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Original article

Unfavorable prognostic role of tumor-infiltrating lymphocytes in hormone-receptor positive, HER2 negative metastatic breast cancer treated with metronomic chemotherapy

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ABSTRACT

Background: High levels of tumor-infiltrating lymphocytes (TILs) in primary triple negative and HER2-positive breast cancer (BC) have been associated with an improved patients' outcome. The role of TILs in Luminal (hormone receptor positive and HER2 negative) tumors remains to be elucidated. Moreover, the association between TILs and prognosis in the metastatic setting is still unknown.
Patients and methods: We evaluated the relationship between TILs and time to progression (TTP) in metastatic BC patients enrolled in a prospective phase II trial of metronomic chemotherapy, that used cyclophosphamide 50 mg daily, capecitabine 500 mg thrice daily and vinorelbine 40 mg orally three times a week (VEX combination).
Results: Of the 108 ER + BC patients enrolled in the VEX trial, 92 (85%) had sufficient tumor tissue and were assessed for TILs in H&E stained slides. TILs were evaluated in 38 primary BC samples and 54 metastatic sites. High (≥10%) TILs levels were significantly correlated with high Ki-67 labeling index. At multivariable analysis, each 10% increase in TILs strongly predicted a worse TTP (HR: 1.27, p = 0.008). VEX trial patients, categorized by a 3 tiers system (0–4%, 5–9% and >10% TILs) showed significantly different progression free survival curves (p = 0.011).
Conclusions: High TILs levels are significantly associated with a worse TTP in Luminal metastatic BC patients treated by metronomic chemotherapy. Our data confirm the reliability of TILs as a biomarker in the BC metastatic setting. The putative unfavorable prognostic role of TILs in Luminal BC patients might have clinical utility if validated by further studies.

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1. Introduction

Immunity plays a pivotal role in cancer shaping and elimination, and a positive correlation between the extent of tumor infiltrating lymphocytes (TILs) and better clinical outcome has been convincingly demonstrated in a number of solid human malignancies, such as lung cancer, melanoma and colorectal adenocarcinoma. The prognostic value of TILs has been proven in thousands of triple-negative (TN) and HER2+ breast cancer (BC) patients, both in the adjuvant and neoadjuvant setting [1–8]. Moreover, recently issued standardized guidelines, ring studies and validation analyses in routine patient samples have improved the robustness and the reproducibility of TILs evaluation [9–11]. Less straightforward data have been reported thus far in estrogen receptor positive (ER+) BC patients. In their pivotal studies, Loi et al. evaluated TILs levels in 1670 ER+, HER2- BC patients enrolled in the BIG 2–98 and FinHER trials, failing to find any significant correlation with outcome [1,2]. In a recent meta-analysis on twenty-five published studies including 22,964 BC patients, Mao et al. found that TILs indicated a

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survival benefit in TN and HER2+ BC patients, but not in ER + patients (HR: 1.01; 95% CI: 0.94–1.07 for DFS; HR: 1.09; 95% CI: 0.98–1.21 for OS) [12]. Interestingly, recent data from a retrospective study on lobular BC patients suggested that the TILs were associated with a worse prognosis [13]. Denkert et al. found that a high content of TILs conferred a significantly ($p = 0.003$) higher likelihood of pathological complete response in HR + BC patients enrolled in the GeparDuo and GeparTrio Trials of neoadjuvant chemotherapy [3]. Likewise, Issa-Nummer retrospectively evaluated TILs in pre-chemotherapy biopsies of 209 HR+, HER2- BC patients from the GeparQuinto Trial, finding that pCR rates were significantly ($p = 0.002$) higher in lymphocyte predominant BC (LPBC, 28.2%) than in non-LPBC patients (8.2%) [14].

There are no data on the prevalence and clinical relevance of TILs in Luminal BC metastatic setting. Our group recently reported toxicity and efficacy data of a phase II study of oral metronomic chemotherapy with vinorelbine, cyclophosphamide plus capecitabine (VEX) in HR positive, HER2 negative, metastatic BC patients [15]. In addition to its antiangiogenic effect, metronomic therapy could exert an immunological action through anti-tumor immunity modulation and tumor dormancy induction [16–20]. Taking advantage of the samples collected within the VEX trial, we firstly interrogated the clinical relevance of TILs in metastatic Luminal BC patients treated by metronomic chemotherapy.

2. Materials and methods

2.1. Study patients

The VEX study was a prospective phase II trial in which metastatic BC were assigned to metronomic chemotherapy with cyclophosphamide 50 mg daily, capecitabine 500 mg thrice daily and vinorelbine 40 mg orally three times a week. Pre- or postmenopausal women with histologically or cytological (cell block) proven, metastatic BC, ER >1% and/or progesterone receptor (PgR) > 1% and negative HER2 status [21] were eligible. Patients with HER-2 overexpressed tumors, were also eligible if they had received previous trastuzumab therapy for advanced disease, and/or a treatment with any HER2-targeted therapy. Patients with a disease measurable by RECIST 1.1 criteria or bone lesions, lytic or mixed (lytic and sclerotic), in the absence of measurable disease as defined by RECIST 1.1 criteria were eligible. Patients were included regardless of any primary and/or adjuvant therapies, or previous lines of chemotherapy and endocrine therapy received for advanced disease. Patients were distributed into two groups: those who received the study treatment as a first-line therapy (naïve group) and those who had already received some treatment for advanced MBC (pre-treated group). Patients were required to attend monthly visits during the VEX treatment period and a radiological evaluation every 12 weeks. Treatment was continued in the absence of progression or relevant toxicities.

2.2. Pathologic assessment

All the patients had pathological evaluation on primary tumors or metastatic sites. ER and PgR immunoreactivity was assessed by the FDA-approved ER/PR PharmDX kit (Dako). The prevalence of ER/PgR positive invasive cancer cells, independent of their staining intensity, was quantitatively annotated in the original reports [22]. HER2 immunoreactivity was assessed using the HercepTest (Dako): in the original report, the prevalence of positive cells and the type and intensity of immunostaining were detailed [23]. Ki-67 labeling index was assessed by the MIB-1 monoclonal antibody (Dako, 1:200), by counting at least 500 invasive cancer cells at the tumor periphery, irrespective of staining intensity and without focusing on hot-spots, as recommended by the International Ki-67 in Breast Cancer Working Group [24].

TILs were evaluated in full-face hematoxilyn and eosin (H&E) sections from the most recent available surgical or bioptic sample, blinded of clinical information. In primary tumor samples, TILs were carefully evaluated following the criteria proposed by the International TILs Working Group [9]. Briefly, all mononuclear cells (including lymphocytes and plasma cells) in the stromal compartment within the borders of the invasive tumor were evaluated and reported as a percentage value. TILs outside of the tumor border, around DCIS and normal breast tissue, as well as in areas of necrosis, if any, were not included in the scoring. Given the lack of standardized criteria for TILs evaluation in metastatic samples, we adopted the main principles of TILs WG guidelines, taking care of some caveats potentially mining the consistence of our evaluation. As an example, we evaluated large nodal metastasis with a sufficient amount of desmoplastic fibrous tissue, excluding small lymph node metastatic deposits surrounded by autochthonous lymphocytes.

2.3. Statistical analyses

We arbitrarily categorized patients in three groups according to TILs level using the typical cut-off values (less than 5; 5–9; 10 or more). Difference of the distribution of patients' characteristics according to TILs level was assessed with the Mantel-Haenszel test for trend. Clinical benefit was defined as stable disease, partial response or complete response for more than 6 months. TTP was calculated from the date of initiation of VEX to the date of progression, or the date of last VEX treatment. Overall survival (OS) was calculated from the date of initiation of VEX to the date of death. Progression free survival and OS plots were drawn using the Kaplan–Meier method. The log-rank test was used to assess the survival difference between patients groups. Univariable and multivariable Cox proportional regression analysis was used to assess the association between clinico-pathological characteristics and progression or death. Univariable and multivariable logistic regression analysis was used to assess the association between clinico-pathological characteristics and clinical benefit. The association between TILs levels and clinical outcome was evaluated considering TILs both as a categorical variable and as a continuous variable, providing the risk associated with a 10% TILs expression increase. All the analyses were performed with the SAS software (version 9.2, Cary NC). All p-values were two-sided.

3. Results

3.1. TILs and tumor characteristics

TILs were evaluated in 92 of the 108 (85.1%) available tumor samples from the VEX trial population which had sufficient tissue for the analysis. In particular, TILs were registered in 38 primary BC samples and in 54 metastatic sites (lymph nodes, liver, lung, skin and bone marrow). The median value of TILs was 6% (IQR: 3–10%) in the overall population, 6% (IQR: 3–13%) in primary tumors and 5.5% (IQR: 3–8%) in metastatic deposits. Table 1 shows the clinico-pathological characteristics of the population under study and their relationship with TILs levels. Among the variables analyzed, TILs values were significantly ($p = 0.004$) correlated only with tumor proliferation as assessed by Ki-67.

3.2. TILs and prognosis

Efficacy data previously reported in our VEX trial [20] were updated for patients which were assessed for TILs status in the present study (Supplementary Table 1). Median TTP was 21.9

Table 1
Patients characteristics according to TILS.

	All	TILs			P-value
		<5%	5-9%	≥10%	
Total	**92**	**37**	**31**	**24**	
Pre treatment					
No	41	15	14	12	
Yes	51	22	17	12	0.47
Age at primary BC					
Mean (SD)	47.4 (10.0)	48.5 (410.5)	47.9 (10.4)	45.1 (8.6)	0.41
<50 years	58	23	19	16	
≥50 years	34	14	12	8	0.74
Age at first metastasis					
Mean (SD)	52.5 (10.1)	53.3 (10.5)	52.8 (10.8)	50.8 (8.7)	0.64
<50 years	40	14	12	14	
≥50 years	52	23	19	10	0.13
Type of metastatic sites					
Non-Visceral	33	13	12	8	
Visceral	7	3	1	3	
Both	52	21	18	13	0.80
Number of pretreatments					
0	41	15	14	12	
1-2	39	16	13	10	
3+	12	6	4	2	0.32
Pretreatment type					
Only hormone therapy	36	11	16	9	
Chemotherapy	15	11	1	3	0.07
Menopausal status					
Pre	38	13	14	11	
Post	54	24	17	13	0.39
Performance status (ECOG)					
0	83	34	27	22	
1	8	3	3	2	
2	1	0	1	0	0.93
T (Primary)					
pT1	25	8	6	11	
pT2	36	11	18	7	
pT3	12	6	4	2	0.08
pTx	19	12	3	4	
N (# Positive lymph-nodes)					
0	17	6	6	5	
1-2	23	6	12	5	
3+	32	14	9	5	0.65
Unknown	20	11	4	5	
ER (Primary)					
Mean (SD)	81.8 (22.2)	84.4 (15.2)	81.5 (25.3)	77.9 (27.1)	0.55
<50%	5	1	2	2	
≥50%	84	35	28	21	0.33
Unknown	3	1	1	1	
PgR (Primary)					
Mean (SD)	49.4 (36.9)	44.0 (34.6)	52.7 (39.3)	53.7 (37.8)	0.52
<50%	43	20	13	10	
≥50%	46	16	17	13	0.35
Unknown	3	1	1	1	
Ki67 (Primary)					
Mean (SD)	26.0 (13.7)	22.5 (10.4)	24.1 (10.5)	34.0 (18.5)	**0.004**
<20%	26	13	10	3	
20–30%	31	12	12	7	
≥30%	29	10	7	12	**0.03**
Unknown	6	2	2	2	
HER2 (Primary)					
0/+/++	89	36	29	24	
+++	3	1	2	0	0.62

In bold are highlighted variables reaching statistical significance.

Table 2
Univariable analysis of factors associated with disease progression.

Variable	Category	HR (95% CI)	P-value
TILs	5-9% vs <5%	1.49 (0.81–2.76)	0.20
	≥10% vs <5%	**2.36 (1.30–4.28)**	**0.005**
	Per 10% increase	**1.27 (1.08–1.49)**	**0.003**
Age at primary BC	≥50 vs. <50 years	1.40 (0.84–2.36)	0.20
	(per year)	1.02 (0.99–1.05)	0.18
Age at first metastasis	≥50 vs. <50 years	1.18 (0.71–1.96)	0.52
	(per year)	1.03 (1.00–1.06)	0.05
Age at initiation of VEX	≥50 vs. <50 years	1.44 (0.84–2.47)	0.18
	(per year)	**1.03 (1.01–1.06)**	**0.01**
Interval primary - metastasis	1-4 vs. 0 year	1.41 (0.64–3.16)	0.39
	≥5 vs. 0 year	1.90 (0.91–3.98)	0.09
	(per year)	1.05 (0.99–1.10)	0.09
Interval metastasis - VEX	**≥1 vs. 0 year**	**2.12 (1.24–3.63)**	**0.006**
	(per year)	**1.11 (1.01–1.22)**	**0.03**
Number of metastatic sites	2 vs. 1	1.26 (0.64–2.50)	0.51
	≥3 vs. 1	1.83 (0.94–3.56)	0.07
Extension of metastatic sites	**visceral vs. non-v**	**1.81 (1.07–3.08)**	**0.03**
Pretreatment	Yes vs. No	1.41 (0.85–2.35)	0.18
Pretreatment	1-2 lines vs. none	1.30 (0.74–2.26)	0.36
	≥3 lines vs. none	1.75 (0.85–3.60)	0.13
Pretreatment type	HT alone	1.60 (0.92–2.78)	0.10
	CT ± HT	1.08 (0.51–2.29)	0.84
Precedent antracycline	yes vs. no	1.32 (0.81–2.16)	0.27
Precedent Taxanes	yes vs. no	1.07 (0.63–1.81)	0.82
Performance status	ECOG 1–2 vs. 0	1.16 (0.50–2.71)	0.73
pT (primary)	pT2 vs. pT1	0.63 (0.35–1.15)	0.13
	pT3 vs. pT1	0.38 (0.15–0.94)	0.04
	pTx vs. pT1	0.94 (0.48–1.87)	0.87
pN (primary)	1-2 vs. 0	0.96 (0.41–1.77)	0.67
	≥3 vs. 0	0.63 (0.31–1.28)	0.20
	Unkn. vs. 0	0.95 (0.44–2.04)	0.88
pM (primary)	M+ vs. M-	0.59 (0.29–1.20)	0.14
ER (primary)	per 10% increase	0.93 (0.82–1.05)	0.26
PgR (primary)	per 10% increase	0.96 (0.90–1.04)	0.32
Ki67 (primary)	**per 10% increase**	**1.38 (1.11–1.71)**	**0.004**
HER2 (primary)	+++ vs. 0/+/++	1.79 (0.43–7.43)	0.42
Neoadjuvant therapy	yes vs. no	1.71 (0.81–3.62)	0.16
Adjuvant therapy	yes vs. no	1.58 (0.80–3.13)	0.19
Adjuvant CT	yes vs. no	1.34 (0.78–2.32)	0.29
Adjuvant HT	yes vs. no	1.41 (0.64–3.12)	0.40
ER (biopsy)	per 10% increase	1.02 (0.91–1.15)	0.74
PgR (biopsy)	per 10% increase	0.99 (0.92–1.07)	0.83
Ki67 (biopsy)	**per 10% increase**	**1.30 (1.04–1.64)**	**0.02**

In bold are highlighted variables reaching statistical significance.

months (95% CI: 14.2–29.9) in the naïve group and 12.8 months (95% CI: 9.2–22.6) in the pretreated group. Overall, TTP for patients with TILs <5%, 5–9% and ≥10% was 26.02 months (95% CI: 12.3–35.1), 19.8 months (95% CI: 10.3–26.1) and 9.3 months (95% CI: 5.6–15.8), respectively (Supplementary Table 1). At univariable analysis, TILs levels per 10% increase ($p = 0.003$), the interval between diagnosis of metastatic disease and start of VEX (>= 1 year vs. < 1 year, $p = 0.006$), the type of metastatic site (visceral vs. non visceral, $p = 0.03$) and Ki-67 values on primary tumors (10% increase, $p = 0.004$) were significantly correlated with a worse disease progression (Table 2). At multivariable analysis, each 10% increase of TILs maintained its unfavorable prognostic role, being associated with a 27% increased risk of disease progression (HR 1.27, 95% CI: 1.07–1.50, $p = 0.008$) (Table 3). The adverse prognostic role of TILs was ascertained irrespective of the site of TILs assessment (primary tumor or metastatic sites, $p = 0.04$) (Table 3). Fig. 1 shows progression free survival (PFS) curves according to TILs in the overall population: at 24 months, PFS for patients with 0–4% and ≥10 TILs in the overall population was 57.2% and 18.5%, respectively. Patients PFS curves in naïve and pre-treated and in accordance with tumor sample type evaluated (primary tumor or metastatic sample) are displayed in Supplementary Figs. 1 and 2.

4. Discussion

It has been convincingly demonstrated that highly aggressive

Table 3
Multivariable analysis of factors associated with disease progression.

Variable		All samples (N = 92)		Only samples in which TILs was evaluated on primary tumors (N = 38)		Only samples in which TILs was evaluated on metastasis (n = 54)	
		HR (95% CI)	P-value	HR (95% CI)	P-value	HR (95% CI)	P-value
Age at initiation of VEX	per year	**1.03 (1.01–1.06)**	**0.02**	**1.08 (1.02–1.14)**	**0.006**	1.02 (0.99–1.06)	0.24
Interval Met- VEX	≥1 year vs 0	**2.59 (1.48–4.54)**	**0.0009**	**6.92 (2.27–21.1)**	**0.0007**	1.68 (0.78–3.60)	0.18
Metastatic sites	visceral vs. non-v	**1.77 (1.01–3.09)**	**0.046**	2.26 (0.93–5.49)	0.07	1.72 (0.81–3.64)	0.15
Ki67 (primary)	Per 10% increase	**1.32 (1.04–1.69)**	**0.025**	1.12 (0.72–1.75)	0.61	**1.35 (1.02–1.81)**	**0.04**
TILs	Per 10% increase	**1.27 (1.07–1.50)**	**0.008**	**1.55 (1.02–2.38)**	**0.04**	**1.25 (1.02–1.54)**	**0.04**

In bold are highlighted variables reaching statistical significance.

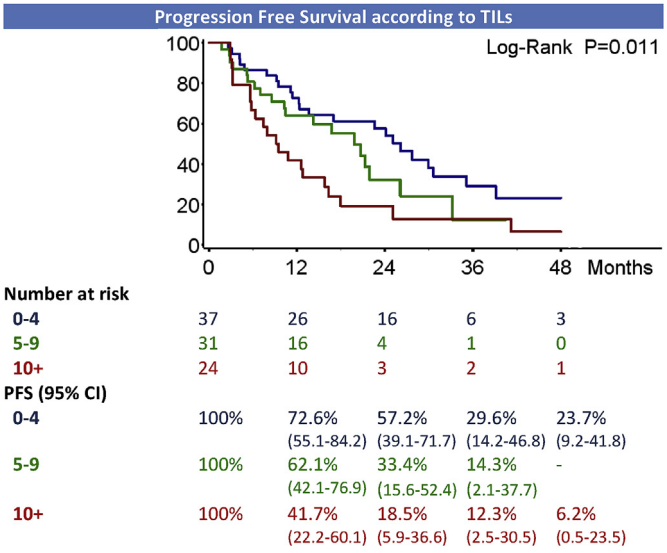

Fig. 1. Progression Free survival according to TILs levels (0–4%, 5–9%, ≥10%) in the overall population.

BC, namely TN and HER2+ BC subtypes, are characterized by high TILs levels and a positive association between TILs and clinical outcome. On the other hand, the relationship of Luminal tumors with the immune system has not yet been fully elucidated. In this study, we confirmed that most luminal BC are characterized by a low lymphocytic infiltration [1,2,13], in contrast to clinically more aggressive BC subtypes. The clinical role of TILs has been usually ascertained in primary tumor samples. Nevertheless, there is evidence that radiotherapy, chemotherapy and target therapy may shape the anti-tumor immune response. In this regard, it is worth noting that TILs levels in post-neoadjuvant chemotherapy residual disease may add prognostic information for BC patients [25]. In the present study, we had the chance to retrospectively interrogate a set of metastatic deposits prospectively collected within a clinical trial. Interestingly, we were able to evaluate most of these samples, providing evidence that TILs analysis is doable in the metastatic setting as well. Our data have been recently confirmed by an independent group that evaluated TILs in 58 metastatic sites from patients with advanced HER2-positive BC within the CLEOPATRA trial [26].

Our study suggests that TILs may represent a biomarker of adverse prognosis in metastatic luminal BC patients. Our data are in line with those recently reported by Desmedt et al. [13], who found that TILs assessed in primary tumors were associated with an unfavorable prognosis for patients with Luminal lobular BC. Collectively, these data lead to hypothesize that luminal BC patients with high levels of TILs could be an ideal target for more aggressive chemotherapy treatment, paving the way for validation studies in the neoadjuvant setting. Tumor mutational burden has been associated with the formation of neo-epitopes and with the magnitude of the immune infiltrate [27–31]. In particular, it has been demonstrated that only a small fraction of cancer somatic mutation results in translation of aberrant proteins recognizable as "non self" peptides by the immune system. Brown et al. interrogated RNA-seq data of 515 patients from six tumor sites from The Cancer Genome Atlas (TCGA), and showed that immunogenic mutation count was positively associated with tumor CD8A expression and survival [27]. These data suggest that a high mutational load would be associated with a higher prevalence of immunogenic peptides, which in turn elicit an immune response, eventually leading to a better survival. Luminal BC seems to escape this simplistic model. Haricharan et al. interrogated 762 invasive BCs from the TCGA dataset, showing that ER-positive tumors with a high mutational load were associated with poorer overall survival (HR = 2.02) [32]. ER has long been thought to have a role in immunosuppression [33]. These data are in keeping with the observation that the median TILs value in our series was 6%, a figure significantly lower than in TN and HER2+ BC [1,2]. Along this line, recent evidences pointed out that Luminal tumors may be less immunogenic than TN and HER + BC, as a result of major histocompatibility complex (MHC) down-regulation. Chung et al. observed that human leukocyte antigen (HLA) ABC and HLA-A expression among BC was significantly associated with ER negativity, high histologic grade and high Ki-67 proliferation index [34]. Likewise, Lee et al. provided evidence that HLA-ABC expression was detectable in 22% of HR+/HER2- and in more than a half of TNBCs, with ER immunoreactivity being inversely correlated to HLA-ABC levels (rho = −0.177, P < 0.001) [35].

It has been proposed that endocrine therapy may modulate the immune microenvironment of hormonal receptor positive tumors. Interestingly, PDL-1 positive hormone responsive metastatic BC patients treated by Pembrolizumab in addition to endocrine therapy showed a manageable safety profileER+ and a 14% overall response rate, supporting further investigations of immune therapies in luminal tumors [36]. Moreover, metronomic therapy may also promote anti-tumor immune response. Generali et al. showed that the combination of hormonal therapy with metronomic cyclophosphamide down-regulated $CD4^{+}CD25^{+}$ regulatory T cells (Treg) in elderly breast cancer patients [37].

The main limitation of the present study include the low number of patients and the heterogeneity of the samples (primary tumor and different metastatic sites) evaluated.

The updated results of VEX combination confirmed the efficacy of this regimen and the possibility to prolong therapy for several months without increase of toxicity.

In conclusion, we showed that high TILs levels are significantly associated with a worse TTP in metastatic Luminal BC patients treated with metronomic chemotherapy. Further validation of the TILs analytic validity and clinical utility in the metastatic setting are warranted.

Conflict of interest statement

The authors have declared no conflicts of interest.
This work has been read and approved by all the authors.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.breast.2017.05.009.

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