead from another cause or living patients at the end of follow-up were treated as censored.

Statistical analysis. Continuous parameters were expressed as mean±standard deviation (SD) or median±IQR. Continuous variables were compared as appropriate using Mann–Whitney or the Kruskal–Wallis test when inter-group comparisons of more than two variables were performed. FOXP3+ and CD8+ stromal T-cell density was classified as high or low in relation to the median value, and survival analyses were performed. The Kaplan–Meier survival analysis and the log-rank test were used to calculate DMFS, DFS and BCSS and evaluate statistical differences. Cox proportional hazards regression models were built for univariate and multivariate survival analyses to estimate the hazard ratios (HRs) of sTILs prognostic effect. All variables with *p*-values of <0.2 from the univariate analysis were included in the multivariate analysis using the Cox proportional hazards model. The event variable was distant metastasis, disease recurrence including both metastasis and local relapse and death due to BC. DMFS, DFS and BCSS cumulative incidence was estimated based on the life-table method using the Gehan's Wilcoxon test for paired analyses. Statistical analysis was performed using SPSS 24.0 for Windows software (SPSS Inc, Chicago, IL, USA). Probability values (two-sided) were considered to indicate statistical significance at $p<0.05$.

Results

FOXP3+ and CD8+ sTILs with regards to BC histopathologic parameters. CD8+ and FOXP3+ sTILs were mainly distributed in the stromal compartment and around the

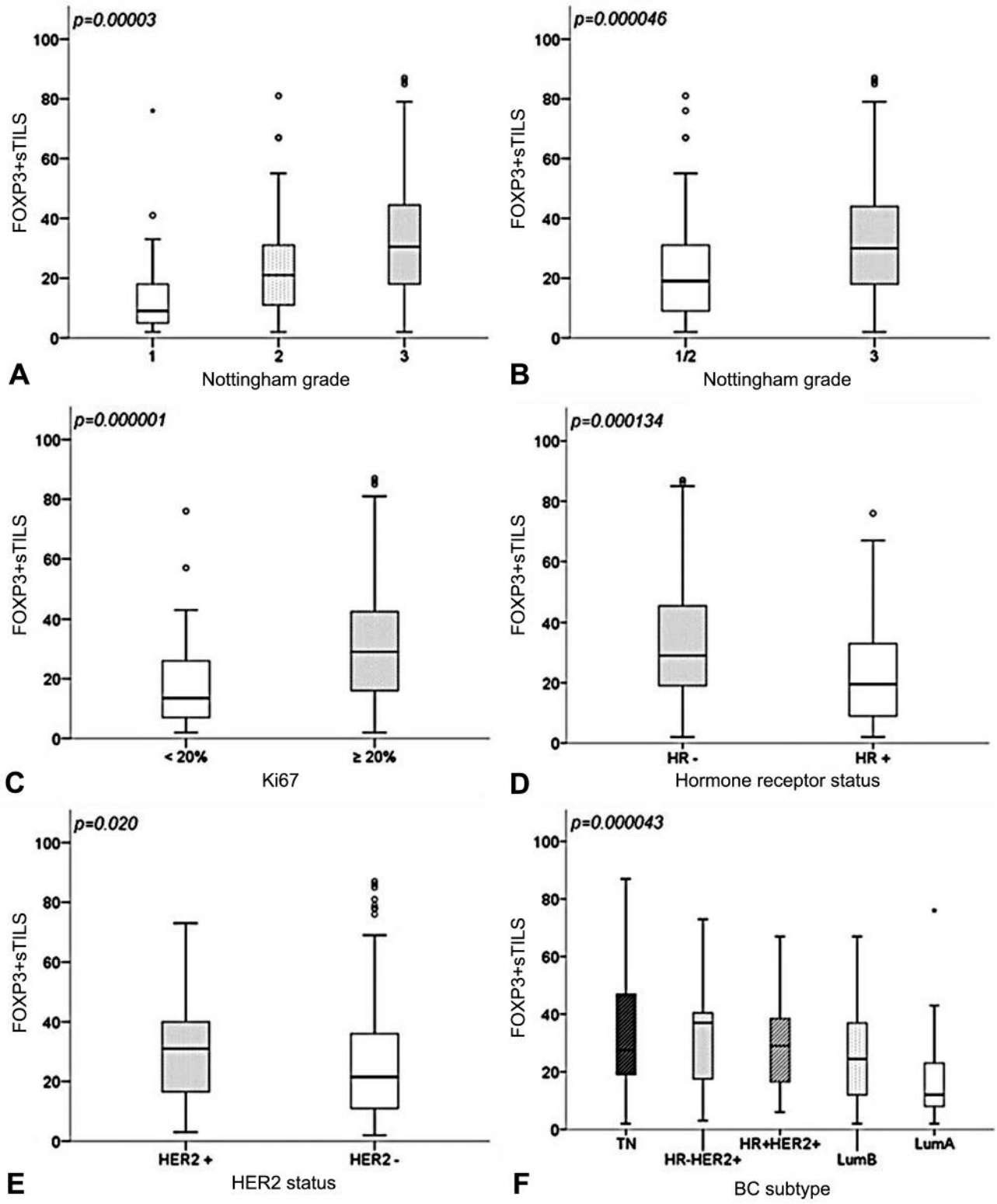

Figure 2. *Boxplot diagrams displaying the number of FOXP3+ TILs according to histologic grade (A, B), Ki67 score (C), Hormone Receptor (HR) status (D), HER2 expression (E), and breast carcinoma (BC) subtypes (F). Median value (bold line across the box), interquartile range (box) which contains the central 50% of values, outliers (circles) and extreme observations (star) according to standard definitions. The whiskers are lines that extend from the box to the highest and lowest values, excluding outliers. p-Value in each diagram refers to the Kruskal–Wallis H test.*

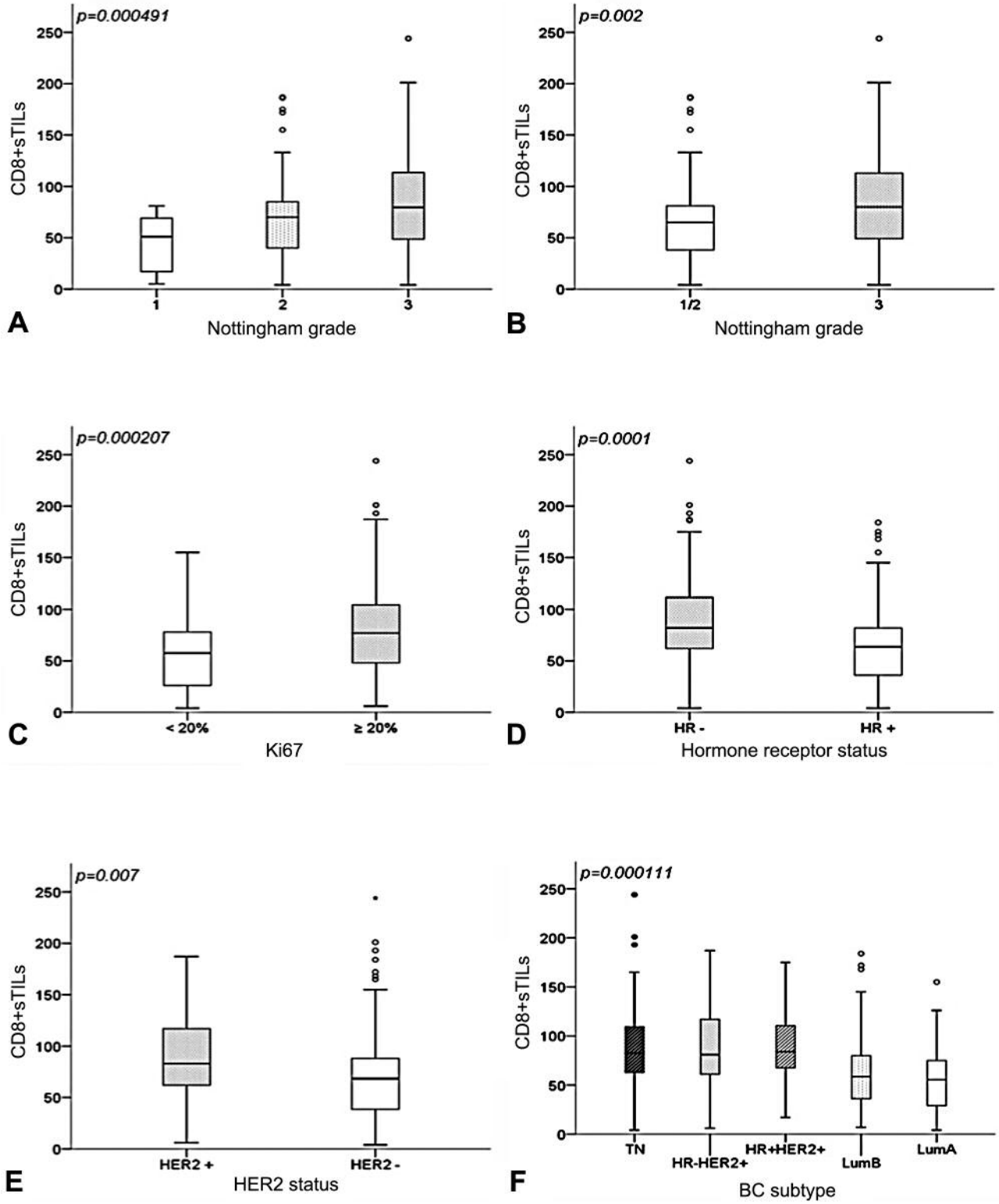

Figure 3. *Boxplot diagrams displaying the numbers of CD8+ TILs according to histological grade (A, B), Ki67 score (C), Hormone Receptor (HR) status (D), HER2 expression (E), and breast carcinoma (BC) subtypes (F). Median value (bold line across the box), interquartile range (box) which contains the central 50% of values, outliers (circles) and extreme observations (star) according to standard definitions. The whiskers are lines that extend from the box to the highest and lowest values, excluding outliers. p-Value in each diagram refers to the Kruskal–Wallis H test.*

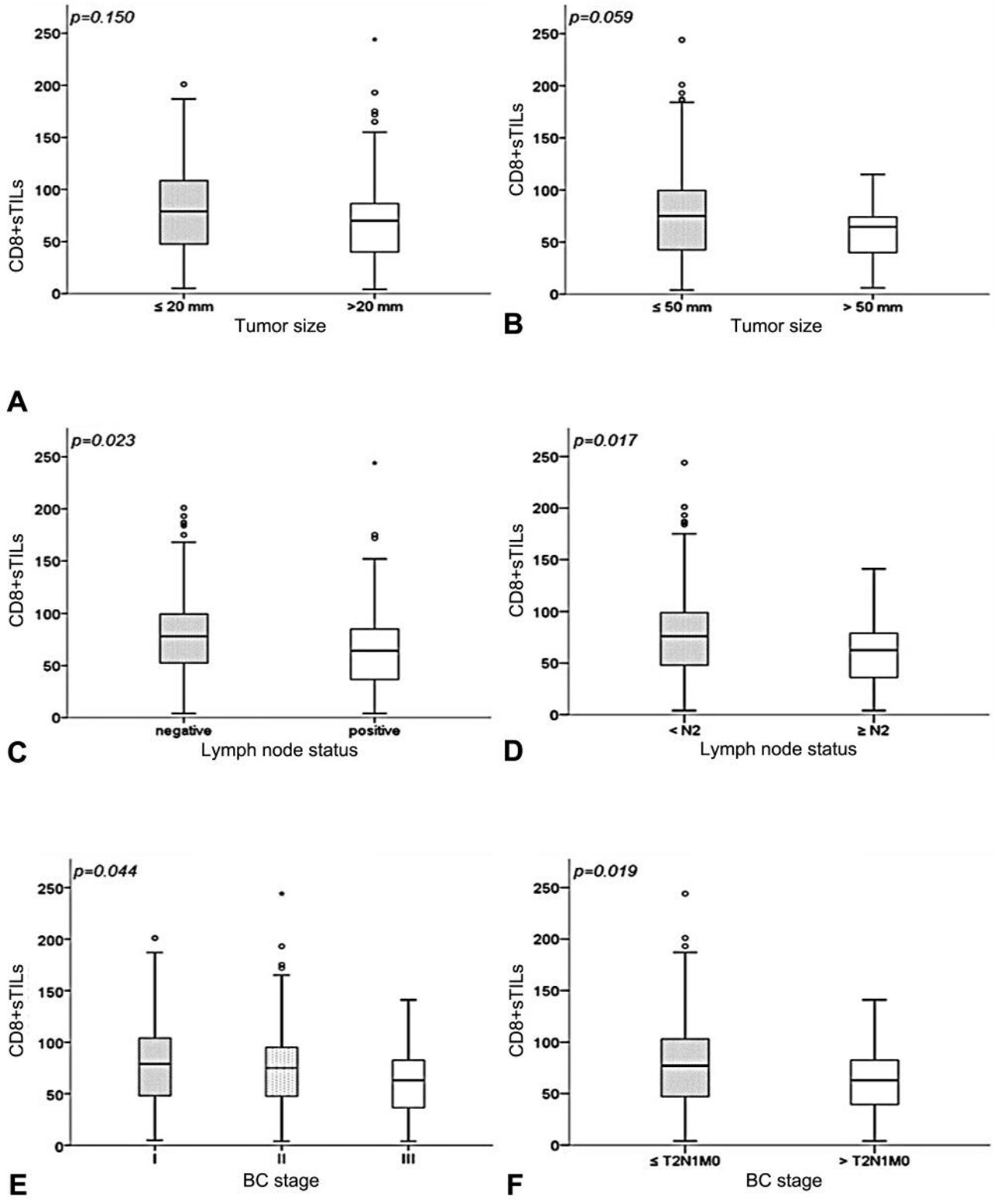

Figure 4. *Boxplot diagrams displaying the numbers of CD8+ TILs according to tumor size (A, B), lymph node status (C, D) and breast carcinoma (BC) stage (E, F). Median value (bold line across the box), interquartile range (box) which contains the central 50% of values, outliers (circles) and extreme observations (star) according to standard definitions. The whiskers are lines that extend from the box to the highest and lowest values, excluding outliers. p-Value in each diagram refers to Kruskal–Wallis H test.*

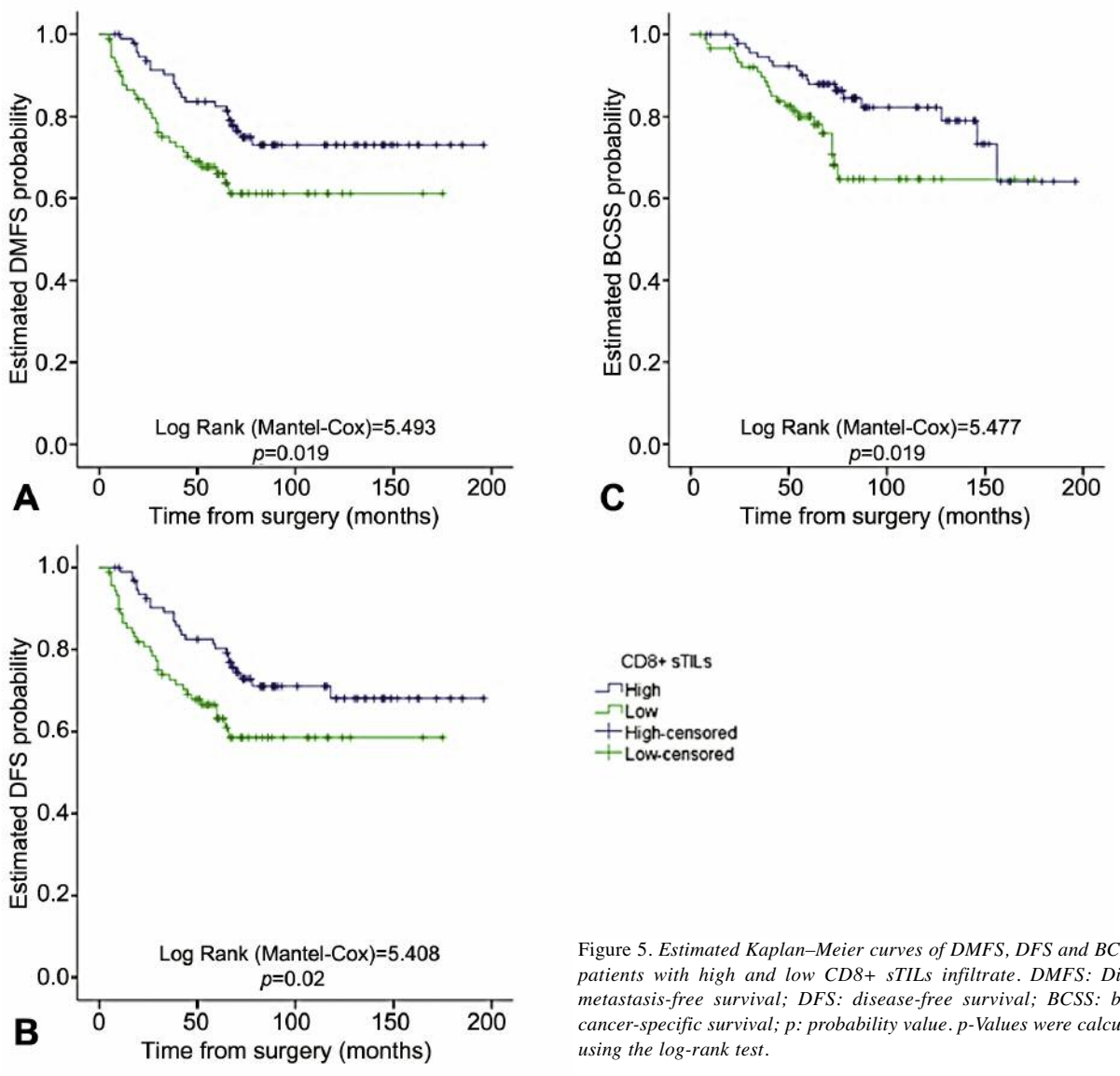

Figure 5. *Estimated Kaplan–Meier curves of DMFS, DFS and BCSS in patients with high and low CD8+ sTILs infiltrate. DMFS: Distant metastasis-free survival; DFS: disease-free survival; BCSS: breast cancer-specific survival; p: probability value. p-Values were calculated using the log-rank test.*

invasive margin of the tumor, similarly to the distribution pattern of the lymphocytes by conventional H-E staining. There was a significant heterogeneity in the distribution of the inflammatory infiltrate in the tumor stroma, within the cancer cell nests and around the invasive margin of the tumor. The median count of FOXP3+ cells per 0.0625 mm^2 of stromal area was 24 (IQR=12-39) and for CD8+ cells 73 (IQR=40-95) and the median CD8/FOXP3 TILs ratio was 3 (IQR=1.92-4.81). The associations between FOXP3+ and CD8+ cell counts and the immunohistochemical surrogate subtypes, stage, histopathologic and clinical parameters are summarized in Table I and depicted in Figures 1-4.

FOXP3+ and CD8+ sTILs' density differed between Nottingham grade groups (1/2/3: $p<0.001$ for both cells types) and high *vs*. low Ki67 proliferation index ($p<0.001$, for both cells types), corresponding to increased infiltration in cases with higher histologic grade and proliferation. Results are demonstrated in Figures 2A-C and 3A-C. No differences were observed for FOXP3+ and CD8+ sTILs score regarding lymphatic vessel invasion ($p=0.752$, $p=0.508$) and blood vessel invasion ($p=0.237$, $p=0.948$).

sTILs density among immunohistochemical surrogate BC subtypes. FOXP3+ and CD8+ sTILs density was significantly different between BC subtypes ($p<0.001$ for both cell types). HR– BCs were profoundly infiltrated by FOXP3+ and CD8+ sTILs ($p<0.001$ for both cells types) compared to HR+ BCs (Figures 2D and D). Tregs and CD8+ T-cells were more

Table I. *sTILs in relation to clinicopathological parameters of breast carcinoma (BC) patients.*

		FOXP3+ sTILs/0.0625 mm^2		CD8+ sTILs/0.0625 mm^2		CD8+/FOXP3+ sTILs ratio	
	N	Median (Q1, Q3)	*p*-Value	Median (Q1, Q3)	*p*-Value	Median (Q1, Q3)	*p*-Value
Age							
≥50	149	23 (12-37)	0.223	73 (40-89.5)	0.270	2.9 (1.97-4.85)	0.861
<50	57	29 (9.5-45.5)		74 (43.5-112)		3.08 (1.86-4.68)	
Nottingham grade							
1	18	9 (4.75-20.75)	0.00003	51 (17-70)	0.000491	3.61 (1.66-5.31)	0.075
2	93	21 (11-32)		70 (40-85)		3.5 (2-5.4)	
3	96	30.5 (18-44.75)		79.5 (48.25-115.25)		2.69 (1.8-3.92)	
Nottingham grade							
1+2	110	19 (9-31)	0.000046	65 (37.75-81.25)	0.002	3.5 (2-5.36)	0.036
3	97	30 (18-44.5)		80 (48.5-115)		2.79 (1.81-3.97)	
Nottingham grade							
1	18	9 (4.75-20.75)	0.002	51 (17-70)	0.001	3.61 (1.66-5.31)	0.564
2+3	189	26 (13.5-39.5)		75 (47-99.5)		2.92 (1.93-4.71)	
Ki67							
<20%	58	13.5 (7-26.25)	0.000001	57.5 (25.75-78.25)	0.000207	3.62 (2.39-5.26)	0.052
≥20%	148	29 (16-42.75)		77 (48-104.75)		2.87 (1.82-4.61)	
Tumor size							
≤2 cm	60	26.5 (13.25-38)	0.873	79 (47.25-110.75)	0.150	3.5 (2.21-4.86)	0.126
>2 cm	147	24 (12-39)		70(40-87)		2.86 (1.85-4.81)	
Tumor size							
≤5 cm	179	26 (12-40)	0.458	75 (40-100)	0.059	3.13 (1.92-4.88)	0.290
>5 cm	28	22 (9.75-36)		64.5 (40-74)		2.51 (1.9-3.97)	
Lymph node metastasis							
Negative	107	24 (12-38)	0.780	78 (52-100)	0.023	3.6 (2.16-5.21)	0.007
Positive	100	24 (12.25-39)		64 (36.25-85)		2.56 (1.82-4.1)	
Lymph node status							
<N2	161	26 (12.5-40)	0.352	76 (48-99.5)	0.017	3.19 (1.95-4.81)	0.145
≥N2	46	21.5 (9-38.25)		62.5 (35.75-79.75)		2.37 (1.81-4.86)	
Stage at diagnosis							
I	45	26 (10.5-36.5)	0.401	79 (48-110.5)	0.044	3.714 (2.33-5.114)	0.092
II	107	27 (13-43)		75 (47-99)		3.128 (1.82-4.875)	
III	55	23 (9-38)		63 (36-83)		2.413 (1.871-4.611)	
Early (up to T2N1M0)/Locally advanced							
≤T2N1	144	26 (12-40)	0.629	77 (47-103)	0.019	3.4 (1.99-4.89)	0.051
>T2N1	63	23 (11-38)		63 (39-83)		2.4 (1.81-4.61)	
Lymphatic vessel invasion							
No	168	24.5 (12-38.75)	0.752	73.5 (47-95)	0.508	3.08 (1.99-4.89)	0.322
Yes	39	24 (14-39)		62 (40-91)		2.84 (1.81-4.46)	
Blood vessel invasion							
No	186	23.5 (12-38.25)	0.237	73 (40-95)	0.948	3.1 (1.96-4.83)	0.138
Yes	21	30 (16.5-46)		63 (42.5-98)		2.2 (1.61 -4.24)	
ER status							
ER–	87	29 (19-46)	0.000134	82 (62-113)	0.0001	2.59 (1.89-4.48)	0.118
ER+	120	19.5 (9-33)		63.5 (36-82.5)		3.2 (1.94-5.27)	
PR status							
PR–	101	29 (17.5-45.5)	0.000068	81 (58.5-111.5)	0.000115	2.59 (1.9-4.44)	0.088
PR+	106	18.5 (8.75-33)		63.5 (34.75-80.25)		3.28 (1.94-5.88)	
HER2 status							
CERB2–	160	21.5 (11-36.5)	0.020	68.5 (38.25-88)	0.007	3.13 (1.86-4.8)	0.532
CERB2+	47	31 (16-40)		83 (62-119)		2.54 (1.98-4.89)	

sTILs: Stromal tumor-infiltrating lymphocytes; HR: hormone receptor status; HER2: human epidermal growth factor receptor 2.

abundant in HER2+, including luminal/ HER2+ BCs (p=0.02, p=0.007, respectively) versus HER2-negative BCs (Figures 2E and E), and in TNBCs *versus* luminal tumors (p=0.001, for both cells types). The HR–/HER2+ BCs had higher infiltration of Tregs (p=0.039) and CD8+ sTILs (p=0.047), as compared to all the remaining subtypes, but both cell types did not differ significantly between HR–/HER2+ BCs and TNBCs (FOXP3+ sTILs; p=0.929, CD8+ sTILs; p=0.947), or

between HR–/HER2+ and HR+/HER2+ (FOXP3+ sTILs; p=0.529, CD8+ sTILs; p=0.919).

sTILs density between early and locally advanced BCs. Distinct patterns of immune infiltration were observed in relation to tumor stage (early *vs*. locally advanced), lymph node (LN) metastasis (positive *vs*. negative) and lymph node status (≥N2 *vs*. <N2). CD8+ sTILs were remarkably diminished in LN positive BCs (p=0.023), in patients with 4 or more positive lymph nodes (p=0.017) (Figures 4C and D) as well as in locally advanced BC (p=0.019) (Figures 4E and F). Although a trend for decreasing CD8+ sTILs was observed with increasing tumor size (>5 cm *vs*. ≤5 cm), this association was of borderline statistical significance (p=0.059). No statistically significant difference was observed regarding FOXP3+ sTILs in relation to tumor stage (>T2N1 *vs*. ≤T2N1, p=0.629), lymph node status (positive *vs*. negative, p=0.780; ≥N2 *vs*. <N2, p=0.352) and tumor size (>5 cm *vs*. ≤5 cm, p=0.458). Nonetheless, lower CD8+/FOXP3+ sTILs ratio was observed among locally advanced BCs as compared to early BCs (p=0.051).

Clinical outcomes. All BC patients were regularly monitored until death or their last appointment. Among all enrolled patients, the 5- and 10-year DMFS rate was 74% and 64%, respectively. The 5- and 10-year DFS rate was 73% and 61%, respectively. The 5- and 10-year BCSS rate was 84% and 74%, respectively Among all patients, the median time to disease recurrence was 30 (IQR=17-52) months and to distant metastasis 29.5 (IQR=16.25-45.5) months.

Tumor stage and clinical outcome. The subgroups of early and locally advanced BCs differed significantly in DMFS (log-rank test=15.465, p<0.001, HR=2.788, 95%CI1.633-4.760, p<0.001), DFS (log-rank test=13.446, p<0.001, HR=2.520, 95%CI=1.509-4.208, p<0.001) and BCSS (log-rank test=12.034, p=0.001, HR=2.880, 95%CI=1.540-5.384, p=0.001).

DMFS rate according to sTILs and tumor stage. The 5- and 10-year DMFS rate was 83% and 73%, respectively for early BC and 56% and 46%, respectively for locally advanced BC (Wilcoxon Gehan=16.599, p<0.001). The 5- and 10-year DMFS rate was 83% and 71% for high CD8+ sTILs in contrast to 65% and 56% in BCs with low CD8+ sTILs (Wilcoxon Gehan=7.069, p=0.008). DMFS was found comparable among low and high FOXP3+ sTIL BC subgroups (Wilcoxon Gehan=0.069, p=0.794).

DFS rate according to sTILs and tumor stage. The 5- and 10-year DFS was 80% and 69%, respectively for early BC and 56% and 43%, respectively for locally advanced BC (Wilcoxon Gehan=14.497, p<0.001). In the high CD8+ sTILs subgroup the 5- and 10-year DFS was 81% and 68%, respectively being higher than the corresponding values of 64% and 53% in the low CD8+ sTIL BCs (Wilcoxon Gehan=7.178, p=0.007). FOXP3+ sTILs did not impact on DFS (Wilcoxon Gehan=0.000464, p=0.983).

BCSS rate according to sTILs and tumor stage. Stratification according to BC stage revealed that for early BC, the 5- and 10-year BCSS rate was 90% and 83%, respectively and for locally advanced BC 72% and 56%, respectively (Wilcoxon Gehan=10.528, p=0.001). The 5- and 10-year BCSS rate was 89% and 83%, respectively in BCs with high CD8+ sTILs, while in BCs with low CD8+ sTILs it was 78% and 63%, (Wilcoxon Gehan=5.621, p=0.018). On the other hand, notable differences of cumulative 5- and 10-year proportional BCSS between high and low FOXP3+ sTIL subgroups were not observed (Wilcoxon Gehan=1.502, p=0.220).

CD8+ sTILs and clinical outcome. The relationship between CD8+ sTILs and survival was analyzed by Kaplan–Meier analysis and the log-rank test (Figure 5). Comparison of BCs with low and high CD8+ sTIL infiltrates, documented significantly shorter DMFS (log-rank test=5.493, p=0.019, Figure 5A), DFS (log-rank test=5.408, p=0.02, Figure 5B) and BCSS (log-rank test=5.477, p=0.019, Figure 5C) among the BC patients with low CD8+ sTILs.

The median BCSS time was 61 months (IQR=49.75-73) for patients with low CD8+ sTILs, significantly shorter than that of patients exhibiting high CD8+ sTILs (77 months, IQR=67-130) (HR=2.128, 95%CI=1.115-4.060, p=0.022). Furthermore, the low CD8+ sTIL group had shorter DMFS (median 55.5 months, IQR=29.75-67.25) compared to the high CD8+ sTIL group (median 74 months, IQR=65-121) (HR=1.898, 95%CI=1.099-3.276, p=0.021), and shorter DFS (median 55.5 months, IQR=28.5-67.25) compared to high CD8+ sTILs expression group (median 74 months, IQR 28.5-67.25) (HR=1.837, 95%CI=1.091-3.095, p=0.022). Stratification of the entire group of BC patients, according to HR status, revealed that CD8+ sTILs show a major impact on HR- BCs as represented in Table II.

FOXP3+ sTILs and clinical outcome. Survival analysis was conducted with regards to FOXP3+ sTIL high and low-density BC subgroups. The mean DMFS, DFS, and BCSS were comparable between the two groups. Treg levels did not affect significantly DMFS (log-rank test=0.008, p=0.929) (HR=1.025, 95%CI=0.600-1.750, p=0.929), DFS (log-rank test=0.017, p=0.896) (HR=0.966, 95%CI=0.579-1.613, p=0.896) or BCSS (log-rank test=3.087, p=0.079) (HR=0.570, 95% CI=0.302-1.076, p=0.083).

Survival analysis was also conducted by splitting the patient population according to HR status (Table II). Cox proportional hazards regression analysis revealed that the prognostic significance of FOXP3+ sTILs was associated with HR- tumor

Table II. *Cox univariate analyses of clinicopathological variables for DMFS, DFS, BCSS in patients with invasive BC stratified by hormone receptor (HR) status.*

	HR–				HR+			
	CD8+ sTILs (low *vs.* high)		FOXP3+ sTILs (low *vs.* high)		CD8+ sTILs (low *vs.* high)		FOXP3+ sTILs (low *vs.* high)	
	HRs (95%CI)	*p*-Value	HRs (95%CI)	*p*-Value	HRs (95%CI)	*p*-Value	HRs (95%CI)	*p*-Value
DMFS	4.401 (1.935-10.012)	0.000410	2.055 (0.936-4.513)	0.073	1.103 (0.527-2.310)	0.794	0.583 (0.280-1.213)	0.149
DFS	4.402 (1.935-10.015)	0.000409	2.067 (0.941-4.538)	0.071	1.040 (0.526-2.055)	0.911	0.659 (0.335-1.296)	0.227
BCSS	3.696 (1.481-9.224)	0.005	2.898 (1.183-7.099)	0.02	1.600 (0.632-4.051)	0.321	1.172 (0.478-2.872)	0.729

CI: Confidence interval; HR–: hormone receptor negative; HR+: hormone receptor positive; HRs: hazard ratios; sTILs: stromal tumor-infiltrating lymphocytes; DMFS: distant metastasis free survival; DFS: disease free survival; BCSS: breast cancer specific survival.

status. On the HR- BC subgroup, high FOXP3+ sTILs showed a favorable effect on BCSS (HR=0.345, 95%CI=0.141-0.845, p=0.02) in contrast to the lack of impact on BCSS among HR+ BCs (HR of 0.853, 95%CI=0.348-2.091, p=0.729).

Multivariate Cox regression analysis. On multivariate analysis, high histologic grade, lymph node metastases and HER2 overexpression were powerful indicators of decreased DMFS, DFS and BCSS. Low numbers of CD8+ sTILs represented an independent predictive factor for DFS (HR=2.221, 95%CI=1.256-3.930, p=0.006), BCSS (HR=2.353, 95%CI=1.132-4.893, p=0.022), and DMFS (HR=2.504, 95%CI=1.382-4.539, p=0.002) (Tables III-V).

Discussion

A standardized evaluation protocol has been proposed by the "International TILs Working Group on BC" for the study of TILs on H-E stained sections of BC (28, 29). Nonetheless, the evaluation of immunohistochemically outlined TIL subpopulations is still not standardized, blurring the importance of the findings and the comparison of results among related studies. In the present study, in order to evaluate FOXP3+ and CD8+ TILs, a protocol as close as possible to the concepts of the H-E TILs protocol was implemented. Thus, full sections were used to provide the opportunity for assessment of the average level of TILs, avoiding hot spots, depleted foci or areas of uncertain derivation, as is often the case with random cores used in tissue microarrays. Immunostained lymphocytes within the intratumoral stroma were then examined, but in order for the results between tumors to be more accurately comparable, it was decided to examine TILs in the same prespecified surface area of stroma for all tumors. This surface was 0.0625 mm^2, equaling to the surface covered by a 10x10 square grid on a 40× high power field. Since the stained cells, particularly FOXP3, are relatively few, the evaluation by the proposed methodology for H-E TILs, implementing a semi-quantitative estimation of percentage of stromal area, would produce results crowded in the lower percentages of surface area, possibly inaccurate and inadequate for stratification. Thus, T-cell subpopulations were quantified using digitized images of several areas of the tumor, corresponding to average TIL concentrations. For facilitation and accuracy of counting, a software device was employed, allowing to click and mark each counted cell of the image, and at the end of counting, export the total number in the appropriate program for further analysis. *Via* this methodology we attempted to standardize our evaluation process. Importantly, this method can be reproduced without the need of a digitized system. By using a 10×10 grid at 40× magnification, immunostained cells can be counted in intratumoral stroma while keeping track of the number of the corresponding surface in terms of the grid's small squares. The count can continue until the total area evaluated corresponds to the entire surface (100 small squares) of the grid, providing standardization of the evaluated intratumoral stromal surface.

In the present study, CTLs outnumbered Tregs and constitute the predominant lymphocyte type, as reported (43-45). FOXP3+ TILs were associated with high tumor grade, and in particular with grade 3 BC, in agreement to previous reports (2, 33, 34, 38, 39, 46-51) and contrary to others (52, 53). They were also associated with high Ki67, similarly to previous reports (32, 51, 52, 54). Tregs were not found to relate to tumor size, as in previously studies (2, 33, 50, 51, 53), although some investigations link higher numbers of Tregs with larger tumor size (38, 39, 49). Higher numbers of FOXP3+ Tregs have been associated with lymph node metastases (32-34, 39, 40, 46, 48, 49, 52, 54-56), a finding not observed in this and other studies (2, 38, 47, 51, 53, 57). With regards to CD8+ sTILs, they were more numerous in grade 3 tumors in agreement with previous reports (51, 58, 59), although contrasting data have also been reported (32, 50). Lymph node-positive and locally advanced BCs displayed decreased numbers of CD8+ sTILs compared to early BCs in line with previous studies (32, 56, 60) and unlike others (50).

Table III. *Univariate and multivariate analyses of variables associated with DFS.*

Variables	Univariate analysis			Multivariate analysis[a]		
	HR	95%CI	*p*-Value	HR	95%CI	*p*-Value
Tumor size (>5 cm *vs*. ≤5 cm)	2.113	1.132-3.945	0.019	1.318	0.676-2.569	0.418
Nodal status (positive *vs*. negative)	3.019	1.662-5.483	0.000285	2.733	1.491-5.009	0.001
Histologic grade (3 *vs*. 1,2)	1.779	1.035-3.055	0.037	2.294	1.301-4.042	0.004
CD8+ sTILs (low *vs*. high)	1.898	1.099-3.276	0.021	2.504	1.382-4.539	0.002
FOXP3+ sTILs (low *vs*. high)	0.976	0.572-1.667	0.929			
ER status (negative *vs*. positive)	1.342	0.783-2.301	0.284			
PR status (negative *vs*. positive)	1.280	0.748-2.190	0.367			
HER2 status (positive *vs*. negative)	1.795	1.010-3.192	0.046	2.256	1.188-4.283	0.013
Age[b]	1.018[c]	0.997-1.040	0.097	1.031	1.009-1.055	0.007

HRs: Hazard ratios; CI: confidence interval; sTILs: stromal tumor-infiltrating lymphocytes; HR: hormone receptor status; HER2: human epidermal growth factor receptor 2; DFS: disease free survival. [a]The multivariate analysis was adjusted for age, tumor size, histologic grade, lymph node status and hormone receptor status; [b]Analysis was performed by the continuous variable; [c]The HRs of the continuous variables are shown as a unit ratio.

Table IV. *Univariate and multivariate analyses of variables associated with BCSS.*

Variables	Univariate analysis			Multivariate analysis[a]		
	HRs	95%CI	*p*-Value	HRs	95%CI	*p*-Value
Tumor size (>5 cm *vs*. ≤5 cm)	2.053	1.127-3.743	0.019	1.384	0.729-2.628	0.321
Nodal status (positive *vs*. negative)	2.734	1.565-4.775	0.00041	2.523	1.433-4.444	0.001
Histologic grade (3 *vs*. 1,2)	1.436	0.860-2.396	0.167	1.862	1.086-3.195	0.024
CD8+ sTILs (low *vs*. high)	1.837	1.091-3.095	0.022	2.221	1.256-3.930	0.006
FOXP3+ sTILs (low *vs*. high)	1.035	0.620-1.727	0.896			
ER status (negative *vs*. positive)	1.088	0.644-1.838	0.753			
PR status (negative *vs*. positive)	1.022	0.610-1.714	0.934			
HER2 status (positive *vs*. negative)	1.548	0.880-2.724	0.130	1.910	1.020-3.577	0.043
Age[b]	1.022[c]	1.001-1.043	0.036	1.033	1.011-1.055	0.003

HRs: Hazard ratios; CI: confidence interval; sTILs: stromal tumor-infiltrating lymphocytes; ER: estrogen receptor; HER2: human epidermal growth factor receptor 2; BCSS: breast cancer specific survival. [a]The multivariate analysis was adjusted for age, tumor size, histologic grade, lymph node status and hormone receptor status; [b]Analysis was performed by the continuous variable; [c]The HRs of the continuous variables are shown as a unit ratio.

The relation between higher density of FOXP3+ sTILs in HR– and Her2+ BCs shown here agrees with previous studies (2, 34, 39, 40, 46-49, 51-53, 57). A strong association between high FOXP3+ sTILs and TNBCs was also demonstrated in concordance with prior reports (34, 39, 53), although data from other studies showed lower FOXP3+ Tregs in TNBCs (32) or no significant association (50). Furthermore, higher density of CD8+ T-cells was observed in HR- BCs, Her2+ BCs and TNBCs, in agreement with published studies (21, 47, 51, 59, 61). Contrary to the above, Huang and colleagues (56) did not note association between CD8+ TILs and ER/PR/HER2 status. The decreased lymphocytic infiltrate in ER+ BCs may relate to the effect of ER (62), which has been shown to promote a Th2 immune response and decrease MHC class II expression in breast cancer cells (63). After stratification of the HER2+ BC patients by HR status, comparable levels of FOXP3+ and CD8+ sTILs were found in both HER2+ subgroups. Our findings suggest a possible overrunning effect of HER2 protein overexpression compared to that of the ER, on the tumor lymphocytic infiltrate.

CD8+ TILs represent a marker of immune response against tumor, directly triggering apoptosis of the target cell *via* the perforin/granzyme A/B system or through FAS ligand expression. Nonetheless, TNBCs and HER2+ BCs grow despite high CTL infiltration, a finding suggesting that their mere presence is not reflecting effective immune function. FOXP3+ Tregs possibly contribute to the immune evasion of TNBC (64) *via* inactivation of CTLs, although functional exhaustion through immune checkpoint activation offers an alternative explanation. The abundance of CD8+ TILs in TNBCs and HER2+ BCs provides reasonable ground for their exploitation, *via* their reactivation through immunotherapeutic targeting of immune checkpoints.

Table V. *Univariate and multivariate analyses of variables associated with DMFS.*

Variables	Univariate analysis			Multivariate analysis[a]		
	HRs	95%CI	*p*-Value	HRs	95%CI	*p*-Value
Tumor size (>5 cm *vs.* ≤ 5 cm)	2.230	1.112-4.472	0.024	1.478	0.695-3.142	0.310
Nodal status (positive *vs.* negative)	2.422	1.242-4.725	0.009	2.440	1.207-4.932	0.013
Histologic grade (3 *vs.* 1,2)	1.779	0.946-3.348	0.074	2.675	1.372-5.218	0.004
CD8+ sTILs (low *vs.* high)	2.128	1.115-4.060	0.022	2.353	1.132-4.893	0.022
FOXP3+ sTILs (low *vs.* high)	1.754	0.929-3.311	0.083	1.573	0.795-3.111	0.193
ER status (negative *vs.* positive)	1.265	0.666-2.403	0.473			
PR status (negative *vs.* positive)	1.211	0.639-2.294	0.557			
HER2 status (positive *vs.* negative)	1.847	0.962-3.545	0.065	2.427	1.181-4.985	0.016
Age[b]	1.033[c]	1.008-1.059	0.010	1.052	1.023-1.081	0.000297

HRs: Hazard ratios; CI: confidence interval; sTILs: stromal tumor-infiltrating lymphocytes; HR: hormone receptor status; HER2: human epidermal growth factor receptor 2; DMFS: distant metastasis free survival. [a]The multivariate analysis was adjusted for age, tumor size, histologic grade, lymph node status and hormone receptor status; [b]Analysis was performed by the continuous variable; [c]The HRs of the continuous variables are shown as a unit ratio.

In the clinical outcome analysis, high CD8+ TILs were associated with significantly prolonged BCSS, DFS and DMFS either in the entire group or the HR- subgroup, but not in the HR+ patients, in accordance to published reports (25, 34, 43, 51, 55, 56, 59, 65). Total CD8+ TILs, namely intraepithelial and stromal TILs, are also shown as a prognostic factor for better OS, DFS and BCSS, especially in TNBC (66, 67). In certain studies, however, higher numbers of either inflammatory cells or total CD8+ TILs have been associated with either worse cancer-specific survival or DFS (68, 69) or with no impact to RFS (70).

The prognostic value of naturally-occurring CD4+CD25+ FOXP3+ Tregs, in BC remains controversial. Due to the suppressive effects on the activity of effector T cells and resulting tumor evasion from the host antitumor response (71), high levels of Tregs are expected to relate with poor prognosis (47, 72). Increased FOXP3+ TILs, indeed, have been associated with unfavorable BCSS (53), OS (2, 46, 47, 52, 73-75), relapse-free survival (RFS) (32, 46, 49) in BC, with poor OS in ER+ BC but not in ER- tumors (57), and are considered strong prognostic factor for DMFS but not for local recurrence risk (52, 76). However, recent studies have challenged this idea and showed that Foxp3+Tregs were associated with improved outcome in BCs (77, 78) or bared no impact on OS (79), either regarding the entire group or the HR+ subgroup (43). The present study showed that in the HR- subgroup, higher levels of FOXP3+ Tregs were associated with favorable BCSS, as previously reported (34, 35). This finding suggests a subtype-dependent relationship between Tregs and BC prognosis and two possible explanations are offered. Firstly, the favorable prognostic effect of FOXP3+ TILs in HR- BC may be primarily due to the concomitant abundance of CD8+ T-cell infiltration (33). Secondly, Tregs require a close contact with target cells to exert suppression (36), and as indicated in one study, fewer than 20% of CD4+FOXP3+ lymphocytes are in direct contact with CD8+ TIL in TNBCs (33), therefore, Tregs in HR- BCs, although present, may not exert significant suppression on CTLs.

This study highlighted the differences in TILs density during stage progression of BC, demonstrating that locally advanced BCs show a detrimental reduction of CTL counts, which parallels the adverse clinical outcome, emphasizing their importance in tumor surveillance. The differences with regards to TILs between BC subtypes with their abundance in HER2+ BC and TNBC confirms previous studies and supports their therapeutic exploitation *via* immunotherapeutic manipulations in these subtypes. The positive prognostic effect of CD8+ CTLs and of FOXP3+ Tregs in ER- BC is in agreement with a large body of the literature and provides support for the use of the employed standardized method of evaluation of immunohistochemically detected TILs subpopulations.

Conflicts of Interest

None of the Authors have any conflict of interest related to this study.

Authors' Contributions

EP, EK conceptualized and designed the study, carried out the experimental analysis. All authors contributed to acquisition and interpretation of data, critical revision and drafting of the manuscript.

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