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ORIGINAL ARTICLE



# Evaluation of tumor-infiltrating lymphocytes, PD-L1, and *PIK3CA* mutations and association with prognosis in HER2-positive early stage breast cancer

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**ABSTRACT**

**Background:** Tumor-infiltrating lymphocytes (TILs) have predictive and prognostic potential in HER2-positive breast cancer (HER2+ BC). Programmed death-ligand 1 (PD-L1) is an immune checkpoint protein, with important roles in the tumor microenvironment, possibly in both tumor and immune cells (ICs), providing rationale for targeting with immune-checkpoint therapy. *PIK3CA* mutations are oncogenic, activating mutations, which are also of relevance in breast cancer. Herein, we investigate the frequency of TILs, PD-L1 and *PIK3CA* mutations, and whether these factors influence outcome, in early HER2+ BC.

**Materials and methods:** Stromal TILs (sTILs) and PD-L1 expressions were assessed using full tumor-sections and TMA, respectively, from 236 patients with HER2+ BC. TILs were assessed, according to a standardized method, as continuous measurement and according to three predefined categories: low (0–10%), intermediate (11–59%), and high (60–100%). PD-L1 immunohistochemistry (Ventana SP263) was evaluated and positivity defined as $\geq$1% expression in tumor and ICs. *PIK3CA* mutations (exons 9 and 20) were determined by pyrosequencing.

**Results:** Fourteen percent of patients had high sTILs and 25% had a *PIK3CA* mutation. PD-L1 expression was more frequent in ICs (68%) than tumor cells (24%). Patients with low sTILs had a significantly worse overall survival (multivariate: HR 2.80; 95% CI 1.36–5.78; $p = .02$).

**Discussion:** Patients with low sTILs had a significantly poorer survival, despite adequate treatment with adjuvant therapy.

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HER2-positive breast cancer; tumor-infiltrating lymphocytes; TILs; PD-L1; PIK3CA mutation

## Background

Breast cancer was once considered non-immunogenic, but growing evidence demonstrates use of tumor-infiltrating lymphocytes (TILs) as a marker of immunogenicity, particularly in certain molecular subtypes [1,2]. TILs have potential as a biomarker, and may soon be part of routine pathology practice, as studies show their prognostic and predictive significance, especially in triple negative breast cancer (TNBC) and HER2-positive breast cancer (HER2+ BC), where TILs are found at higher levels. TILs may be predictive for benefit from chemotherapy, immune checkpoint inhibitors (ICIs), and other targeted therapies like trastuzumab, including the neoadjuvant setting [3,4]. St. Gallen International Consensus recognizes their prognostic value, and advises they should be used alongside other prognostic variables in order to inform treatment options [5].

Programmed cell protein-1 (PD-1) and its ligand, programmed death-ligand 1 (PD-L1), are immune-checkpoint proteins and the PD-1/PD-L1 pathway is important in tumor immune evasion. PD-L1 is expressed by tumor cells (TCs) and various immune cells (ICs) [6,7]. Research demonstrates PD-L1 may also have predictive and prognostic value in breast cancer, albeit with varying results. Some studies suggest favorable clinical outcomes, whilst others suggest the converse or no association at all [8]. Two randomized phase 3 trials, IMpassion 130 and KEYNOTE-355, have shown treatment benefit (progression-free survival) using ICI in TNBC, with cutoffs of PD-L1 $\geq$ 1% on ICs and combined positive score $\geq$1, respectively [9,10]. The phase II PANACEA trial showed PD-L1 combined positive score $\geq$1 was predictive of response in patients with advanced, trastuzumab-resistant HER2+ BC treated with pembrolizumab [11].

Furthermore, *phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PIK3CA)* mutations may also be of importance in breast cancer as they are common oncogenic, activating mutations with varying prognostic significance, possibly affected by subtype and treatment regimens [12,13]. Enhanced signalling through P13K/Akt/mTOR pathway is an important strategy for survival and therapeutic resistance in all breast cancer subtypes and contributes to trastuzumab and endocrine therapy resistance in HER2+ and hormone receptor positive (HR+) breast cancer, respectively [14,15].

Conversely, clinical relevance of TILs in carcinoma *in situ* (CIS) of the breast has not been fully ascertained. Here, TILs

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are also likely to indicate a form of active adaptive immune response. High TILs have been consistently correlated with adverse histopathological factors, and while a few recent studies have demonstrated a link between high TILs and shorter recurrence-free interval, the majority have not demonstrated strong association with clinical outcome in CIS. Evidence of an immune response in CIS suggests that immune-based treatments may be effective and that the immune response could play a role in more accurate DCIS risk stratification [16–18]. Additionally, studies show that anti-HER2 dendritic cell-based vaccinations have demonstrated activity in pre-operative HER2+ DCIS patients [19].

Collectively, we deemed it important to evaluate the immunogenicity of HER2+ BC, as the majority of studies on this subject focus on TNBC, while HER2+ BC is also one of the most immunogenic subtypes. Therefore, the objectives of this study were to investigate the frequency of TILs in HER2+ BC and associated CIS, together with PD-L1 expression and *PIK3CA* mutations, and whether these factors influence outcome, in early HER2+ BC patients.

## Materials and methods

This study was designed as a formal prospective-retrospective study, that was performed on tumor tissue from patients enrolled in the national DBCG-05H protocol [12]. These patients underwent intended curative surgery for early-stage HER2+ BC, had a histologically verified diagnosis of primary invasive breast cancer and completed a minimum of four series of adjuvant chemotherapy before trastuzumab administration for 12 months. Patients with HR + disease received additional adjuvant antihormonal therapy post-chemotherapy for 5 years. HER2-positive status was defined according to national, contemporary guidelines: immunohistochemical staining for HER2 of 3+ and/or FISH positive (ratio >2.0) [20]. Other requirements were high-risk disease according to DBCG guidelines, age <70 years, performance status of less than 2 and normal cardiac function. Treatment has already been outlined in a previous study from the same cohort, but is briefly now explained [12]. According to DBCG guidelines, seven cycles of CEF (cyclophosphamide 600 mg/m$^2$, epirubicin 60 mg/m$^2$, and fluorouracil 600 mg/m$^2$) were recommended before trastuzumab administration. Dates of administration of first trastuzumab treatment ranged from 11 January 2006 to 1 February 2008. Trastuzumab was administered as an initial loading dose of 8 mg/kg followed by 6 mg/kg every 3 weeks, totaling 17 series. Thereafter, patients were followed every half year for 4 years and the following 5 years once a year with clinical examination. Patients with hormone receptor-positive disease further received adjuvant antihormonal therapy (tamoxifen and/or aromatase inhibitor) after chemotherapy. Figure 1 shows the availability of patient material. Formalin-fixed paraffin-embedded (FFPE) primary breast tumors were included in the study. From each FFPE block, one full tissue HE-stained section (4-μm thick) was evaluated for stromal TILs (sTILs). TILs were assessed according to the International Immuno-Oncology Biomarker Working Group's (WG) guidelines [1]. Briefly, all stromal mononuclear cells were evaluated as TILs within the borders of the invasive tumor. Interobserver agreement (calculated in a separate study) was acceptable (ICC 0.93) when assessed by two pathologists who examined 100 randomly selected full-sections [21]. Both pathologists were blinded to all clinical information. TILs in CIS were assessed according to WG's update, which are similar to those for invasive carcinoma but with some modifications [22]. Notably, any type of circumferential sTIL infiltration was assessed and when multiple CIS lesions were present, TILs were evaluated in all lesions and the mean value taken without focusing on hotspots. For both invasive cancer and CIS, TILs were assessed as a continuous measurement, and in 10% intervals, except values <10%, which were evaluated as 1% or 5%, forming the basis for three predefined categories: low (<10%), intermediate (10–59%) and high (60–100%). Additionally, from each FFPE block, two 2 mm TMA cores, taken from each tumors invasive front, were evaluated immunohistochemically for PD-L1 expression, which was assessed manually according to contemporary best practice using PD-L1 SP263 Ventana-assay and positivity defined as ≥1% membranous and/or cytoplasmic expression in TCs and ICs. One TMA core was assessed when it was the only one available/suitable, and the core with the highest value was considered most representative. This study follows the recommendations for tumor marker prognostic studies (REMARK) [23]. *PIK3CA* mutational analysis has been outlined in an earlier study using the same patient cohort [12] and the data are now re-presented with a longer follow-up. The study was approved by the Ethical Committee of Region Southern Denmark (approval no. KF 02-284444).

### *Statistical analysis*

Descriptive statistics were utilized to summarize patient characteristics. Follow-up time was quantified in terms of a Kaplan–Meier estimate of potential follow-up.

Overall survival (OS) was defined as the time elapsed from surgery until death of any cause and complete follow up for OS was achieved by retrieval of data from Denmark's civil registration system. Invasive disease-free survival (IDFS) was defined as the time elapsed from surgery until invasive breast cancer recurrence irrespective of localization, new invasive breast cancer involving the same or the contralateral breast, second non-breast invasive cancer or death of any cause as first event. Patients without an IDFS event were censored at latest visit date with maximum 10 years of follow-up. IDFS and OS rates were estimated according to the Kaplan–Meier method, and univariate comparisons between groups were made using the log-rank test. The effects of PIK3CA mutations, TILs and PD-L1 in TCs and ICs on IDFS and OS were quantified in terms of hazard ratios (HRs) and estimated unadjusted and adjusted using the Cox proportional hazard models. Nodal status (0 vs. 1–3 vs. >3) and *PIK3CA* mutational status were included in the multivariate model. Tumor size was tested but was found not to be significant and thus not included in the multivariate model. No corrections for multiple testing were done. The assumptions

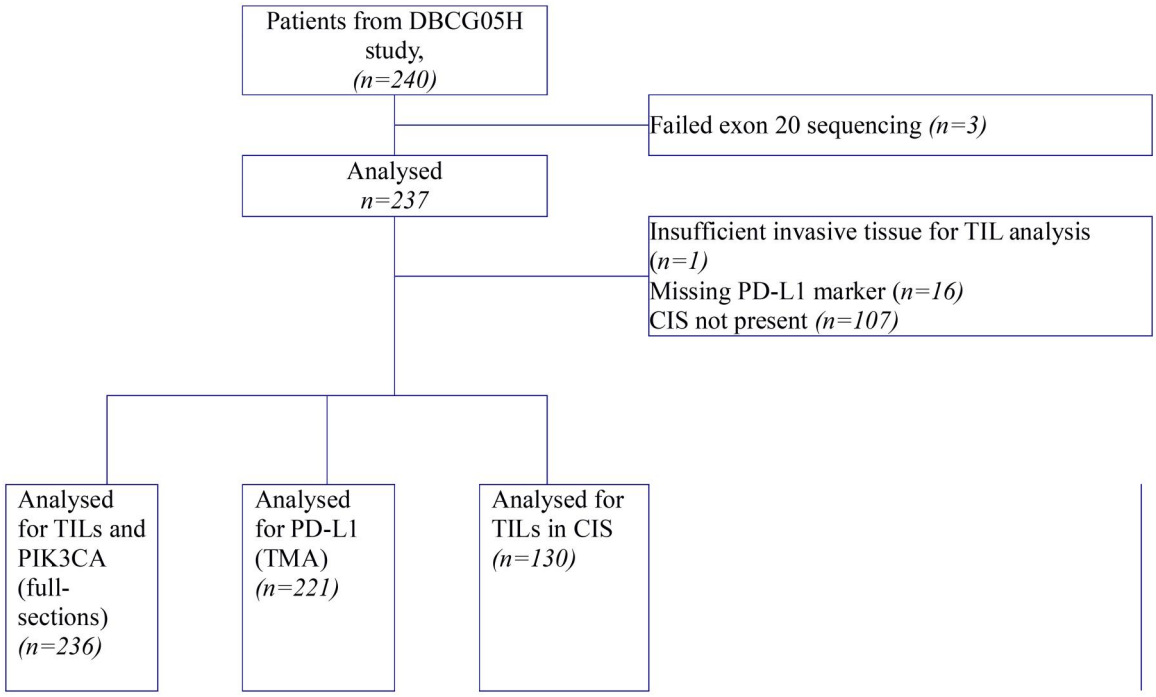


**Figure 1.** Flow diagram illustrating patient material.

of proportional hazards were assessed by the Schoenfeld residuals. Differences in distribution of patient and tumor characteristics between subgroups were assessed with Chi-square test or Fisher's exact test where appropriate (unknowns not included). $p$ Values are two-sided and $p \leq .05$ was considered significant. Statistical analyses were performed with the use of SAS software, version 9.4 (SAS Institute, Cary, NC).

## Results

### Patient characteristics

Of the 240 patients, 236 were available for sTILs and *PIK3CA* analysis in invasive cancer on full tumor-sections, and 221 were available for PD-L1 on TMA, while 130 patients were available for sTILs analysis in CIS co-existent with carcinoma.

Among the 240 patients assessable for biomarker analysis (Figure 1), 60 had died, and 61 IDFS events had been recorded. IDFS events comprised 51 patients with breast cancer recurrence, four patients diagnosed with a new invasive contralateral breast cancer, six patients diagnosed with a second primary non-breast invasive cancer, and one patient had died as first event. Median estimated potential follow-up time was 13.3 years for OS and 9.2 years for IDFS.

All patients had a minimum of four cycles of chemotherapy before trastuzumab administration. Two hundred and fifteen patients received a minimum of seven cycles of CEF. All patients received EC (epirubicin and cyclophosphamide), and only five patients did not receive fluorouracil. Nine patients received docetaxel-containing therapy (sequentially after EC/CEF), outside of protocol. Thirty-nine patients (16%) received <17 series of trastuzumab, 16 of whom had breast cancer recurrence during therapy, five stopped treatment due to toxicity, three due to patient wishes and 15 for unknown reasons.

High, intermediate and low sTILs were observed in 14%, 78% and 8% of invasive cancers, respectively, and the median sTILs value was 20% (interquartile range 10–40%). For CIS co-existent with carcinoma, there was a comparable distribution; high, intermediate, and low sTILs were observed in 12%, 83%, and 5%, respectively, with the same median sTILs value and interquartile range as the purely invasive cancers. Twenty-five percent of patients had a *PIK3CA* mutation (data not shown in Table 1).

PD-L1 expression was seen in 204 (92%) cases, being nearly three times so frequent in ICs (68%) than TCs (24%).

### Association of sTILs and PD-L1 with clinicopathological characteristics

Table 1 shows baseline characteristics according to sTILs (in both invasive cancer and CIS co-existent with carcinoma) and PD-L1. sTILs in CIS co-existent with carcinoma were significantly associated with premenopausal status ($p = .05$) and smaller invasive tumor size ($p = .0002$). PD-L1 expression was significantly associated with fewer lymph node metastases ($p = .02$) and higher malignancy grade ($p = .05$) in TCs and ICs, respectively.

### Survival analyses

Kaplan–Meier's curves of OS and IDFS according to *PIK3CA*, sTILs in both invasive cancer and CIS co-existent with carcinoma and PD-L1 are presented in Figure 2. Univariate and multivariate HR estimates for OS and IDFS by marker status are presented in Table 2. In univariate analysis, OS was significantly poorer among patients with a *PIK3CA* mutation (HR

**Table 1.** Patient and tumour characteristics by sTILs and PD-L1 status.

| | %sTILs in invasive cancer | | | | | %sTILs in CIS | | | | | PD-L1 in TCs | | | | PD-L1 in ICs | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *N* | <10 *N* (%) | 10–59 *N* (%) | 60–100 *N* (%) | *p* | *N* | <10 *N* (%) | 10–59 *N* (%) | 60–100 *N* (%) | *p* | *N* | 0% *N* (%) | ≥1% *N* (%) | *p* | 0% *N* (%) | ≥1% *N* (%) | *p* |
| Total | 236 | 19 (8) | 185 (78) | 31 (14) | | 130 | 7 (5) | 108 (63) | 15 (12) | | 221 | 167 (76) | 54 (24) | | 71 (32) | 150 (68) | |
| Menopausal status | | | | | .28 | | | | | .05 | | | | .42 | | | .77 |
| Pre | 143 | 10 (53) | 117 (63) | 16 (50) | | 85 | 4 (57) | 72 (67) | 9 (60) | | 137 | 101 (60) | 36 (67) | | 45 (63) | 92 (61) | |
| Post | 93 | 9 (47) | 68 (37) | 16 (50) | | 45 | 3 (43) | 36 (33) | 6 (40) | | 84 | 66 (40) | 18 (33) | | 26 (37) | 58 (39) | |
| Positive nodes | | | | | .14 | | | | | .87 | | | | .02 | | | .38 |
| 0 | 76 | 7 (37) | 53 (29) | 16 (50) | | 41 | 3 (42) | 32 (30) | 6 (40) | | 73 | 51 (30) | 22 (41) | | 21 (30) | 52 (35) | |
| 1–3 | 82 | 5 (26) | 67 (36) | 10 (31) | | 46 | 2 (29) | 40 (37) | 4 (27) | | 78 | 55 (33) | 23 (42) | | 23 (32) | 55 (37) | |
| >3 | 78 | 7 (37) | 65 (35) | 6 (19) | | 43 | 2 (29) | 36 (33) | 5 (33) | | 70 | 61 (37) | 9 (17) | | 27 (38) | 43 (28) | |
| Tumor size (mm) | | | | | .26 | | | | | .0002 | | | | .28 | | | .58 |
| 0–20 | 117 | 8 (42) | 91 (49) | 18 (56) | | 69 | 3 (42) | 58 (54) | 8 (53) | | 110 | 88 (53) | 22 (41) | | 37 (52) | 73 (49) | |
| 21–50 | 106 | 8 (42) | 86 (47) | 12 (38) | | 55 | 2 (29) | 48 (44) | 5 (33) | | 102 | 72 (43) | 30 (55) | | 30 (42) | 72 (48) | |
| >50 | 13 | 3 (16) | 8 (4) | 2 (6) | | 6 | 2 (29) | 2 (2) | 2 (14) | | 9 | 7 (4) | 2 (4) | | 4 (6) | 5 (3) | |
| Histology | | | | | .80 | | | | | n/a | | | | 1 | | | .27 |
| Ductal | 228 | 18 (95) | 179 (97) | 31 (97) | | 129 | 7 (100) | 107 (99) | 15 (100) | | 213 | 161 (96) | 52 (96) | | 67 (94) | 146 (97) | |
| Other | 8 | 1 (5) | 6 (3) | 1 (3) | | 1 | 0 | 1 (1) | 0 | | 8 | 6 (4) | 2 (4) | | 4 (6) | 4 (3) | |
| Malignancy grade | | | | | .40 | | | | | .97 | | | | .28 | | | .05 |
| I | 11 | 1 (5) | 9 (5) | 1 (3) | | 9 | 0 | 8 (7) | 1 (7) | | 9 | 9 (5) | 0 | | 4 (6) | 5 (3) | |
| II | 88 | 10 (53) | 69 (37) | 9 (28) | | 40 | 3 (43) | 33 (31) | 4 (27) | | 81 | 61 (37) | 20 (37) | | 33 (46) | 48 (32) | |
| III | 126 | 7 (37) | 99 (54) | 20 (63) | | 80 | 4 (57) | 66 (61) | 10 (66) | | 123 | 92 (55) | 31 (57) | | 31 (44) | 92 (62) | |
| Unknown | 11 | 1 (5) | 8 (4) | 2 (6) | | 1 | 0 | 1 (100) | 0 | | 8 | | | | 3 (4) | 5 (3) | |
| ER status | | | | | .34 | | | | | .37 | | | | .37 | | | .12 |
| Positive | 117 | 10 (53) | 95 (51) | 12 (37.5) | | 63 | 5 (71) | 52 (48) | 6 (40) | | 111 | 81 (48) | 30 (56) | | 41 (58) | 70 (47) | |
| Negative | 119 | 9 (47) | 90 (49) | 20 (62.5) | | 67 | 2 (29) | 56 (52) | 9 (60) | | 110 | 86 (52) | 24 (44) | | 30 (42) | 80 (53) | |

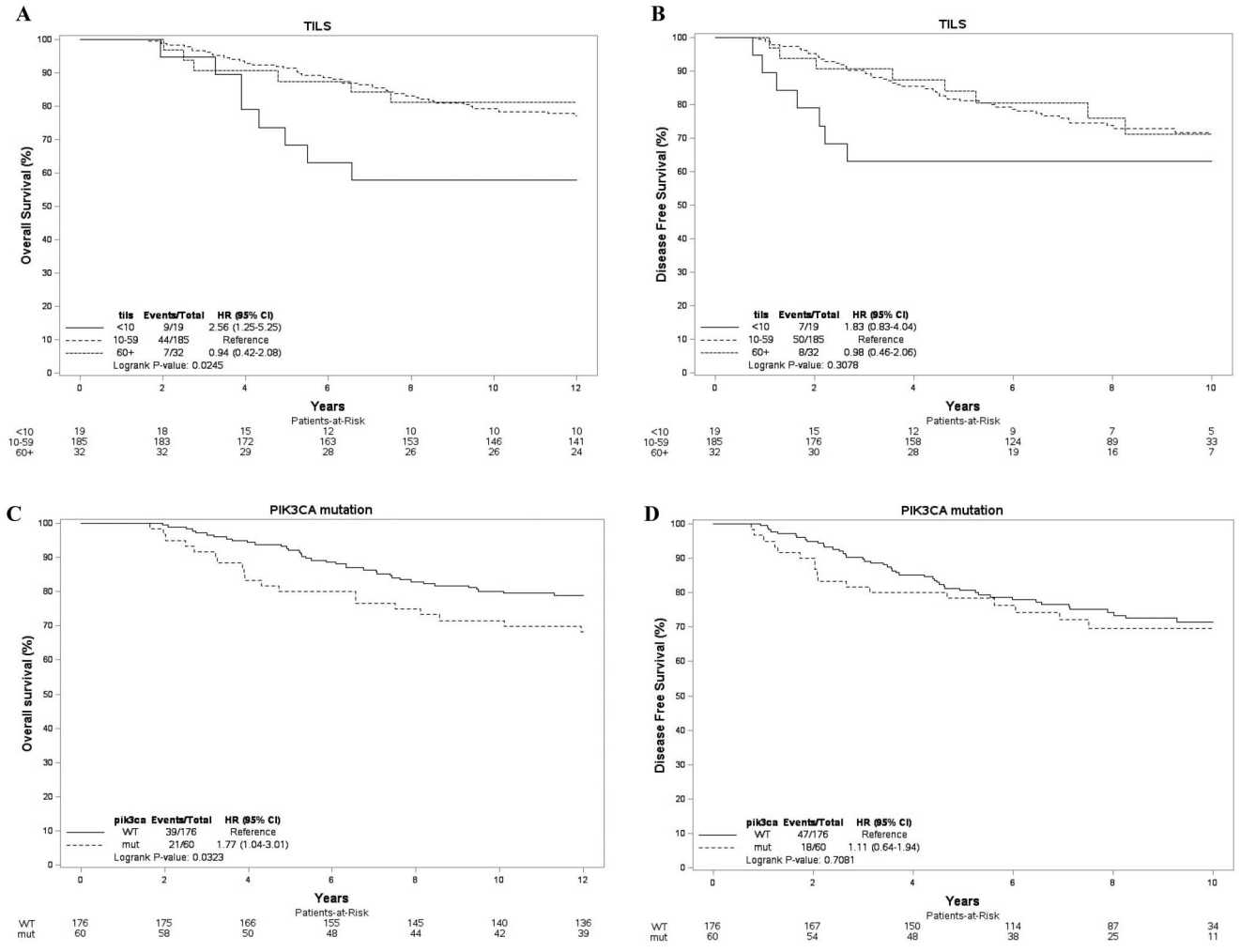


**Figure 2.** Kaplan–Meier's curves of overall survival and invasive disease-free survival in patients with early-stage HER2-positive breast cancer treated with adjuvant trastuzumab. (A, B) sTILs in invasive cancer (<10% vs. 10–59% vs. ≥60%). (C, D) PIK3CA mutation status (wild type (WT) vs. mutation (mut) (exon 9 or 20)).

**Table 2.** Univariate and multivariate HR estimates for OS and IDFS by marker status.

| | OS, univariate | | | OS, multivariate[a] | | |
|---|---|---|---|---|---|---|
| | HR | (95% CI) | *p* | HR | (95% CI) | *p* |
| PIK3CA mutational status[b] | | | .03 | | | .14 |
| Mutation vs. WT | 1.77 | (1.04–3.01) | | 1.49 | (0.88–2.54) | |
| sTILs, invasive cancer[b] | | | .03 | | | .02 |
| <10 vs. 10–59 | 2.56 | (1.25–5.25) | | 2.80 | (1.36–5.78) | |
| 60+ vs. 10–59 | 0.94 | (0.42–2.08) | | 1.26 | (0.55–2.88) | |
| sTILs, CIS[c] | | | .28 | | | .28 |
| <10 vs. 10–59 | 2.18 | (0.65–7.27) | | 2.29 | (0.68–7.68) | |
| 60+ vs. 10–59 | 1.71 | (0.65–4.49) | | 1.74 | (0.62–4.89) | |
| PD–L1 in tumor cells | | | .09 | | | .43 |
| >0% vs. 0% | 0.52 | (0.25–1.11) | | 0.73 | (0.34–1.58) | |
| PD-L1 in stroma cells | | | .29 | | | .40 |
| >0% vs. 0% | 0.74 | (0.43–1.29) | | 0.79 | (0.45–1.37) | |
| | IDFS, univariate | | | IDFS, multivariate | | |
| | HR | (95% CI) | *p* | HR | (95% CI) | *p* |
| PIK3CA mutational status[b] | | | .71 | | | .92 |
| Mutation vs. WT | 1.11 | (0.64–1.94) | | 0.97 | (0.55–1.71) | |
| sTILs, invasive cancer[b] | | | .32 | | | .20 |
| <10 vs. 10–59 | 1.83 | (0.83–4.04) | | 2.02 | (0.91–4.50) | |
| 60+ vs. 10–59 | 0.98 | (0.46–2.06) | | 1.34 | (0.61–2.92) | |
| sTILs, CIS[c] | | | .73 | | | .65 |
| <10 vs. 10–59 | 0.61 | (0.08–4.51) | | 0.65 | (0.09–4.77) | |
| 60+ vs. 10–59 | 1.33 | (0.51–3.44) | | 1.49 | (0.55–4.05) | |
| PD–L1 in tumor cells | | | .32 | | | .86 |
| >0% vs. 0% | 0.71 | (0.36–1.41) | | 0.94 | (0.46–1.90) | |
| PD-L1 in stroma cells | | | .38 | | | .51 |
| >0% vs. 0% | 0.78 | (0.45–1.36) | | 0.83 | (0.48–1.44) | |

CI: confidence interval; HR: hazard ratio; OS: overall survival; IDFS: invasive disease-free survival; WT: wildtype.
[a]Adjusted for positive nodes (0 vs. 1–3 vs. 4+) and PIK3CA (mutation vs. WT). Tumor size was tested but was found not to be significant.
[b]$n = 236$.
[c]$n = 130$.

1.77; 95% CI 1.04–3.01; $p = .03$) and among patients with low sTILs (<10%) in cancer (HR 2.56; 95% CI 1.25–5.25; $p = .03$), while there was a tendency towards better OS among patients with ≥1% PD-L1 expression in TCs (univariate: HR 0.52; 95% CI 0.25–1.11, $p = .09$). There were no significant associations with IDFS for all markers. When adjustments were made for other prognostic factors (nodal status and *PIK3CA* mutation) in multivariate analysis (tumor size was tested but was found not to be significant in multivariate analysis), patients with low sTILs had a significantly higher risk of dying (HR 2.80; 95% CI 1.36–5.78; $p = .02$). The association between *PIK3CA* mutation and survival became non-significant, and the associations between sTILs in CIS co-existent with carcinoma, and PD-L1, with survival remained non-significant.

## Discussion

The purpose of this study was to investigate the frequency of TILs in HER2+ BC and associated CIS, together with PD-L1 expression and *PIK3CA* mutations, and whether these factors influence outcome, in early HER2+ BC patients.

We confirm higher levels of sTILs are associated with better prognosis and re-present findings from a previous study using the same cohort, now with longer follow-up, that *PIK3CA* mutations are associated with poorer prognosis, with the latter only being significant in univariate analysis. PD-L1 expression was very common in our cohort, especially in ICs, and associated with fewer lymph node metastases and higher malignancy grade, with a tendency towards better prognosis.

We confirm the relatively high rates of 'lymphocyte-predominant breast carcinoma' (>50–60% of tumor area occupied by TILs), and the association between higher sTILs and better OS in a cohort of early stage HER2+ BC treated with adjuvant chemotherapy, trastuzumab and antihormonal therapy. Interestingly, some previous studies have only demonstrated such an association in patients treated with chemotherapy alone, where anti-HER2 therapy was not used [24,25], suggesting an added role of anti-HER2 therapy. Indeed, a link has been shown between high TILs and increased trastuzumab benefit [26], further corroborating the complex mechanisms of action of this monoclonal antibody as it both interferes directly with the HER2-signalling pathway and has an immune-mediated role. Current perspectives regard TILs as a solid prognostic and predictive 'backbone' in evaluation of tumor microenvironment (TME) activity, so that it may even be applied as a single biomarker, or in any case as an essential element in combination with other relevant biomarkers such as PD-L1 [27]. Other potentially relevant biomarkers involved in the complex TME include CD8, CD4, FOXP3 and LAG3 [27], forming scope for further study.

PD-L1 expression was frequent in this study, being more common in ICs (68%) than TCs (24%). In the literature, PD-L1 expression in breast cancer varies widely from 0 to 83% [8], possibly reflecting well-documented ongoing issues and lack of standardised consensus with the assessment of this biomarker [8,28]. Indeed, PD-L1 positivity in ICs is known to be higher in SP263 and 22C3 assays than SP142 [28]. In the present study, the methodology was selected to largely mirror expert opinion at the time of study initiation. Namely, both TCs and ICs were evaluated as per manufacturer instructions, SP263 assay was selected as it is used in breast cancer clinical trials and seems to be relatively comparable with most other relevant assays. The ≥1% staining cut-off for PD-L1 positivity was chosen as this seems to be one of the most clinically relevant strategies [8,9,28]. Of note, recent research demonstrates that there may be a difference between the predictive role of baseline PD-L1 expression for ICI in early-stage compared with advanced TNBC, as the KEYNOTE-355 trial demonstrated significant improvement in progression-free survival in patients with metastatic TNBC with a CPS ≥ 10, whereas the KEYNOTE-522 trial showed longer event-free survival with ICI in all early TNBC subgroups independent of PD-L1 expression [29], creating further discussion regarding the role of PD-L1 in breast cancer.

To our knowledge, there are only few previous studies to date, exclusively assessing PD-L1 expression in HER2+ BC. In one study expression was identified in TCs and ICs in 6% and 12% of cases, respectively [30]. Li et al. showed PD-L1 expression in tumor was 25.7% for clone 28-8 and 11.5% for clone 22C3, but did not include frequency in ICs [31]. Whilst these overall trends are somewhat comparable with our findings, variation in methodologies may account for these differences. The association of PD-L1 expression in TCs with

fewer lymph node metastases in our study supports the findings of Hou et al. indicating PD-L1 may convey strong host antitumor immunity [7,30,32]. Conversely, Muenst et al. demonstrated association between expression and positive lymph node status in a mixed breast cancer population, suggesting the biological behavior of PD-L1 may vary according to subtype [6]. Moreover, our finding of PD-L1 expression in ICs correlating with higher malignancy grade corroborates other studies reporting association with higher risk clinical parameters [32,33]. This may be attributed to the higher mutation rate of these tumors and that in breast cancer to date, clinical benefit has only been linked with PD-L1 expression in ICs, not TCs [28]. The link between PD-L1 expression and prognosis continues to be ambiguous. In our study, there was a tendency towards better OS among patients with PD-L1 expression in TCs, although this did not reach statistical significance. This is consistent with results of the few other studies evaluating HER2+ BC specifically [30,31,33,34], along with studies assessing TNBC and other subtypes [9]. Indeed, the relationship may vary according to breast cancer subtype, with some studies only showing a correlation in ER/PR negative, HER2+ or TNBC subsets [8]. Whilst associations have been made from these studies, it must be emphasised that the agreement of standardised PD-L1-evaluation method will bring clearer conclusions; such clarity is fortunately gradually en route [3,28].

In addition, recent studies have shown that a combination of several histopathological parameters may be able to identify immunogenic tumours, which potentially may be suitable for treatment de-escalation. TILs and PD-1 and/or PD-L1 may have a synergistic effect, whereby a combination of both immunological biomarkers may identify the most immunogenic tumours more effectively than either marker alone [35,36]. Stenmark Tullberg et al. demonstrated that 'high risk' tumours with an activated immune response (defined as sTILs $\geq$10% and PD-1 and/or PD-L1 $\geq$1% in lymphocytes) are associated with a reduced risk of ipsilateral breast tumour recurrence in stage I–IIA breast cancer. Indeed, this risk reduction may be equivalent to the benefit gained by treatment with adjuvant radiotherapy [37]. There may therefore be subsets of tumours, which are suitable for treatment de-escalation, despite having aggressive histopathological features [38–41]. This exciting concept of 'immune responsiveness' has much scope for further study, including in HER2+ BC, preferably in a larger cohort to enable significant conclusions to be drawn.

*PIK3CA* mutations were significantly associated with poorer OS with univariate analysis. This finding corroborates both those from an earlier study using the same cohort [12], and a recent large pooled analysis, specifically for the HER2+ subgroup [13]. This may be clinically significant as data suggest activity of Akt serine/threonine kinase inhibitors (part of the PI3K/Akt/mTOR pathway) with standard neoadjuvant treatment in HER2+ BC [14]. Furthermore, reports suggest that genetic alterations, like *PIK3CA* mutations can induce PD-L1 overexpression [34], again highlighting the complex interplay in the TME.

The frequency of higher TILs in CIS was greater in our cohort than the largest series to date [16]. This may be partly because HER2+ DCIS shows higher TILs frequency, and partly because we evaluated TILs in CIS co-existent with carcinoma, an entity, which contains denser TILs than in pure non-invasive DCIS; pointing towards the role of immune response in DCIS progression, an observation that could be exploited, potentially providing more treatment options in the pre-invasive stage [16–19,42]. The association between sTILs in CIS co-existent with carcinoma and premenopausal status is consistent with other studies demonstrating TILs correlating with adverse clinicopathological factors. Interestingly, there was a converse association between sTILs in CIS co-existent with carcinoma and invasive tumor size, suggesting a robust TME in high-risk DCIS may confer a protective role [43]. Our exploratory observations can form the basis for further studies with the aim of identifying patients who can potentially be targeted with ICI in early HER2+ disease.

There is emerging evidence that TILs, PD-L1, and *PIK3CA* mutations may also be associated with treatment benefits from antibody–drug conjugates. Trastuzumab emtansine (T-DM1) may be effective in advanced, pre-treated HER2+ BC with *PIK3CA* mutations, where other standard therapies have not been of benefit [44]. There may also be potential for combination immunotherapy treatment strategies such as antibody–drug conjugates and ICI; the randomized phase II KATE2 trial suggested a benefit in progression-free survival, in exploratory analyses, in pretreated HER2+ PD-L1-positive tumors with T-DM1 and atezolizumab, compared to no benefit in the PD-L1-negative subset [45].

Strengths of this study besides the long follow-up period, include the uniform treatment regimen of adjuvant trastuzumab and chemotherapy, since any differences in outcome could not have been due to treatment regimen. Conversely, the relatively small patient number is a limitation as this leads to effect size with greater variation and reduces the power in the study, as well as limiting the possibility of adjusting for all standard parameters in the multivariate analysis. Therefore, validation in a larger patient sample size is warranted to establish an effect independent of standard prognostic parameters. Use of TMA and lack of availability of data for hormone receptor status and HER2 expression in CIS components are other limitations.

To conclude, the present study of HER2-positive early-stage breast cancer patients demonstrates that patients with lower levels of sTILs have a significantly poorer survival. This conclusion is drawn from a relatively small cohort and we recommend validation for an independent effect in a larger sample size, in order to obtain a more precise estimate and with all relevant parameters included in an adjusted analysis.

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## Author contributions

All authors contributed to the study conception and design. Data collection and analysis was performed by Frances Reznitsky. Statistical analysis was performed by Danish Breast Cancer Group's secretariat. The first draft of the manuscript was written by Frances Reznitsky and all authors contributed on drafts of the manuscript. All authors read and approved the final manuscript.

## Disclosure statement

The authors declare there are no conflicts of interest

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## Data availability statement

The data that support the findings of this study are available from the corresponding author, [FR], upon reasonable request.

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