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REVIEW

# Tumor-infiltrating lymphocytes in HER2-positive breast cancer: potential impact and challenges

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**Introduction:** In this review, we evaluate the role of stromal tumor-infiltrating lymphocytes (sTILs) as a biomarker in human epidermal growth factor receptor 2 (HER2)-positive breast cancer, exploring the prognostic and predictive potential in various treatment settings.

**Methods:** Data from multiple clinical trials in the early and metastatic settings, focusing on TILs' correlation with pathologic complete response (pCR), progression-free survival (PFS), and overall survival across early and metastatic HER2-positive breast cancer were summarized. This review also discusses TILs' assessment methods, interobserver variability, and emerging technologies to assess TILs.

**Results:** TILs have been identified as a highly reproducible biomarker that predicts pCR in patients receiving neoadjuvant therapy and serves as a prognostic indicator for long-term outcomes in several breast cancer subtypes, including HER2-positive. Studies indicate that higher TIL levels correlate with better recurrence-free survival rates. Despite these findings, there is no consensus on the optimal TIL threshold for clinical decision making, and further research is required on how to incorporate TILs into routine clinical practice.

**Conclusions:** TILs represent a promising biomarker in HER2-positive breast cancer, particularly in early disease settings. This assessment could guide treatment de-escalation or intensification, tailoring therapies to individual patient profiles. Due to their prognostic importance, TILs can be added to pathology reports. However, further validation in clinical trials is essential for the widespread adoption of TILs in clinical practice.

**Key words:** HER2-positive breast cancer, stromal tumor-infiltrating lymphocytes, biomarkers, pathologic complete response, neoadjuvant therapy

## INTRODUCTION

Human epidermal growth factor receptor 2 (HER2)-expressing tumors account for 15%-20% of all breast cancers.[1,2] Patients with HER2-positive breast cancers historically had worse outcomes relative to those with other breast cancer subtypes.[1] The development of HER2-directed therapies changed the natural history of early and metastatic disease.[1,3,4] Even though HER2 is an excellent prognostic and predictive biomarker in breast cancer, the disease is heterogeneous.[5,6] There is a need to identify novel biomarkers that could allow clinicians to personalize treatment for patients—intensifying regimens for those likely to have worse outcomes and de-escalating for those with a better prognosis.

Several clinical questions highlight the need for additional biomarkers when treating patients with HER2-positive breast cancer. For example, for patients with stage II-III disease, the current standard of care is neoadjuvant therapy [preoperative docetaxel, carboplatin, trastuzumab, pertuzumab (THCP)], which allows clinicians to assess the individual's response to treatment.[4,7] This is relevant as pathologic complete response (pCR) has shown to be an excellent surrogate biomarker of good long-term outcomes in HER2-positive disease.[8] Moreover, adjuvant treatment can be modified based on the patient's response to the preoperative treatment, and HER2-directed treatment can be intensified for those with residual disease (RD) after neoadjuvant systemic therapy.[9] Despite the significant improvements achieved with HER2-directed therapies in early breast cancer, many patients relapse. Identifying biomarkers to determine which patients are likely to relapse and those

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that have excellent long-term outcomes will allow clinicians to tailor therapy to maximize the pCR rates while limiting toxicities.

For patients with metastatic disease (stage IV), the current first-line treatment is docetaxel, trastuzumab, and pertuzumab (THP) based on the phase III CLEOPATRA study.[10,11] Studies are ongoing to assess the role of other novel agents in the first-line setting, such as trastuzumab deruxtecan (T-DXd—NCT04784715). It is important to identify biomarkers to determine which patients may benefit from more intensive treatments and those who can continue to receive the CLEOPATRA regimen, which has been associated with excellent cancer-related outcomes and significant benefits in quality of life relative to other cancer therapies.[12,13]

One promising biomarker being studied is the morphologic evaluation of tumor-infiltrating lymphocytes (TILs).[14] In this review, we discuss the evidence for using TILs in HER2-positive breast cancer and the potential advantages and uncertainties of this biomarker use for research and in the clinic.

## TUMOR IMMUNE MICROENVIRONMENT

The innate and adaptive immune systems play a critical role in the development and progression of malignancies.[14,15] Immune surveillance prevents tumor development, and neoplastic transformation often occurs in the setting of immune escape.[14,15] Understanding the relationships between cancer and immune cells is critical to shed light on mechanisms of tumor progression, how immune markers can function as prognostic or predictive biomarkers, and to guide drug development.

It is important to consider the type and number of immune cells surrounding the tumor and the physical location of these cells in relation to the tumor cells. In terms of the immune infiltrate present in the tumor microenvironment (TME), lymphoid and myeloid cells can be associated with 'hot' or 'cold' tumors, which have been associated with response to immunotherapies and chemotherapy.[14,16] Hot or inflamed tumors have higher numbers of M1 macrophages, natural killer cells, CD8+ T cells, and CD4+ helper T cells (common cytokines include interferon gamma and interleukin 21).[14,17] The T-cell populations play an important role. Tumors with CD8+ T cells with features of tissue memory and high expression of immune checkpoint proteins and effector proteins have been associated with improved outcomes.[17] In contrast, cold or immunosuppressed tumors have more M2 macrophages, dendritic cells, and Treg lymphocytes [common cytokines include interleukin 10 and tumor necrosis factor (TNF) beta] (Figure 1A).[14] Notably, the concept of 'cold' versus 'hot' tumors has limited application in clinical practice, as definitions and cut-offs of immune infiltration vary, and there is no clear boundary between when a cold tumor becomes hot and vice versa.

Understanding the location of the immune cells in relation to the tumor is also critical. Tumors can be classified as immune desert, immune excluded, or immune inflamed (Figure 1B).[18] Immune-desert tumors have limited immune cells, immune-excluded tumors have immune cells in a separate compartment than the tumor cells considered mostly to be at the periphery, and immune-inflamed tumors contain tumor and an extensive amount of immune cells in the same compartment.[18] This spatial characterization has shown to be prognostic in how breast cancers respond to immune checkpoint inhibitors (ICIs).[18,19] The definitions for these terms vary in the literature, as well as the cut-offs, precluding defining clear boundaries between these phenotypes, which have limited reproducibility and, therefore, limited use in clinical practice.

## TUMOR-INFILTRATING LYMPHOCYTES

The association between TILs and breast cancer outcomes has been studied in triple-negative breast cancer (TNBC) and HER2-positive breast cancer. Patients with tumors with high TIL percentages have better long-term outcomes. The guidelines from the European Society of Medical Oncology (ESMO) suggest the use of TILs as a biomarker for early-stage TNBC and HER2-positive breast cancer.[20-25] The prognostic value of TILs in hormone receptor (HR)-positive breast cancer remains controversial. Morphologically speaking, TILs are defined as lymphocytes and plasma cells, not neutrophils, eosinophils, or macrophages. TILs include the B cells and T lymphocytes, including cytotoxic (CD8), usually more predominant than helper (CD4) or regulatory T cells within and around tumor cells.[16] TILs can be classified as intratumoral (iTILs) or stromal (sTILs). While iTILs are mononuclear cells within the tumor nest in direct contact with tumor cells, sTILs are dispersed in the stroma and do not have direct contact with cancer cells.[14,21]

Current guidelines from the International Immuno-Oncology Biomarker Working Group (IIOBWG) on Breast Cancer (www.tilsinbreastcancer.org) recommend the assessment of sTILs, as this assessment is highly reproducible.[16] Multiple studies have shown that sTILs can be used as a prognostic biomarker, while iTILs are less abundant and difficult to identify on hematoxylin and eosin (H&E) sections. Currently, sTILs are analyzed for prognostic purposes. In this review, we will focus on the sTILs as defined by the IIOBWG.

TILs can be evaluated using H&E tumor sections, which are standard for diagnosing breast cancer.[14,21] H&E stains are widely available and low-cost, making TILs a particularly attractive biomarker, especially in resource-poor settings.[14] In addition to H&E slides, immunohistochemistry can be used to subtype the lymphocytes using markers such as CD3, CD8 (Figure 1C), CD4, and CD45. However, this is not a necessary step for the assessment of sTILs. sTILs can be assessed in formalin-fixed and paraffin-embedded biopsy or surgical specimens. All mononuclear cells are included in the assessment, and sTILs are often reported as the percentage (Figure 1D) of TILs on the stromal surface.[14] The prognostic effect of sTILs appears to be linear. When using sTILs as a categorical variable, studies have used different

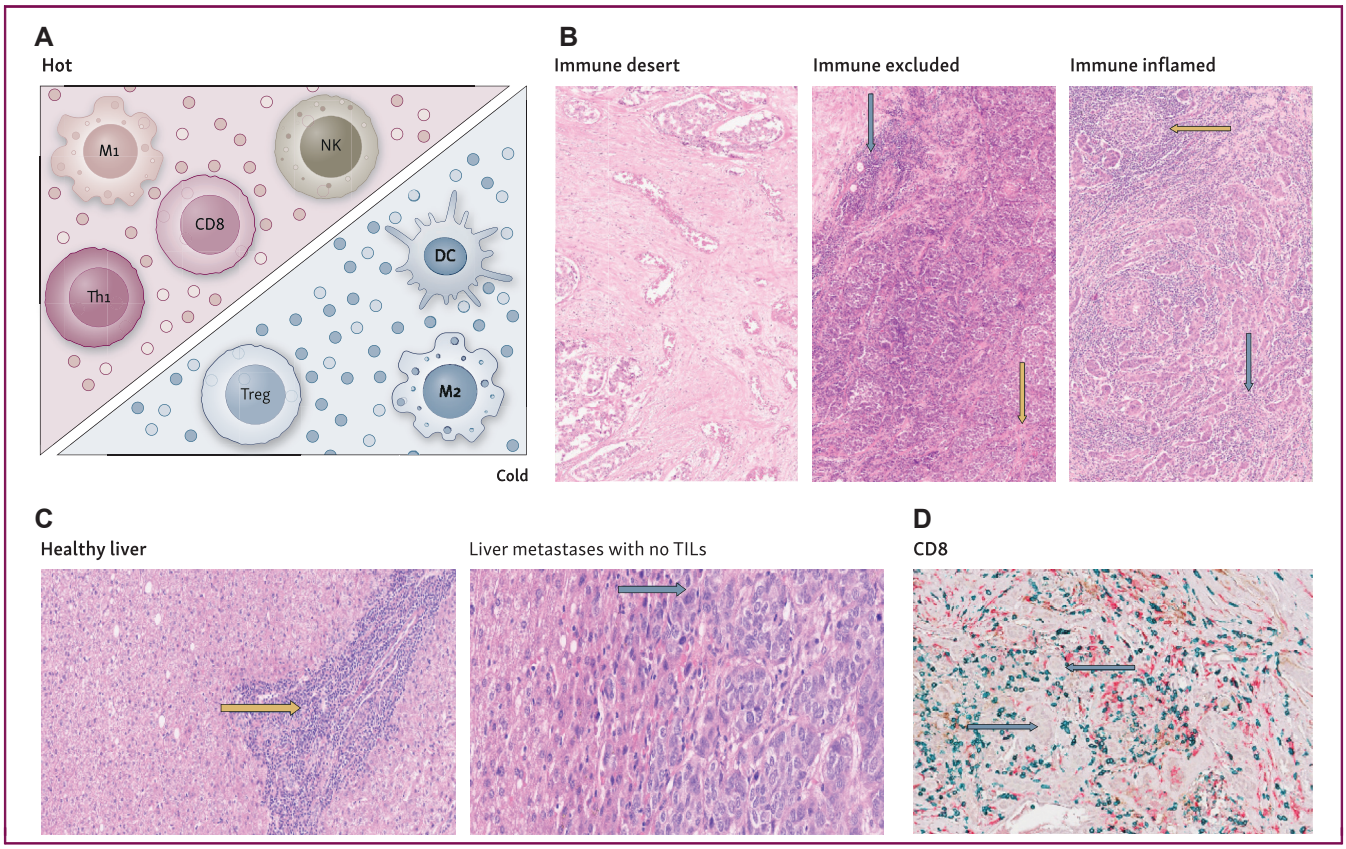


**Figure 1. Characteristics of the tumor microenvironment: categorization by cell types and spatial characteristics.** (A) Hot or inflamed tumors have higher numbers of M1 macrophages, natural killer cells, CD8+ T cells, and CD4+ helper T cells (common cytokines include interferon gamma and interleukin 21). In contrast, cold or immunosuppressed tumors tend to have more M2 macrophages, dendritic cells, and Treg lymphocytes (common cytokines include interleukin 10 and TNF-beta). (B) Illustrates immune-desert (limited immune cells), immune-excluded [TILs at the periphery (blue arrow), not in the center of the tumor (yellow arrow)], and immune-inflamed tumors [a lot of TILs (blue arrow), and in this case also having a tertiary lymphoid structure in the middle of the tumor (yellow arrow)]. (C) Healthy liver image with a high number of immune cells next to the liver metastasis with no TILs. This illustrates not only that most metastases are immune-deprived but will affect any genomic signature in HER2-positive that includes an immune cell component. (D) Multiplex immunohistochemistry images, with CD8-stained TILs (green color), CD163-stained macrophages (yellow), and PD-L1-stained macrophages, surrounding invasive cancer cell nests of invasive ductal breast cancer (arrow). Created with BioRender.com.

DC, dendritic cells; HER2, human epidermal growth factor receptor 2; NK, natural killer; PD-L1, programmed death-ligand 1; TIL, tumor-infiltrating lymphocyte; TNF, tumor necrosis factor.

cut-offs of sTILs. Recent studies have used a cut-off of sTILs $\geq$50%-60% to define tumors as lymphocyte-predominant breast cancers (LPBC) which appears to be a good predictor of long-term outcomes.[16] However, this cut-off has not been used uniformly, because an optimal cut-off has not been established. Prior studies have shown that the cut-off may vary by breast cancer subtype (including HR status in HER2-positive breast cancer) and the clinical question being asked. Additionally, the research question and goal play an important role. Therefore, a cut-off must be established for each of the potential applications of TILs, likely conducting prospective-retrospective analysis of large randomized clinical trials. An alternative is to use TILs as a continuous variable, which has shown to correlate with long-term outcomes in HER2-positive breast cancer and TNBC. Ongoing efforts to incorporate software in TIL assessment, including artificial intelligence tools, are under way.[26,27]

Pathologists can be trained to assess sTILs with guidelines published to maximize reproducibility.[14] The ring studies, which included up to 32 pathologists, assessed the reproducibility [interclass correlation coefficient (ICC)] of sTIL assessment. In these studies, ICC-concordance rates at multiple cut-offs were >0.8 which are considered by the KI67 International Working Group as reliable for use in clinical practice.[28] These studies identified several limitations faced by pathologists when assessing TILs.[28] These can be technical problems with the slide (poor quality slides, histological artifacts, crush artifact, out-of-focus scan) or with the selection of scoring areas, all causing discordance in the assessment between pathologists.[28] The heterogeneity of sTIL distribution and the quality of the H&E stains were identified as a significant contributing factor in all these studies, which can be overcome by averaging multiple tumor areas.[28]

As mentioned, understanding the spatial measures of TILs is critical; however, there is no standardized approach.[29] A report from the IIOBWG provides recommendations for spatial characterization of TILs in breast cancer using computational methods.[29] This report suggests that novel imaging platforms could be used to improve the quantification and spatial characterization of sTILs over H&E slides using segmentation, subregion sampling, density

estimation, and calculation of the sTIL score. However, a major caveat is that different computational methods on TILs measure different spatial metrics, causing discrepancies. Prospective studies to validate these computational methods are critical.[30]

## TUMOR-INFILTRATING LYMPHOCYTES IN HER2-POSITIVE EARLY BREAST CANCER

### *Neoadjuvant studies*

Several studies (summarized in Table 1) have assessed the role of sTILs as a potential biomarker in early HER2-positive breast cancer. It is important to note that all studies were prospective-retrospective studies entailing level 1B evidence (results are reproduced across trials). None of the studies were powered to assess sTILs as they were *post hoc* or based on secondary endpoints.

CHER-lob was a phase II study in which 121 patients with stage II-III operable breast cancer were randomized to receive chemotherapy [paclitaxel → fluorouracil, epirubicin, cyclophosphamide (FEC)] with lapatinib (L) versus H or the combination (HL).[31] sTILs were significantly associated with recurrence-free survival (RFS) (hazard ratio 0.978 with each 1% increment in TILs).[31] Also, the intrinsic breast cancer subtype, Luminal A, was an independent predictor of improved RFS.

A combined analysis of the GeparQuattro [neoadjuvant epirubicin + cyclophosphamide → docetaxel ± capecitabine and H if HER2-positive ($n = 445$ patients with stage I-III primary breast cancer)] and the GeparQuinto trials [neoadjuvant epirubicin + cyclophosphamide → docetaxel with L or H ($n = 615$ patients with stage II-III HER2-positive breast cancer)] assessed the role of sTILs.[32,33] sTILs were assessed as a continuous variable with 10% increases, and tumors with at least 60% sTILs were defined as LPBC.[34] sTILs were an independent predictor of pCR [10% increase in sTILs, the odds ratio (OR) was 1.12, $P = 0.002$]. In this analysis, LPBC had higher pCR rates relative to non-LPBC. LPBC had a better disease-free survival (DFS) than non-LPBC ($P = 0.058$). The prognostic benefit of sTILs was noted in the H-treated group but not in the L-treated subgroup in GeparQuinto. Notably, in patients with HER2-positive, HR-positive breast cancer, TILs had more prognostic relevance than predictive relevance (for pCR).

**Table 1. Selected neoadjuvant studies assessing the role of TILs in early HER2-positive breast cancer**

| Trial(s) | Sample size in sTIL analysis (HER2-positive) | Eligibility and regimen | sTIL cut-off | Positive association with pCR | Positive association with long-term outcomes |
|---|---|---|---|---|---|
| Cher-LOB[31] | 102 | Stage II-IIIa FEC with H or L or HL | Continuous variable (1% increase) | N/A | ✓ Recurrence-free survival |
| GeparQuattro[34] | 178 | T4 or N3 versus T1-3 and N0-2 EC→TH ± cape | Continuous variable (10% increase) or ≥60% (lymphocyte predominant) | ✓ | ✓ Disease-free survival for high-risk tumors |
| GeparQuinto[34] | 162 | cT3 or CT4 or ER+/PR− or ER+ or PR+ which are cN+ EC→T + H or L | Continuous variable (10% increase) or ≥60% (lymphocyte predominant) | × | ✓ Disease-free survival for high-risk tumors |
| GeparSixto[36] | 266 | Stage II-III Paclitaxel + doxorubicin H and L ± carboplatin | Continuous variable (10% increase) or ≥60% (lymphocyte predominant) | ✓ | N/A |
| PAMELA[76] | 148 at baseline, 134 at day 15 | Stage I-IIIa HL | 0%-20%, 21%-40%, ≥41% or continuous variable (1% increase), or ≥50% | ✓ | N/A |
| CALGB 40601[47] | 230 | Stage II-III THL versus TH versus TL | 0%-20%, 21%-40%, ≥41% or continuous variable (1% increase), or ≥50% | ✓ | ✓ Event-free survival |
| NeoALTTO[49,63] | 387 | cT2 or above, any N Paclitaxel +H or L or HL → FEC after surgery | 0%-5%, 6%-13%, 14%-30%, ≥31% or continuous variable (1% increase) | ✓ | ✓ Event rate |
| TBCRC006[77] | 59 | >3 cm or >2 cm if palpable nodes HL | ≥60% | ✓ | N/A |
| PREDIX HER2[78] | 172 | >2 cm or node + THP versus T-DM1 | ≥10% | ✓ | N/A |
| TRYPHENA[52] | 225 | Stage I-III FEC + HP→ T + HP versus FEC → T + HP versus TCHP | 0%-7%, 8%-14%,15%-32%, ≥33%, continuous variable (10% increase) | × | ✓ Event-free survival |
| NeoSphere[53] | 243 | Stage I-III TH versus TP versus THP versus HP | <5%, 6%-49% or ≥50%, continuous variable | × | N/A |
| TRAIN2[54] | 389 | Stage II-III FEC - HP → TCHP versus TCHP | ≤14, >14 OR ≤10, 11-59, ≥60 (%) | × | ✓ Invasive disease-free survival |

Green: positive association, red: negative association, gray: not studied/not applicable.
AC→T, doxorubicin, cyclophosphamide followed by paclitaxel; cape, capecitabine; EC→T, epirubicin + cyclophosphamide → docetaxel; ED, epirubicin and docetaxel; ER, estrogen receptor; FEC, fluorouracil, epirubicin, cyclophosphamide; H, trastuzumab; HER2, human epidermal growth factor receptor 2; HL, trastuzumab + lapatinib; HR, hormone receptor; L, lapatinib; pCR, pathologic complete response; PR, progesterone receptor; sTIL, stromal tumor-infiltrating lymphocyte; TCHP, docetaxel, carboplatin, trastuzumab, pertuzumab; T-DM1, trastuzumab emtansine; V or D, docetaxel or vinorelbine.

GeparSixto was a study that assessed 595 patients with operable breast cancer treated with paclitaxel and liposomal doxorubicin with or without carboplatin. The study included 273 patients with HER2-positive breast cancer who also received H and L in the neoadjuvant setting with the remainder TNBC.[35] In the intention-to-treat population, increased levels of sTILs predicted pCR ($P < 0.001$)[36] The pCR rate was 59.9% in LPBC and 33.8% in non-LPBC ($P \leq 0.001$). In the HER2-positive cohort, 53 (19.9%) of the tumors had LPBC phenotype. Notably, the effect size measured as OR was in a similar range for LPBC (OR 2.92) and HR status (2.78), suggesting that sTILs could be an additional biomarker in this patient population.

A meta-analysis of 29 neoadjuvant trials included all breast cancer subtypes.[37] When assessing the studies that included patients with HER2-positive breast cancer, increased levels of sTILs were associated with increased response rates to neoadjuvant therapy [OR 2.54, 95% confidence interval (CI) 1.50-4.29]. A threshold of 20% sTILs was associated with higher rates of pCR in HER2-positive disease ($P = 0.035$). Moreover, sTILs were associated with a benefit in survival in HER2-positive breast cancer, and with each 10% increase in sTILs the overall survival (OS) improved (pooled hazard ratio 0.93, 95% CI 0.87-0.99, $P = 0.01$).

### *Adjuvant studies*

Multiple studies have assessed the role of sTILs for patients with early-stage breast cancer receiving adjuvant therapy (summarized in Table 2). NSABP-B31 was a phase III study that compared four cycles of AC → T versus the same regimen with a year of H in 2043 patients with early breast cancer.[38] N9831 was another phase III trial with a similar design.[38] However, it had a third arm in which H was started after the completion of the taxane therapy. This study included 1633 patients. Because of the similarities in the trial designs, the analysis was combined, and these studies showed that H improved DFS in patients with operable breast cancer.[38] sTIL analyses of these studies have been conducted. In NSABP-B31, sTIL as a semicontinuous variable or as a categorical variable (>50%) was associated with improved invasive DFS (IDFS) (hazard ratio 0.42, 95% CI 0.27-0.64, $P < 0.001$ and hazard ratio 0.65, 95% CI 0.49-0.86, $P = 0.003$).[39] In N9831, there was a positive association between sTILs and RFS in patients treated with AC→T, and the test for interaction between H and sTIL was significant ($P = 0.03$).[40]

A patient-level meta-analysis that included 4097 patients from five trials [NSABP-B31, N9831, FinHER (FEC with either docetaxel or vinorelbine ± H[41]), HERA (1 versus 2 years of H after locoregional therapy and at least four cycles of chemotherapy[42]), and PACS-04 (adjuvant epirubicin + docetaxel + H in HER2-positive[43])] was conducted by the Early Breast Cancer Trialists' Collaborative Group, partnering with the IIOBWG.[44] The trials assessed the role of adding H to chemotherapy in the adjuvant setting, except for HERA. The median percentage of sTILs was 13% [interquartile range (IQR) 5%-30%]. Patients with higher sTILs had a lower risk for recurrence (adjusted hazard ratio per 10% sTIL increase 0.87, 95% CI 0.84-0.90, $P < 0.0001$). The 10-year recurrence rates decreased from 30% in those with

**Table 2. Selected adjuvant studies assessing the role of TILs in early HER2-positive breast cancer**

| Trial(s) | Sample size in sTIL analysis (HER2-positive) | Eligibility and regimen | sTIL cut-off | Positive association with long-term outcomes |
|---|---|---|---|---|
| BIG 02-98 [a,79] | 297 | Node + A-CMF, AC-CMF, A-T-CMF, AT-CMF[a] | Continuous variable (10% increase), or ≥50% LPBC | ✓ Increase in TILs was associated with decreased risk of recurrence and decreased risk of death |
| NSABP B-31[39] | 1581 | Node + or >2 cm if ER+/PR+ or >1 cm if ER-/PR-AC→T ± H | Semicontinuous variable, or ≥50% LPBC | ✓ Disease-free survival |
| FinHER[24] | 209 | Node + or ≥2 cm and PR− FEC + V or D ± H | Continuous variable (10% increase) | ✓ Increase in TILs was associated with decreased risk of recurrence |
| ShortHER[51] | 866 | Node+ or node− with one of the following: ≥2 cm tumor, G3, LVI, <35 years of age, ER/PR <10% EC → T + H to complete a year versus docetaxel + H 9 weeks → FEC 9 weeks | Continuous variable (1% increase) or ≥20% | ✓ Distant disease-free survival |
| N9831[40] | 945 | AC→T ± H (Arm A = no H, arm C = H) | Continuous variable (10% increase) or ≥60% | ✓ Recurrence-free survival in arm A |
| APHINITY[56] | 4313 | Stage I-III Chemotherapy + H ± P | Continuous variable and by quartiles | ✓[b] |

Green: positive association, red: negative association, gray: not studied/not applicable.
AC→T, doxorubicin, cyclophosphamide followed by paclitaxel; cape, capecitabine; CMF, cyclophosphamide, methrotexate, 5-fluorouracil; EC→T, epirubicin + cyclophosphamide → docetaxel; ED, epirubicin and docetaxel; ER, estrogen receptor; FEC, fluorouracil, epirubicin, cyclophosphamide; H, trastuzumab; HER2, human epidermal growth factor receptor 2; HL, trastuzumab + lapatinib; HR, hormone receptor; L, lapatinib; LPBC, lymphocyte-predominant breast cancer; LVI, lymphovascular invasion; pCR, pathologic complete response; PR, progesterone receptor; sTIL, stromal tumor-infiltrating lymphocyte; V or D, docetaxel or vinorelbine.
[a]Trial designed before the routine use of trastuzumab, the interaction between increasing sTIL and benefit with anthracycline-only chemotherapy ($P = 0.018$).
[b]'Better outcomes', details unavailable.

sTILs <10% to 15% in those with sTILs of 70% or higher. Also, there was no evidence of a natural cut-off value and there was no group identified with a low risk of recurrence. However, <10% of tumors had sTILs >50%. In this meta-analysis, higher sTILs were associated with lower recurrence rates; however, there was no interaction with trastuzumab. In other words, there was a significant benefit of trastuzumab irrespective of TIL status, although there were few patients with very high TIL levels.

In the APT trial (single-arm, phase II study in which patients with tumors <3 cm and node-negative received adjuvant paclitaxel and H), immune profiling has been assessed.[45] The median sTIL was 5% and 65% of tumors had low (<10%), 28% intermediate (11%-60%), and 7% high (>60%) sTILs. High sTILs were seen with greater frequency in HR-negative cases than in HR-positive cases (11% versus 5%). Given the low event rates in this study, the association between immune markers and long-term outcomes could not be assessed.

A combined PAMELA (LH and endocrine therapy) and CALBG 40601 (paclitaxel + H, L, or HL) neoadjuvant trials analysis assessed immune-related gene expressions and pCR and long-term outcomes.[46,47] sTILs (as a continuous variable) were significantly associated with pCR (OR 1.01, 95% CI 1.01-1.02, $P = 0.02$). sTILs were not associated with event-free survival (EFS). In contrast, several immune signatures were assessed and correlated with pCR and EFS. NeoALTTO assessed paclitaxel + H, L, or the combination followed by surgery then adjuvant FEC.[48] Patients with tumors with sTILs of at least 5% had higher pCR rates, independent of treatment arm (OR 2.6, 95% CI 1.26-5.39, $P = 0.01$).[49] Additionally, every 1% increase in TILs was associated with a 3% decrease in recurrence events (hazard ratio 0.97, 95% CI 0.95-0.99, $P = 0.002$).

ShortHER was a randomized, prospective study in which 1254 patients were randomized to 9 weeks versus 1 year of H in combination with anthracycline and taxane-containing chemotherapy. The study did not meet its non-inferiority endpoint for the 9-week regimen.[50] A sTIL analysis included 866 patients and revealed that sTILs were significantly associated with distant disease-free survival (DDFS), using TILs as continuous or categorical variables.[51] With a cut-off of 20% TIL, the 10-year DDFS was 89.8% for those with ≥20% TILs and 85% for those with <20% (hazard ratio 0.49, 95% CI 0.28-0.86, $P = 0.012$).[51] There was a numerical improvement in OS for those with higher sTILS, which was not statistically significant.[51] In this study, tumors with higher sTIL benefitted from a shorter duration of H, which is consistent with the findings from the FinHER trial.[24,51] This suggests that similar outcomes may be achieved with shortened treatment for patients with higher sTILs as a potential opportunity for treatment de-escalation.

Neoadjuvant studies that include dual HER2-directed monoclonal antibodies, the current standard of care, revealed no association between TILs and pCR rates. TRYPHENA was a study in which 225 patients with operable, locally advanced, or inflammatory breast cancer were randomized to receive concurrent anthracycline and taxane-containing therapy with HP, sequential anthracycline and taxane-containing therapy with HP or docetaxel, carboplatin + HP.[52] In contrast to the previously mentioned studies with H or L, in TRYPHENA sTILs had no association with pCR rates ($P = 0.17$). However, sTILs correlated with EFS, at a median follow-up of 4.7 years, and for each 10% increase in sTILs there was a 25% reduction in the hazard ratio for EFS ($P = 0.5$), after adjusting for clinicopathological features and pCR rates. In NeoSphere, patients were randomized to four groups. They received neoadjuvant therapy (TH, THP, HP, TP).[53] sTILs as a continuous or categorical variable was not associated with pCR rates, similar to what was reported in TRYPHENA. TRAIN2 compared FEC-HP → TCHP versus TCHP and again showed no association between pCR and TILs.[54,55] Patients with tumors with sTILs >60% had a 3-year IDFS of 100%, irrespective of nodal status, HR status, or pCR. APHINITY was a phase III study in which 4805 patients were randomized to receive adjuvant chemotherapy + H ± P or placebo.[56] sTIL analysis was carried out in 4313 participants, and this analysis showed that TILs as a continuous variable was associated with a favorable prognosis ($P = 0.001$) and those with the highest quartile of sTILs derived a greater benefit with pertuzumab ($P = 0.04$). The reason why the use of dual HER2-directed monoclonal antibodies with HP showed no association between TILs and pCR remains elusive.

### *Potential use of sTIL in early HER2-positive breast cancer—the role of sTILs in risk stratification*

Several biomarkers have been studied to determine which patients are more likely to achieve a pCR to tailor their initial treatment. It is possible that, ultimately, a combination of these biomarkers will improve risk stratification, treatment, and patient outcomes.

In terms of the prognostic value of sTILs, the evidence so far suggests that sTILs are for HER2-positive disease, what histological grade is for luminal disease, and may be considered to be used in a similar manner. The histological grade is never used alone to determine the prognosis for an individual patient, but together with other clinicopathological variables (such as HR status and lymphovascular invasion), grade provides an additional piece of information.

Higher TILs are associated with higher rates of pCR for patients treated with HER2-directed therapies other than pertuzumab, which is a significant limitation as this antibody is part of the current standard neoadjuvant regimens.[54,55] However, there is an EFS benefit when pertuzumab is part of the regimen given to patients with high sTILs, despite not being associated with pCR. Notably, in most of these studies, residual cancer burden (RCB) was unknown, and RCB status may affect any outcome assessment when using biomarkers.[57] These findings question the notion of whether pCR is a good surrogate endpoint for biomarker incorporation in early HER2-positive breast cancer.

There is robust evidence of the use of sTILs as a prognostic biomarker in this setting; it is a pragmatic and widely available biomarker that does not require special stains, which makes it particularly attractive. Adding TILs in pathology reports may provide clinicians with useful prognostic information for their patients. However, more studies are needed on how to combine it with other prognostic variables, which may include clinicopathological characteristics and/or other novel biomarkers, to better risk stratify patients.

Other assays, such as HER2DX, an expression profile panel, have shown promise.[58,59] HER2DX includes 27 genes that form four signatures (immunoglobin/B plasma cells, proliferation, luminal differentiation, HER2 expression) and provide a risk and a pCR score.[58] Notably, this assay includes a 14-gene immune signature that focuses more on immunoglobulins and B/plasma cells and has a limited focus on the entire lymphocytic infiltrate. Therefore, using HER2DX in combination with sTIL assessment could provide additional information on the immune infiltrate and may provide a more robust assessment than either alone. For example, if a patient's tumor has very low or very high TILs, the added value of a molecular assay may be limited. In contrast, for those with intermediate sTIL scores, genomic assays such as the HER2DX may indeed provide complementary information. Limitations of HER2DX include the cost of the assay, which has been seen in other genomic assays in breast oncology, and the availability of the assay globally based on cost and regulatory limitations.[60] A more comprehensive and integral use of molecular assays such as HER2DX with morphology may potentially allow a more cost-effective approach to the incorporation of these molecular assays in daily practice. Ongoing prospective studies are incorporating HER2DX for additional validation that may result in the clinical implementation of this test (NCT05912062, NCT06446882).

Assessing the breast cancer intrinsic subtypes using gene expression profiling has shown to be prognostic in HER2-positive early breast cancer.[61] A pooled analysis of the CALBG 40601, Neo-ALTTO, and NSABP-B41 trials revealed that the majority of early HER2-positive breast cancers were HER2-enriched (57.9%), followed by luminal B (15.0%), luminal A (9.9%), basal-like (8.8%), and normal-like (8.3%).[62] Notably, the distribution differed by HR status. In the HR-negative group, 77.9% of the tumors were HER2-enriched, and there were no luminal A or B tumors.[62] In the HR-positive group, 44.7% of the tumors were HER2-enriched, 18.4% luminal A, and 27.7% luminal B.[62] A subsequent analysis of these studies revealed a higher proportion of TILs in HER2-enriched and basal-like (median 30, IQR 15-60) than in luminal A or B tumors (median 20, IQR 10-30) ($P < 0.001$).[47] There were differences in outcomes by subtypes; pCR was significantly associated with EFS in patients with HER2-enriched and basal-like tumors but not in those with luminal A or B disease.[62] These findings suggest that TILs could be used in combination with the tumor-intrinsic subtypes to provide additional granularity. Moreover, in settings of limited resources, TILs could be used in combination with tumor size, nodal status, and HR status to optimize patient treatments. For example, a potential application for TILs is to guide escalation or de-escalation of treatment when used in combination with other clinicopathological features, such as tumor size, nodal status, and HR status.

A biomarker analysis of the NeoALTTO and CALBG 40601 trials assessed other immune markers, which included RNA sequencing and the assessment of B- and T-cell receptor repertoires.[63] A model that incorporated the B- and T-cell receptor repertoires, pCR, clinicopathological information, and a set of gene signatures was successful in predicting EFS. HER2-EveNT, a prognostic score derived from this analysis, can be used to identify patients with immune-enriched tumors that do not exhibit a pCR and yet have an excellent EFS (5-year EFS >90%).[63] Models like this are promising as they consider multiple aspects of tumor biology. However, they are difficult to implement in routine clinical practice due to cost and the need for special testing. To date, none of these biomarkers have been used for clinical decision making, and ongoing studies will likely help determine how and whether these interesting biomarkers will fit into clinical practice.

Another critical clinical question concerns adjuvant therapy for those who exhibit a pCR. Clinicians often continue H based on initial clinical staging. However, it is possible that biomarkers such as baseline sTILs can help determine the optimal duration of H in the post-neoadjuvant setting for patients who exhibit a pCR. Conversely, for patients with RD after neoadjuvant therapy, there is a need to determine which patients may benefit from additional therapy to trastuzumab emtansine (T-DM1). Small-molecule tyrosine inhibitors and novel antibody drug conjugates are being studied and perhaps to determine which patients could forgo T-DM1. In those with RD, the combination of sTILs and RCB may provide additional prognostic information to guide decision making.[64] Collecting data about biomarkers for this higher-risk patient population is imperative to continue to optimize their treatment.

Finally, studies have suggested a different effect of TILs for those with HER2-positive disease, depending on their HR status. More research is needed to determine how this biomarker may be influenced by HR expression and what the clinical implications may be.

## TILS IN HER2-POSITIVE METASTATIC BREAST CANCER

Secondary TIL analyses have also been carried out in metastatic studies (summarized in Table 3). In CLEOPATRA the role of docetaxel plus trastuzumab (TH) with or without P was assessed in patients with metastatic HER2-positive breast cancer and led to the approval of THP, which is the current first-line standard of care.[11] Based on the TIL analysis, there was no statistical association between sTILs and progression-free survival (PFS).[65] However, each 10% increase in sTILs was statistically associated with prolonged OS (hazard ratio 89, 95% CI 0.83-0.96, $P = 0.0014$). The

**Table 3. Selected studies assessing the role of TILs in metastatic HER2-positive breast cancer**

| Trial | Sample size in TIL analysis | Regimen | TIL cut-off | Association with PFS | Association with OS |
|---|---|---|---|---|---|
| CLEOPATRA[65] | 678 | TH ± P | Continuous variable (10% increase) | × | ✓ |
| MA.31[66] | 614 | T + H versus L | 5% | ✓[a] | N/A |
| PANACEA[69] | 50 | H + pembrolizumab | Continuous variable (1% increase) | ✓ | N/A |
| KATE2[80] | 190 | T-DM1 ±atezolizumab | 5% | ✓ | N/A |

Green: positive association, red: negative association, gray: not studied/not applicable.
H, trastuzumab; HL, trastuzumab + lapatinib; OS, overall survival; P, pertuzumab; PFS, progression-free survival; sTIL, stromal tumor-infiltrating lymphocyte; T-DM1, trastuzumab emtansine; TH, trastuzumab + docetaxel.
[a]Only for tumors with low CD8+ sTILs.

treatment effect of pertuzumab did not differ by sTILs for PFS or OS.

In the Canadian Cancer Trials Group MA.31, patients with metastatic disease received a taxane with H or L.[66] A sTIL percentage >5 was present in 35% of cases, but it did not show prognostic effects on PFS.[66] Of note, tumors with low CD8+ sTILs had a higher risk for progression with lapatinib compared with trastuzumab (hazard ratio 1.36, 95% CI 1.40-6.17, $P = 0.02$), and there was no difference for those with high CD8+ sTILs.

ICIs have changed the treatment paradigm of multiple malignancies, including TNBC.[67,68] These agents have been studied in HER2-positive breast cancer in the PANACEA and KATE2 studies.[69,70] PANACEA was a single-arm study in which 52 patients with H-resistant breast cancer received the program cell death protein 1 inhibitor pembrolizumab with H.[69] The objective response rate was 15%. Forty of the patients had programmed death-ligand 1 (PD-L1)-expressing tumors, and only patients in that group responded to therapy, and the combination of sTILs and PD-L1 appeared to be the best predictor of response to this combination. KATE2 studied the combination of T-DM1 with atezolizumab or placebo.[70] There was no difference in PFS; however, as was shown in PANACEA, patients with HER2-positive, PD-L1-positive tumors appeared to benefit from the combination, and all biomarkers of T-cell activation and quantity, including sTILs, were enriched in the PD-L1-positive subgroup.[71] In PANACEA, the median sTIL count was lower than what has been reported in early breast cancer studies, which is consistent with prior studies that have shown that primary breast tumors have higher TIL quantities than metastatic tumors across breast cancer subtypes.[69] PD-L1-positive tumors had higher sTILs than PD-L1-negative tumors. The site of metastatic disease biopsied also revealed different sTIL quantities, with lung and breast lesions having higher TILs and skin and liver, lower. Similar to TNBC, sTIL is a predictive biomarker of response to ICI in metastatic HER2-positive breast cancer in these limited studies. Therefore, several studies are ongoing to confirm these findings. If these studies confirm TILs' predictive value, incorporating them into HER2-positive ICI selection will be uniquely challenging, as selection must also account for HER2 status, PD-L1 expression, and TIL levels.

A retrospective analysis by Taurelli Salimbeni and colleagues included 80 patients with stage IV HER2-positive breast cancer.[72] A total of 72.5% of the patients had TILs >10% (high TIL). There was no association with PFS or OS and sTILs, possibly due to small sample size.

Further research is needed to determine how to incorporate sTILs and other immune biomarkers in clinical practice for the treatment of patients with advanced HER2-positive breast cancer. Hence, it is important to continue to educate and train pathologists for TILs assessment, using publicly available training schemes. Since it has been established that primary tumors have more immune markers than metastatic sites, it is important to determine how the timing and location of biopsies may impact patient outcomes.[73] For example, in the CLEOPATRA study, nearly 80% of the cases that were TIL-scored were assessed in the primary tumors, still showing a prognostic significance in the advanced and metastatic setting. Additionally, there is variation in the expression of immune markers by tumor sites, which must be considered when making clinical decisions. The TME and other immune markers change with cancer-directed therapies, which will have to be considered as well, especially when studying the use of ICIs and other therapies with immune mechanisms in HER2-positive breast cancer.

Additionally, other novel HER2-directed therapies that rely in the host immune system and antibody-dependent T-cell mediated cytotoxicity, such as vaccines, chimeric antigen receptor T cells (CAR-T), and bispecific antibodies (BiTES), are being developed. It is imperative to analyze sTILs and other immune markers in these settings in order to help identify which patients could benefit from these therapies.

## FUTURE OF TILS

In this review, we present the available data for using TILs as a prognostic biomarker in HER2-positive breast cancer. In Figure 2, we present a summary of possible future applications of sTILs in clinical practice. sTILs are an accessible and highly reproducible prognostic and predictive biomarker.

A concerted effort is being made to determine cut-offs for sTILs and to continue to train pathologists globally. It is also critical to determine how the TME and spatial characterization of the immune cells impact the patient's prognosis and response to therapy. More research is needed to determine whether combining genomic and morphologic tumor characteristics may increase this

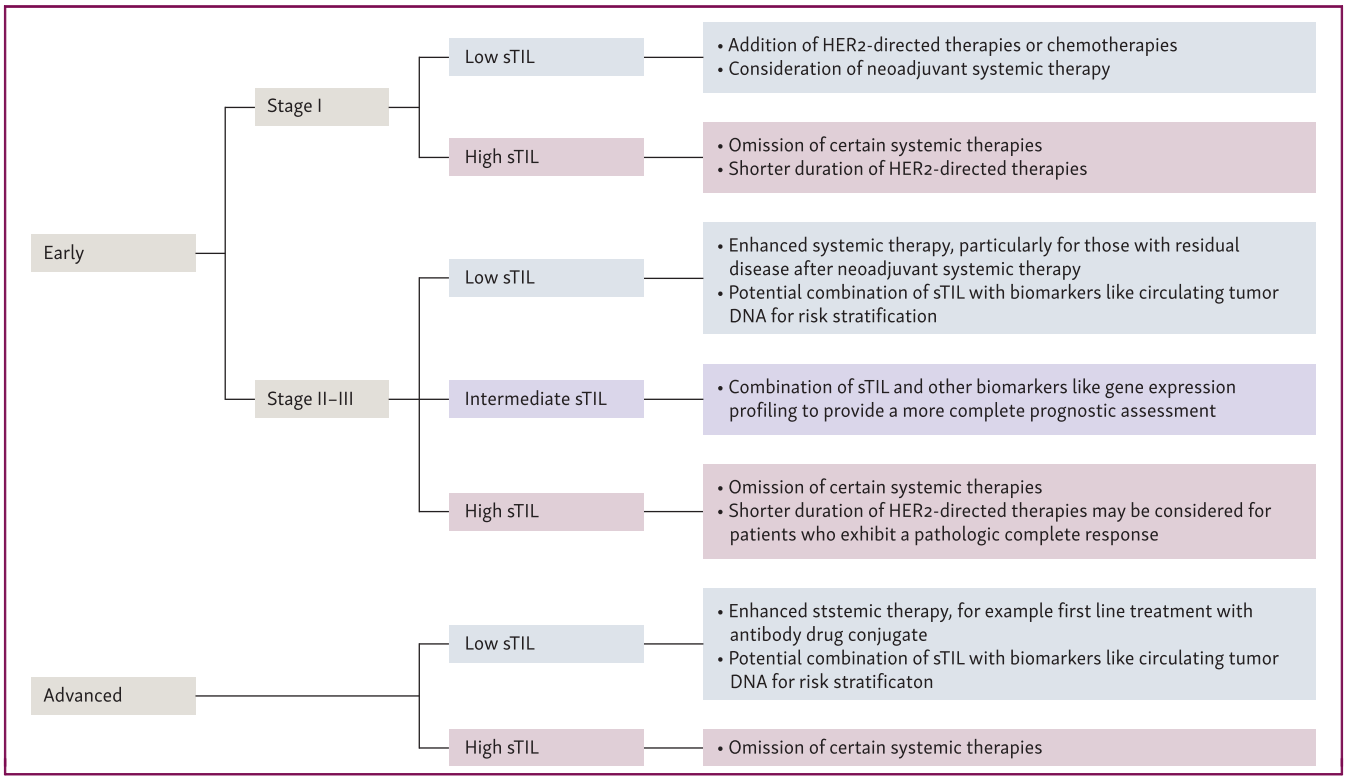


**Figure 2. Potential future applications of sTIL in clinical practice.** Created with BioRender.com.
sTIL, stromal tumor-infiltrating lymphocyte.

biomarker's prognostic and whether it has predictive value. Machine learning models (MLMs) may provide pathologists with an additional tool to improve the reproducibility of TIL testing.[74] Several models have been implemented with variable levels of success, and some are able to provide information about the spatial location of the TILs.[75] Limitations of implementing MLMs in patient care include cost, comparability between assays, and concerns about maintaining confidentiality, neither of which should be taken lightly.

Finally, more information about how this biomarker can be used with novel agents, such as antibody—drug conjugates, is critical for incorporating TIL assessment into clinical practice. In general, TILs can provide useful prognostic information to clinicians on their patients, hence integration in pathology reports may be considered. However, more studies are needed on how exactly this may impact treatment decisions.

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