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Histopathological Evaluation of Tumor-Infiltrating Lymphocytes (TILs) as Predictive Biomarker for Hormone Receptors Status, Proliferative Activity and Clinical Outcome in Her-2 Positive Breast Cancer

Giuseppe Angelico [1,†], Giuseppe Broggi [2,†], Rosario Caltabiano [2], Angela Santoro [1], Saveria Spadola [1], Nicoletta D'Alessandris [1], Giulia Scaglione [1], Michele Valente [1], Damiano Arciuolo [1], Alejandro Martin Sanchez [3], Gianluca Franceschini [3,4], Riccardo Masetti [3,4], Antonino Mulè [1] and Gian Franco Zannoni [1,5,*]

1 Unità di Gineco-Patologia e Patologia Mammaria, Dipartimento Scienze della Salute della Donna, del Bambino e di Sanità Pubblica, Fondazione Policlinico Universitario A. Gemelli IRCCS, L.go Francesco Vito 1, 00168 Rome, Italy; giuangel86@hotmail.it (G.A.); angelasantoro1@hotmail.it (A.S.); saveriaspadola@hotmail.it (S.S.); ndalessandris@gmail.com (N.D.); scaglione.giulia90@gmail.com (G.S.); dr.valente.m@gmail.com (M.V.); damiano.arciuolo@policlinicogemelli.it (D.A.); antonino.mule@policlinicogemelli.it (A.M.)

2 Department G.F. Ingrassia, Section of Pathology, University of Catania, 95123 Catania, Italy; giuseppe.broggi@gmail.com (G.B.); rosario.caltabiano@unict.it (R.C.)

3 Multidisciplinary Breast Center, Dipartimento Scienze della Salute della donna e del Bambino e di Sanità Pubblica, Fondazione Policlinico Universitario A. Gemelli IRCCS, Largo Agostino Gemelli 8, 00168 Rome, Italy; martin.sanchez@policlinicogemelli.it (A.M.S.); franceschinigianluca@gmail.com (G.F.); riccardo.masetti@policlinicogemelli.it (R.M.)

4 Istituto di Semeiotica Chirurgica, Università Cattolica del Sacro Cuore, 00168 Rome, Italy

5 Istituto di Anatomia Patologica, Università Cattolica del Sacro Cuore, 00168 Rome, Italy

* Correspondence: gianfranco.zannoni@unicatt.it

† These authors have equally contributed.

Abstract: Background: In the present study, we evaluated the prognostic value of TILs as well their relation with clinicopathological factors in patients affected by HER-2 positive breast cancer. **Methods**: We evaluated 47 patients with a histologically confirmed diagnosis of invasive breast carcinoma showing an immunohistochemically confirmed (score 3+) amplification of the c-erbB-2 gene for the presence of TILs and categorized in three predefined groups of low (0–10% immune cells in stromal tissue within the tumor), intermediate (11–40%), and high TILs (>40%). **Results**: Low, intermediate and high TILs were found in 17/47 (36%), 23/47 (49%) and 7/47(15%) cases, respectively. It was found that 6/47 cases treated with adjuvant chemotherapy plus trastuzumab underwent progression of the disease; none of these cases exhibited high TILs. It was found that 12/47 patients with a prognostically unfavorable stage of III and IV showed low and intermediate levels of TILs, while high TILs were never observed. A significant association between intermediate/high-levels of TILs, elevated KI 67 index and hormone receptors nuclear staining was observed. High concordance in TILs distribution was observed between preoperative breast biopsies and surgical samples. **Conclusions**: We observed a positive correlation between the TILs and the response to both adjuvant and neoadjuvant treatments in HER-2 positive BC. High TILs were also related to increased KI-67 index and to the expression of hormone receptors.

Keywords: breast cancer; HER2; tumor infiltrating lymphocytes; KI-67; hormone receptors; immune microenvironment; target therapy

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1. Introduction

Breast cancer (BC) is the most frequent malignancy in women [1–3]; it exhibits a wide geographic variability, with higher incidence rates in the most economically advanced

countries [1–3]. With respect to age groups, the incidence of BC tends to increase up to the menopausal years (around 50–55 years), slow down and then start to rise again after the age of 60 [2]. BC does not represent a homogeneous disease, but a wide spectrum of neoplasms characterized by huge differences in terms of clinical behavior, prognosis and response to treatments [1–3]. In recent years several studies have been carried out in order to identify a series of morphological or molecular parameters that were related to the patient's prognosis (i.e., the clinical course of the disease and the overall survival) or to the response to specific treatments (predictive factors) [1]. Some of these fall into both categories, such as the expression of estrogen receptors (ERs), HER-2, the proliferation index and genetic signatures [4].

The immunogenicity of BC has not traditionally been taken into account either in clinical practice or in scientific research, unlike other types of malignancies, such as melanoma, lung cancer and gynecological neoplasms, for which new therapies, able to stimulate the antitumor immune response have recently been introduced in clinical practice with a significant impact on prognosis [5–8]. However, in recent years numerous clinical studies have been conducted in order to better understand whether the individual immune response was able to influence the clinical behavior of BC and, in other words, whether it was possible to assign to each mammary neoplasms an "immunological grade" that had prognostic and/or predictive significance of therapeutic response [9]. The presence of tumor-infiltrating lymphocytes (TILs), within the tumor and/or in peritumoral sites, has been recognized as an important immunological biomarker that reflected the antitumor immune response in BC, as well as in other malignancies, including ovarian and endometrial carcinomas [9–11].

Several studies have attempted to determine the prognostic value of TILs in breast cancer. In detail, increased TIL levels have been linked to better response to neoadjuvant chemotherapy and with improved survival for patients with triple negative tumors (TNBC) and HER2-positive BC [12]. On the other hand, the prognostic and predictive value of TILs in luminal breast cancer remains poorly understood [12].

TILs are most often found in triple-negative breast cancers, HER2+, and other highly proliferative subtypes [9,13]. Some authors have extensively studied the composition of TILs: 75% of each TIL is made up of T lymphocytes, less than 20% of B lymphocytes, less than 10% of monocytes and less than 5% of natural killer (NK) and NK- T cells [14,15]. TILs are classified into stromal (sTILs) and intratumoral (iTILs) TILs, both located within the tumor tissue; however, while sTILs are composed of lymphocytes dispersed in the tumor stroma, up to and including the invasive front of the tumor, iTILs are made up of lymphocytes properly located within the tumor nests and therefore in direct contact with neoplastic cells [9,14,15]. sTILs represent a more reproducible parameter because they are more frequently encountered and therefore easier to detect in hematoxylin and eosin (H&E)-stained sections with no use of immunohistochemical methods [9,14–16]; to date, there is no standardized method for evaluating TILs in daily histopathological practice. In 2010 a method of evaluating TILs on needle biopsies samples was proposed by Denkert et al. [16] and, since then, it has been widely accepted. In 2014 an International TILs Working Group introduced a set of recommendations for a standardized evaluation of TILs in BC [14]. Based on the abovementioned acquisitions, the aim of the present study was to evaluate the potential prognostic and predictive role of TILs in patients affected by HER-2 positive BC. Since this subset of tumors is eligible to be treated with trastuzumab, a humanized anti-Human Epidermal growth factor Receptor 2 (HER2) receptor monoclonal antibody, we also aimed to evaluate a possible correlation between TILs levels and treatment response. Associations between TILs distributions and clinicopathological factors such as hormone receptors status and Ki-67 proliferative index have also been investigated.

2. Materials and Methods

2.1. Patients Selection

We retrospectively collected information about 47 patients, aged between 40 and 70 years, with a histologically confirmed diagnosis of invasive breast carcinomas showing an immunohistochemically confirmed (score 3+) amplification of the c-erbB-2 gene from the files of our institution. Preoperative breast core needle biopsies (CNB) were also available for all the patients. All patients were treated with adjuvant chemotherapy plus trastuzumab, in particular 6/47 women (mean age: 69.5 years; age range: 53–82 years) were treated also with neo-adjuvant chemotherapy plus trastuzumab. All cases underwent a 2–3-year follow-up.

Expressions of estrogen (ER), progesterone (PR), HER2, and Ki-67 were assessed using immunohistochemistry, as elsewhere reported [4]. HER2-negative tumors were excluded from this study. Tumor proliferative activity was assessed by immunohistochemistry, using the Ki-67 antibody. In detail, at least three high power fields (HPFs) were selected to represent the spectrum of staining seen on the initial overview of the entire section. The Ki67 index was expressed as the percentage of positively stained cells among the total number of invasive cancer cells in the area scored. Scoring involved the counting of at least 500 malignant invasive cells with a 40× object lens.

All slides were prepared by standard techniques and scanned with Aperio ScanScope at ×20. Digital images viewed in ImageScope (Aperio) image viewer which was also utilized to take photomicrographs.

2.2. Histological Evaluation of TILs

The evaluation of the TILs was performed according to the guidelines of the "International Working Group for TILs in Breast Cancer—2014" [14]. In detail, a section of 4–5 µm at a magnification of 200–400× was evaluated for each patient. The evaluation of the TILs was performed by a percentage count of the stromal areas occupied by the lymphocyte and plasma cellular infiltrate, instead excluding the areas occupied by tumor cells. This evaluation considered only the mononuclear infiltrate within the borders of the invasive tumors. Large areas of central necrosis or fibrosis are not included in the evaluation.

The findings were categorized in three predefined groups of low (0–10% immune cells in stromal tissue within the tumor), intermediate (11–40%), and high TILs (>40%) as described by the International Working Group for TILs in Breast Cancer 2014 (14). For the intermediate group different areas at higher magnification were also considered.

TILs levels were also evaluated separately for core needle biopsies (CNBs) and surgical samples (SRS) and recorded as CNB-TILs and SRS-TILs. Tumors were categorized in the following subgroups: CNB-High-TILs, CNB-Intermediate-TILs, CNB-Low-TILs, SRS-High-TILs, SRS-Intermediate-TILs and SRS-Low-TILs, according to CNB-TILs and SRS-TILs status.

2.3. Statistical Analysis

Descriptive data were expressed as absolute values, relative percentages and mean or median with standard deviations. The agreement rate in TILs evaluation between CNBs and surgical samples was performed using Cohen's Kappa test (k). The interpretation of the agreement by Kappa value was performed with the intervals: k < 0, less than chance agreement; k = 0.01–0.20, slight agreement; k = 0.21–0.40, fair agreement; k = 0.41–0.60, moderate agreement; k = 0.61–0.80, substantial agreement; and k = 0.81–0.99, almost perfect agreement. p-values less than 0.05 were considered significant. To assess the correlations between TILs and clinicopathological variables, Fisher's exact test was performed using the SPPS Statistics 23 software (Statistical Package for Social Science, SPSS Inc, Armonk, New York, NY, USA). Statistical significance was defined when $p < 0.05$.

3. Results

3.1. Patient's Characteristics and TILs Distribution

The clinical-pathological features of the cohort of patients included in the study are summarized in Table 1.

Table 1. Patients' Characteristics.

Total number of patients enrolled	47
Median age at diagnosis (y)	61
Tumor stage	
I	27 (57.45%)
II	8 (17%)
III	3 (6.38%)
IV	9 (19.15%)
Histological type	
Ductal NOS	42 (89.36%)
Lobular	4 (8.51%)
Metaplastic	1 (2.13%)
Histological grade	
G2	11 (23.4%)
G3	36 (76.6%)
Lymph node status	
Negative	28 (59.57%)
Micrometastases	3 (6.38%)
Macrometastases	16 (34.04%)
TILs (evaluation on CNB/surgical samples)	
Low	19/17
Intermediate	21/23
High	7/7
Hormone receptors (ER, PR)	
positive	28 (59.57%)
negative	19 (40.43%)
Ki-67	
>20%	36 (76.6%)
$\leq$20%	11 (23.4%)
Chemotherapy	
Only Adjuvant	47 (100%)
Neoadjuvant+Adjuvant	6 (12.77%)
Relapse	
Yes	6 (12.77%)
No	41 (87.23%)
Surgical approach	
Quadrantectomy	43 (93.49%)
Mastectomy	4 (8.51%)
Margin status	
Free	47
Ink on Tumor	0

After evaluating the percentage of TILs, all the cases of HER-2 positive breast carcinomas from our cohort were assigned to groups A, B and C, corresponding to the groups of low (0–10%), intermediate (10–40%) and high (40–90%) TILs, respectively, as described by the International Working Group for TILs in Breast Cancer 2014 (14). Low, intermediate and high TILs were found in 17/47 (36%), 23/47 (49%) and 7/47(15%) patients, respectively (Figures 1 and 2) (evaluation of surgical samples). Considering TILs assessment on CNBs from the same patients, low, intermediate and high TILs were found in 19/47 (40.4%), 21/47 (44.6%) and 7/47(15%) cases, respectively.

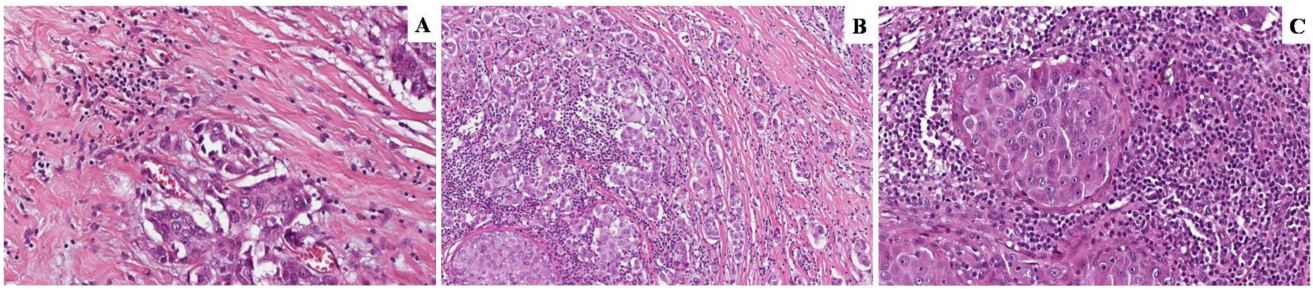

Figure 1. Hematoxylin and eosin (H&E) stained sections illustrating different TILs distribution in our series. (**A**) Invasive breast carcinoma with low TILs (0–10%) in stromal tissue within the tumor (H&E, ×10). (**B**) Another case in our series demonstrating increased TILs in tumor stroma, categorized as intermediate (11–40%) (H&E, ×10). (**C**) High TILs (>40%) in tumor stroma are depicted in this invasive breast cancer case (H&E, ×20).

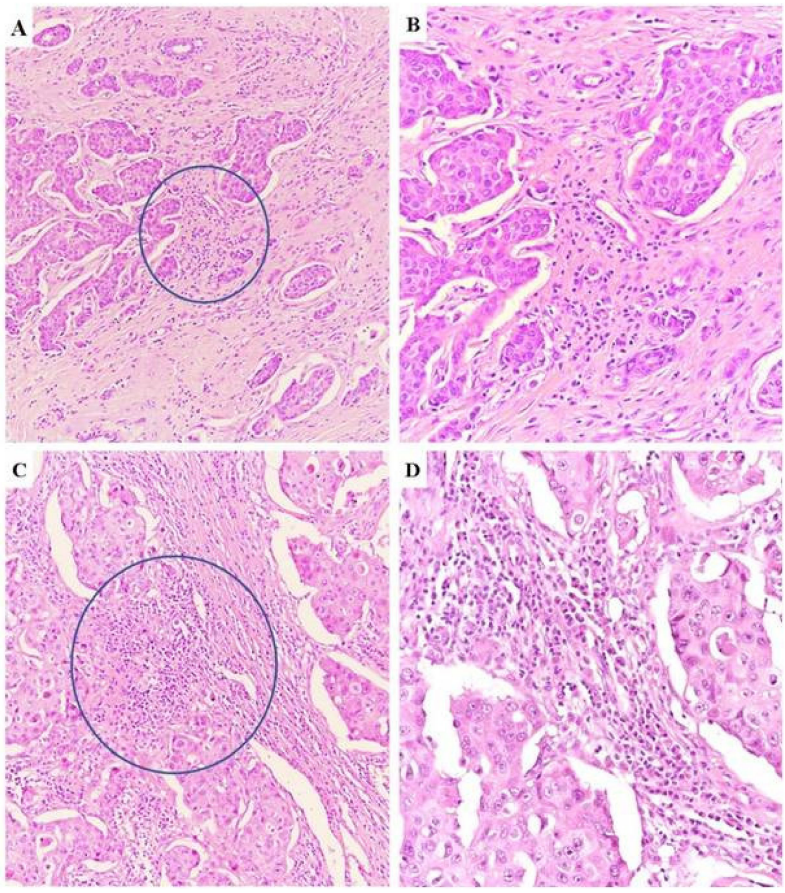

Figure 2. Hematoxylin and eosin (H&E) stained sections illustrating different TILs distribution in our series. (**A**) Invasive breast carcinoma demonstrating low TILs (H&E, ×10). (**B**) Higher magnification of the same case (H&E, ×20) illustrating the TILs distribution in tumor stroma. (**C**) Another invasive breast cancer case categorized as intermediate TILs (H&E, ×10). (**D**) Higher magnification of the same case (H&E, ×20) demonstrating the increased TILs distribution in tumor stroma.

3.2. TILs and Clinicopathological Features

Of the 47 cases of HER-2 positive BC considered here, six were treated with treated with adjuvant chemotherapy plus trastuzumab but underwent progression of disease (local recurrence): the average time to progression for these patients was 10 months; five of the six cases that progressed showed low TILs (0–10%) at the time of the histological diagnosis, in addition to an absent or mild peritumoral lymphocytic infiltrate; the remaining relapsed case TILs levels were intermediate, equal to about 25%, with a moderate peritumoral

lymphocytic infiltrate; notably, none of the 6 relapsed cases exhibited high TILs (40–90%). Considering the surgical management of these patients, two patients underwent mastectomy, while in four patients a more conservative approach was preferred (quadrantectomy). In all patients, clear margins were reported.

Taking into consideration tumor stage, according to The American Joint Committee on Cancer (AJCC) staging, 12/47 patients showed a prognostically unfavorable stage of III and IV (Table 1); this subgroup of patients showed low levels of TILs, while intermediate and high TILs were never observed (Table 2). No other significant correlation has emerged between TILs distribution and clinicopathological variables.

Table 2. Relation between TILs and stage.

	Stage III–IV	Stage I–II	Total
LOW TILs	12 (25.53%)	15 (31.91%)	27
I–HIGH TILs	0	20 (42.55%)	20
Total	12	35	47
p-value		0.0004	

Considering nodal status, 19/47 patients showed metastatic nodes (Table 1); this subgroup of patients showed low levels of TILs in 12 cases, while intermediate–high TILs were observed in 7 cases (Table 3). A positive nodal status was significantly related to low TILs (*p*-value: 0.006)

Table 3. Relation between TILs and nodal status.

	N+	N-	Total
LOW TILs	12 (25.53%)	6 (12.77%)	27
I-HIGH TILs	7 (14.89%)	22 (46.81%)	20
Total	19	28	47
p-value		0.006	

In regard to the correlation between TILs and tumor response to neoadjuvant chemotherapy, our series included only six cases of locally advanced HER-2 positive BC, preoperatively treated with a combination of anthracyclines, taxanes and trastuzumab. All cases examined showed low TILs and none of them underwent tumor pathological complete response (pCR). In addition, 1/6 cases exhibited local disease progression. From an immunohistochemical point of view, in two patients, the neoplasms were luminal HER2 (HR+/HER2+) BCs; in four patients, the neoplasms were pure HER2 (HR-/HER2+, one of which also exhibited immunopositivity for basal CKs—CK14, CK5/6 and focally for CK17, as a basal HER2: HR-/HER2+/basal CKs+).

No statistically significant associations have been observed between TIL status, histotype and pathological grade.

3.3. Relation between TILs, Hormone Receptors and Proliferative Index (Ki-67)

We assessed whether the proportion of TILs was associated with estrogen and progesterone staining patterns as well as Ki-67. Interestingly, a statistically significant association between intermediate and high-levels of TILs and a Ki-67 index <20% was observed (*p*-value: 0.0094) (Table 4).

Table 4. Relation between TILs and proliferative index (Ki-67).

	Ki-67 > 20%	Ki-67 ≤ 20%	Total
LOW TILs	9 (19.15%)	8 (17.02%)	17
I-HIGH TILs	27 (57.45%)	3 (6.38%)	30
Total	36	11	47
p-value		0.0094	

Moreover, the nuclear staining of estrogen and progesterone receptors, observed in 28/47 cases, was significantly related to intermediate–high TILs (*p*-value: 0.0150) (Table 5).

Table 5. Relation between TILs and hormone receptors.

TILs	LOW	INTERMEDIATE–HIGH	Total
HR+/HER2+	6 (12.77%)	22 (46.81%)	28
HR-/HER2+	11 (23.4%)	8 (17.02%)	19
Total	17	30	47
p-value		0.0150	

3.4. Concordance of TILs Evaluation between CNB and Surgical Samples

On the basis of the number of observed agreements (100% of the observations) and the number of agreements expected by chance (53.83, 65% of the observations), the statistical analysis of specific Cohen's kappa values showed almost perfect agreement between CNB and surgical samples in the intermediate and high TILs groups (k = 1000, 95% confidence interval from 1000 to 1.000). On the other hand, two cases classified as low TILs in the CNB evaluation revealed intermediate TILs in breast surgical samples (k = 0.905, 95% CI, from 0.778 to 1000). In detail, all tumors with high TILs on CNBs were confirmed on surgical samples. On the other hand, 2/19 tumors categorized as low TILS on CNB evaluation were upgraded to intermediate TILs on surgical sample examination. This difference is explained by the heterogeneity of TILs' distribution on definitive examination of these tumors (Table 6).

Table 6. TILs distribution in CNB vs. surgical samples.

	TILs		
	LOW	INTERMEDIATE	HIGH
CNB	19	21	7
SURGICAL SAMPLES	17	23	7

4. Discussion

In recent years, the immunogenicity of breast cancer has become subject of great scientific attention, with the aim of better understanding whether it can influence the biological behavior of BC, acquiring prognostic and/or predictive significance of therapeutic response [9,12,14–16]. The presence of TILs is an expression of the antitumor immune response. Many studies have therefore focused on the predictive and/or prognostic impact of TILs in patients with different types of cancers, including BC [9–11]. TILs are classified into stromal and intratumoral TILs, depending on whether we consider the lymphocytes dispersed in the tumor stroma, or the lymphocytes located within the tumor nests [9,14,15]. Due to the absence of a standardized method for evaluating TILs, many studies have evaluated both the sTILs and the iTILs; however, the evaluation of the former was easier to

detect and more reproducible [9,14–16]. In 2014 a series of recommendations for a standardized assessment of TILs in breast cancer was proposed by an international TILs working group [14]; in addition, several studies demonstrated the prognostic and predictive value of TILs in various cohorts of patients; particularly, among triple negative BCs (TNBCs), TILs can be used as a prognostic marker because they positively correlated with the response to adjuvant chemotherapy, in terms of overall survival [9,12,16–18]. Recent studies aimed to investigate the potential prognostic role of TILs in patients affected by HER-2 positive BC treated with chemotherapy plus trastuzumab. In this regard, the FIN-HER study [18] demonstrated that patients affected by BC exhibiting high TILs levels showed a better response to adjuvant chemotherapy with trastuzumab and, consequently, longer overall survival than those affected by BC showing low TILs. On the other hand, Perez et al. [19] reported that HER2-positive lymphocytic-dominant BCs, defined by the presence of at least 60% sTILs, had a slightly worse response when combining trastuzumab with chemotherapy than those treated with chemotherapy alone in terms of relapse-free survival times (80% and 90.9% at 10 years, respectively). Therefore, based on these data, the potential role of TILs in mediating the response to adjuvant chemotherapy plus trastuzumab still remains controversial. Further data from neoadjuvant therapy-based studies [20,21] showed that high rates of TILs were significantly associated with higher rates of pCR; accordingly, TILs appeared as predictive biomarkers of positive response/resistance to neoadjuvant chemotherapy. Post-neoadjuvant treatment TILs have also been found to be associated with breast cancer prognosis [22,23].

The aim of the present study was to demonstrate that the evaluation of TILs was capable of identifying, among 47 patients with HER-2 positive BC, specific subgroups of patients characterized by different prognostic and clinical features. In this regard, we found that only the patients (6/47) who underwent disease progression after adjuvant chemotherapy plus trastuzumab had TILs <25%. Moreover, neoadjuvant treatment failed to achieve a pathological complete response in all BC cases from our series (6/47); interestingly these latter cases exhibited low TILs.

Previous studies have documented high TILs levels in aggressive breast cancer subtypes, and showed a possible relation between high TILs, Ki-67, ER and PR immunohistochemical expression [9,24,25].

Similarly, in the present study, we observed a correlation between TILs distribution, proliferative index (Ki-67) and hormone receptors. In detail, intermediate and high-levels of TILs were significantly related to a Ki-67 index <20%. We selected a 20% Ki-67 cut-off according to previous studies by other authors on TILs in HER-2 positive BC [19,23]; moreover, a Ki-67 index greater than 20% is generally considered the watershed between luminal A and luminal B tumors [3,4].

Regarding hormone receptors' expression, intermediate–high TILs were significantly related to BC cases showing nuclear staining for both estrogen and progesterone receptors. Finally, there are limited data regarding the concordance or the variability of immune profiles between CNBs and whole tumor sections from surgical procedures [26]. Some authors have reported immune-activation after CNB, with TILs being higher in SRS than in CNB [26]; other studies performed on mouse models suggested a biopsy-mediated development of an increasingly immunosuppressive tumor microenvironment [27]. Different hypotheses have been postulated in order to explain the possible reasons of TILs changes: (1) the CNB process as primary cause of immune-activation; (2) the concept of intratumor heterogeneity; (3) younger age and longer surgery time interval as indicators of the higher chance of TILs' increase after CNB [26–28].

In our study, we observed a high level of concordance between preoperative biopsies and surgical samples in TILs' distribution. In detail, almost perfect agreement was observed in the intermediate and high TILs groups.

We can postulate that the reason for this high concordance rate could rely on the adequate bioptical sampling of the neoplastic tissue (at least four bioptic fragments from different points of the tumoral tissue, in almost the totality of our study cases). On the

other hand, two cases were preoperatively classified as low TILs in the CNB evaluation but changed from a low immune status to an intermediate TILs status on surgical samples and experienced a worse breast cancer-free interval. In particular, in these non-concordant cases only 1–2 bioptic fragments were obtained in the preoperative setting. This TILs' change may be explained by: (1) the spatial heterogeneity of lymphocytic infiltrate or the regional segregation of immune cells in some tumors, which can be missed in bioptic cores; (2) the not completely adequate bioptical sampling; (3) a dysfunctional immune infiltrate. The bad prognosis observed in these two cases was quite different from the common view that TILs' increase is related to better survival (especially in TNBCs) but is similar to a recent study on HER2+ tumors in which TILs' heterogeneity was associated with poor prognosis [28].

5. Conclusions

In conclusion, the results from the present study demonstrate a positive correlation between TILs and the response to adjuvant treatments in HER-2 positive BC. Furthermore, we found that all the patients who presented an advanced stage of the disease at the time of the diagnosis showed low levels of TILs. Increased TILs concentration may also be indicative of a subset of tumors with elevated Ki-67 index and with the expression of estrogen and progesterone receptors, which tend to be more responsive to chemotherapy. Our data also support the hypothesis that a subset of HER2+ BC is immunogenic and may harbor a different biological and pathogenetic pathway which includes microsatellite instability, as also documented in tumors of the gynecological tract [7,8,29]. These latter neoplasms may also be treated with immune-modulating therapies.

We are aware of the limitations of our study: the sample size, the retrospective nature and the short follow-up period, which is not fully adequate to assess tumor biology.

Further studies on larger series and with longer more meaningful follow-up length are needed in order to elucidate the prognostic role of TILs as well as their utility as a predictive biomarker for the therapeutic management of BC patients.

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Institutional Review Board Statement: The study complied with the Ethical Principles for Medical Research Involving Human Subjects according to the World Medical Association Declaration of Helsinki. The clinical information had been retrieved from the patients' medical records and pathology reports. Patients' initials or other personal identifiers did not appear in any image. Finally, all samples were anonymized before histology and immunohistochemistry. Therefore, no further ethical approval was necessary to perform the study.

Informed Consent Statement: The non-interventional, retrospective nature of our study did not require any informed consent, even if written informed consent had been obtained from each patient before surgical procedures.

Data Availability Statement: All data are available after reasonable request to the corresponding author.

Conflicts of Interest: The authors declare no conflict of interest.

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