

# Early Stage HER2-Positive Breast Cancers Not Achieving a pCR From Neoadjuvant Trastuzumab- or Pertuzumab-Based Regimens Have an Immunosuppressive Phenotype

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## Abstract

Approximately 30% to 40% of HER2-positive (HER2<sup>+</sup>) early-stage breast cancers do not completely respond to neoadjuvant anti-HER2 therapy. We investigated immune cell-associated markers in 30 early-stage HER2<sup>+</sup> breast cancer tissues treated with neoadjuvant TCH (docetaxel, carboplatin, and trastuzumab) with or without pertuzumab. We identified an immunosuppressive tumor immune microenvironment in cancers with residual disease. These hypothesis-generating results should be validated in a larger cohort.

**Background:** Stromal tumor-infiltrating lymphocytes (TILs) might predict pathologic complete response (pCR) in patients with HER2-positive (HER2<sup>+</sup>) breast cancer treated with trastuzumab (H). Docetaxel (T), carboplatin (C), H, and pertuzumab (P) have immune-modulating effects. Pre- and post-treatment immune biomarkers in cancers treated with neoadjuvant TCH with or without P are lacking. In this study we quantified baseline and changes in TILs, cluster of differentiation (CD) 4<sup>+</sup>, CD8<sup>+</sup>, FoxP3<sup>+</sup>, and PD-L1<sup>+</sup> cells using immunohistochemistry (IHC) and quantified productive T-cell receptor  $\beta$  (TCR $\beta$ ) rearrangements and TCR $\beta$  clonality using next-generation sequencing (NGS) in 30 HER2<sup>+</sup> breast cancer tissues treated with neoadjuvant H with or without P regimens. **Materials and Methods:** Thirty pre- and post-neoadjuvant TCH (n = 4) or TCHP (n = 26) breast cancer tissues were identified. TILs were quantified manually using hematoxylin and eosin. CD4, CD8, FoxP3, and PD-L1 were stained using IHC. TCR $\beta$  was evaluated using NGS. Immune infiltrates were compared between pCR and non-pCR groups using the Wilcoxon rank sum test. **Results:** A pCR occurred in 15 (n = 15; 50%) cancers (TCH n = 2; TCHP, n = 13). Pretreatment TILs, CD4<sup>+</sup>, CD8<sup>+</sup>, FoxP3<sup>+</sup>, and PD-L1<sup>+</sup> cells were not associated with response ( $P = .42$ ,  $P = .55$ ,  $P = .19$ ,  $P = .66$ ,  $P = .87$ , respectively). Pretreatment productive TCR $\beta$  and TCR $\beta$  clonality did not predict response,  $P = .84$  and  $P = .40$ , respectively). However, post-treatment CD4<sup>+</sup> and FoxP3<sup>+</sup> cells (T-regulatory cells) were elevated in the non-pCR cohort ( $P = .042$  and  $P = .082$ , respectively). **Conclusion:** An increase in regulatory T cells in non-pCR tissues suggests the development of an immunosuppressive phenotype. Further investigation in a larger cohort of samples is warranted to validate these findings.

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**Keywords:** ADCC, Antibody-dependent cellular cytotoxicity, FoxP3, Regulatory T cell, T-cell receptor, Tumor immune microenvironment

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# HER2<sup>+</sup> Immunosuppressive Phenotype

## Introduction

HER2 was first identified in 1984 as an oncogene activated by a point mutation in rat neuroblastomas.<sup>1</sup> HER2 proto-oncogene overexpression was later reported to occur in 15% to 30% of invasive breast cancers.<sup>2-4</sup> HER2-positive (HER2<sup>+</sup>) breast cancer is more aggressive and has higher rates of local and distant relapse.<sup>4</sup> The clinical use of trastuzumab has led to significant improvement in the outcomes of patients with early-stage and metastatic HER2<sup>+</sup> breast cancer.<sup>5-9</sup> The additional use of pertuzumab (P) with trastuzumab has been associated with improved survival in patients with HER2<sup>+</sup> breast cancer.<sup>10-12</sup> Mechanistic studies have shown that trastuzumab inhibits HER2 activation by blocking homodimerization with HER2 and P blocks heterodimerization with HER3. Additionally, both antibodies are thought to enhance antibody dependent cellular cytotoxicity (ADCC).<sup>13,14</sup>

Trastuzumab improved pathologic complete response (pCR) and 5-year outcomes when used in combination with chemotherapy in early-stage locally advanced HER2<sup>+</sup> breast cancers.<sup>10,15</sup> Neoadjuvant THP (docetaxel, trastuzumab, and P) further increased pCR rates in early-stage hormone receptor (HR)-negative, HER2<sup>+</sup> breast cancer patients.<sup>16,17</sup> Additionally, the combination of THP with carboplatin (TCHP) yielded pCR rates that were numerically higher, but statistically similar to anthracycline-based regimens using HP (trastuzumab with P).<sup>18</sup> Subsequently, P was approved in combination with trastuzumab for HER2<sup>+</sup> early-stage breast cancer patients. TCHP has become a preferred neoadjuvant regimen in many practices because anthracyclines can be avoided without loss of efficacy. However, 30% to 40% of early-stage HER2<sup>+</sup> patients receiving TCHP will have residual disease at the time of surgery, which portends a poor prognosis. Biomarkers determining who will or will not completely respond to neoadjuvant TCHP are lacking.

Several studies have investigated surrogate markers to identify which early-stage patients with HER2<sup>+</sup> disease might achieve a pCR from TCH (docetaxel, carboplatin, and trastuzumab) or TCHP regimens. An area gaining much interest involves the investigation of tumor-infiltrating lymphocytes (TILs) as a predictive biomarker to chemotherapy response in breast cancer.<sup>19-21</sup> TILs appear to be a biomarker in several breast cancer settings despite a lack of tumor immune microenvironment phenotype specificity. Baseline TILs have been studied in early-stage HER2<sup>+</sup> breast cancer tissues as a predictive biomarker of response from neoadjuvant HP regimens.<sup>20,22-26</sup> Increased regulatory T cells have been associated with lower pCR rates in HER2<sup>+</sup> breast cancers and high levels of cluster of differentiation (CD) 8<sup>+</sup> T-cell exhaustion are associated with shortened disease-free survival in ER<sup>+</sup>/HER2<sup>+</sup> tumors.<sup>27</sup> Additionally, some evidence suggests next-generation sequencing of the T-cell receptor (TCR)- $\beta$  (TCR $\beta$ ) might correlate with TILs in early-stage breast cancer tissues from patients receiving immunotherapy.<sup>28</sup> However, there is debate whether TILs or TCR $\beta$  are predictive or prognostic of TCH or TCHP response in HER2<sup>+</sup> early-stage breast cancers.

The purpose of this study was to evaluate the tumor immune microenvironment and whether this is predictive or prognostic of residual disease after neoadjuvant treatment with TCH or TCHP.

## Materials and Methods

### Tumor Specimen Analysis

We identified 51 HER2<sup>+</sup> early-stage breast cancer patients treated at an academic medical center with neoadjuvant TCH with or without P.<sup>29</sup> After obtaining institutional review board approval, pharmacy records from the electronic health care record system were used to identify breast cancer patients who had received trastuzumab and/or P-based regimens from July 1, 2013 to November 1, 2015. Chart review was used to identify patients who received neoadjuvant TCH with or without P and to obtain demographic and clinical characteristics including age at diagnosis, initial hormone receptor and HER2 status, pretreatment clinical stage, number of cycles of therapy received, and postsurgical pathologic response and staging.

Using American Society of Clinical Oncology/College of American Pathologists guidelines, we defined HER2 positivity if the tissue was HER2<sup>+</sup> according to immunohistochemistry (IHC) of 3+, HER2/CEP17 ratio  $\geq 2$ , or if the HER2/CEP17 ratio was  $< 2$  with HER2 copy number  $\geq 6$  according to fluorescence in situ hybridization analysis.<sup>30</sup> Hormone receptor status was determined using the Dako ER/PR pharmDx kit (Agilent Technologies, Santa Clara, CA) and scored using the Allred system: a proportion score (PS) is given to represent the proportion of positive-staining cells (range, 0-5). An intensity score (IS) is given to represent the estimated average staining intensity of positive cells (range, 0-3). A total score (TS) is obtained from the sum of the PS and IS (range, 0-8). A positive result is determined to be a TS of  $\geq 3$ .<sup>31</sup>

Specific immune markers were chosen in large part because we wished to identify stromal TILs and immunosuppressive phenotypes including regulatory T cells (CD4<sup>+</sup>, FoxP3<sup>+</sup>), checkpoint upregulated cells (PD-L1<sup>+</sup>), and CD8<sup>+</sup> cells. If CD8<sup>+</sup> cells were associated with residual disease then we could consider investigating for CD8<sup>+</sup> T-cell exhaustion. Additionally, because of the relevance of stromal TILs in HER2<sup>+</sup> breast cancer, we chose to determine T-cell receptor changes in our study.

Thirty of the 51 patients (59%) had pre- as well as post-treatment formalin-fixed paraffin-embedded (FFPE) tissue available. Full-faced tumor sections were used for analyses. For patients with HER2<sup>+</sup> disease who fully achieved a pCR, stromal tissue adjacent to the clip was analyzed. The Histowiz IHC platform was used to identify pre- and post-treatment changes in CD4 (4B12; Leica Biosystems, Buffalo Grove, IL), CD8 (4B11; Leica Biosystems), FoxP3 (SP97, Abcam, Cambridge, MA), PD-L1 (E1L3N XP; Cell Signaling Technology, Danvers, MA), and TILs. IHC was performed using a Bond Rx autostainer (Leica Biosystems) with heat-mediated antigen retrieval (Bond ER2) using standardized protocols. Bond Polymer Refine kit (Leica Biosystems) was used for detection. The sections were dehydrated and film coverslipped using TissueTek-Prisma and Coverslipper (Sakura). Aperio AT2 (Leica Biosystems) was used for whole-slide scanning at 40 $\times$ . The images were quantified using Halo image analysis software (Indica Labs, Corrales, NM) using the CytoNuclear module and quantified using optical density units (ODs).

Lymphocyte predominant breast cancer was defined as having stromal TILs  $\geq 50\%$  within the pretreatment tumor specimen.

**Table 1** Patient Characteristics and Responses

Patient Characteristic	n (%)	Receptor Status	pCR, n (%)	No pCR, n (%)
Median Age	50	Total	15 (50)	15 (50)
TCH	4 (14)	ER <sup>+</sup> /PR <sup>+</sup>	2 (14)	7 (50)
TCHP	26 (86)	ER <sup>+</sup> /PR <sup>-</sup>	3 (22)	2 (14)
HR <sup>+</sup>	14 (47)	ER <sup>-</sup> /PR <sup>-</sup>	10 (63)	6 (37)
HER2 3+ only	18 (60)	HER2 3+ only	10 (56)	8 (44)
HER2 2+ and FISH Amplification	6 (20)	HER2 2+ and FISH amplification	1 (17)	5 (83)
HER2 3+ and FISH Amplification	6 (20)	HER2 3+ and FISH amplification	4 (67)	2 (33)

All tumor samples were initially tested for HER2 expression using immunohistochemistry.

Abbreviations: FISH = fluorescent in situ hybridization; HR = hormone receptor; pCR = pathologic complete response.

Pathological complete response was defined as no residual invasive tumor in the breast or axilla, ypT0N0M0 or ypTisN0M0.

DNA extraction of FFPE breast cancer tissue was performed following the DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA). For each sample, genomic DNA was extracted from T cells and then TCR $\beta$  CDR3 regions were amplified and sequenced using ImmunoSEQ (Adaptive Biotechnologies, Seattle, WA).<sup>32</sup> Sequencing was performed using an Illumina HiSeq system (Illumina, San Diego, CA). Multiplexed primers targeting all variable (V) and joining (J) gene segments were used to amplify rearranged V(diversity)J segments for high-throughput sequencing. Synthetic templates mimicking natural V(diversity)J rearrangements were used to measure and correct amplification bias in the multiplex polymerase chain reaction (PCR).<sup>33</sup> After correcting primary sequence errors via a clustering algorithm, CDR3 segments were annotated according to the International ImMunoGeneTics collaboration, identifying which V, D, and J genes contributed to each rearrangement, and classifying them as nonproductive if nontemplated insertions or deletions produced frame shifts or premature stop codons.<sup>34,35</sup> Within each sample, the frequency distribution of productive TCRs was examined and the clonality score calculated as  $(1 - \text{Pielou evenness})$ , with a clonality score of 0 indicating a perfectly diverse repertoire and a clonality score of 1 indicating a monoclonal repertoire.

### Statistical Methods

Baseline immune microenvironment phenotypes were candidate predictors of pCR in univariate logistic regression. Changes in the immune microenvironment from pretreatment tissue samples to post-treatment tissue samples were compared with Wilcoxon signed rank tests. Differences in the central tendency of the immune microenvironment phenotype between response outcomes were compared using Wilcoxon rank sum tests. Pretreatment TCR $\beta$  were

correlated with TILs using Spearman correlation coefficients and evaluated as a predictive biomarker for response using logistic regression.

## Results

Patient characteristics and treatment response data for the 30 patients who had pre- and post-treatment samples available were similar between cohorts (Table 1). The median age of the early-stage breast cancer patients diagnosed with HER2<sup>+</sup> disease in our study was 50 (range, 27-75) years. All tumor samples were initially tested for HER2 expression using IHC. Twenty-four (80%) cancers were identified to be HER2 IHC 3+ and 6 (20%) were determined to be IHC 2+. HER2/chromosome 17 (CEP17) ratios showed HER2 positivity in 6 of the HER2<sup>+</sup> IHC 3+ cancers (range, 7.16-14.06) and all of the IHC 2+ cancers (range, 2.13-8.5). Eighteen HER2 IHC 3+ tumors were not tested for HER2 amplification. Hormone receptors were positive in 14 (47%) cancers. Four patients (14%) received TCH and 26 patients (86%) received TCHP. PCR was achieved in 15 cancers (50%), of which 2 patients (13%) received TCH and 13 (87%) received TCHP. Most patients completed 6 cycles of neoadjuvant TCH with or without P, however, 3 patients (12%) did not complete the entire 6 cycles because of chemotherapy-related adverse events (Table 2). There were no differences in pCR in patients who did not fully complete all 6 cycles of TCH with or without P. Rates of pCR were lower among patients with HR<sup>+</sup>/HER2<sup>+</sup> cancers with 5 (17%) achieving a pCR and 9 (30%) with residual invasive disease (non-pCR). Of the 15 tissue samples with a pCR, 14 (93%) were HER2 IHC 3+ and 1 (7%) was IHC 2+ with a HER2/CEP17 ratio of 2.13. From the 15 cancer samples in the non-pCR group, 10 (67%) were HER2 IHC 3+ and 5 (33%) were HER2 IHC 2+ with HER2/CEP17 ratios showing HER2 amplification (range, 2.23-13.35). Three of the 5

**Table 2** Treatment Regimens With Associated Responses

Treatment Regimen	n (%)	pCR, n (%)	No pCR, n (%)
TCH for 6 Cycles	4 (13)	2 (13)	2 (13)
TCH for 6 Cycles	23 (77)	12 (80)	11 (73)
TCHP for 5 Cycles	1 (3)	—	1 (7)
TCHP for 1 Cycle → THP for 4 Cycles <sup>a</sup>	1 (3)	—	1 (7)
TCHP for 1 Cycles → THP for 2 Cycles → Hp for 3 Cycles	1 (3)	1 (7)	—

Abbreviations: pCR = pathologic complete response; TCHP = docetaxel, carboplatin, trastuzumab, and pertuzumab.

<sup>a</sup>Chemotherapy stopped because of chemotherapy-induced toxicity.

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HER2 IHC 2+ samples were HR<sup>+</sup>; the HER2/CEP17 ratios for these 3 samples were 2.96, 4.37, and 8.5.

### Immunohistochemical Measurement of Immune Cells in Early Stage HER2<sup>+</sup> Breast Cancer Specimens

Lymphocyte-predominant breast cancer was observed in 4 HER2<sup>+</sup> tissue samples with a pCR and 1 with residual disease after receiving neoadjuvant treatment. Pretreatment TILs were non-normally distributed and the central tendency of the median pretreatment TILs (pCR = 20; range 1-80; non-pCR = 10; range 5-70) was not associated with response ( $P = .42$ ; Figure 1). There were no significant associations between pretreatment CD4<sup>+</sup>, CD8<sup>+</sup>, FoxP3<sup>+</sup>, and PD-L1<sup>+</sup> cells and response to neoadjuvant TCH with or without P ( $P = .55$ ,  $P = .19$ ,  $P = .66$ ,  $P = .87$ , respectively).

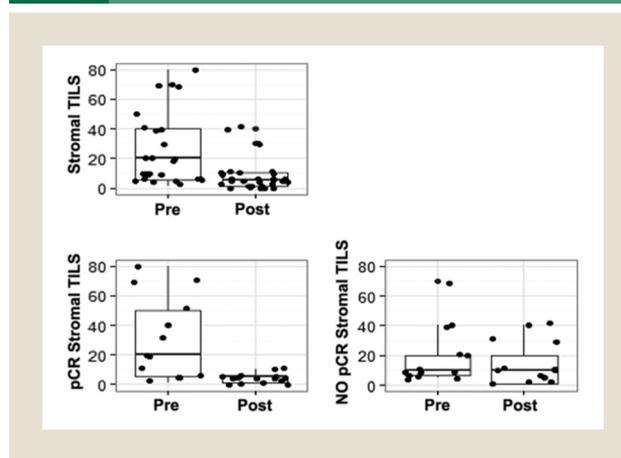
The levels of CD4<sup>+</sup> cells were significantly elevated ( $P = .042$ ) and there was a trend toward elevation of FoxP3 cells ( $P = .082$ ) in the post-treatment specimens with residual disease (Figure 2). CD4<sup>+</sup> and FoxP3<sup>+</sup> represent regulatory T cells. CD8 cells showed a trend toward being more elevated in the post-treatment tissue with residual disease ( $P = .082$ ). PD-L1 was not statistically different between post-treatment cohorts ( $P = .20$ ).

The absolute difference in TILs change between HER2<sup>+</sup> cancers with a pCR compared with residual disease was not statistically significant (pCR median change, -19%; non-pCR median change, -7%;  $P = .07$ ). There were no significant differences in the absolute changes in FoxP3 (pCR median change, 36,364 OD; non-pCR median change, 63,063 OD;  $P = .17$ ) and PD-L1 (pCR median change, 1566 OD; non-pCR median change, 8234 OD;  $P = .18$ ) in the residual disease cohort compared with the pCR group. There were significant increases in CD4<sup>+</sup> and CD8<sup>+</sup> cells detected in specimens with residual disease (pCR median change, -364 OD; non-pCR median change 47,636 OD;  $P = .03$ ; and pCR median change, 14,949 OD; non-pCR median change, 53,692 OD;  $P = .01$ , respectively).

No significant differences were observed between the median pretreatment TILs, CD4, CD8, FoxP3, or PD-L1 in the HR<sup>-</sup>/HER2<sup>+</sup> and HR<sup>+</sup>/HER2<sup>+</sup> cohorts. However, median TILs were increased in the post-treatment tissues with HR<sup>-</sup>/HER2<sup>+</sup> (5; range, 1-40) compared with HR<sup>+</sup>/HER2<sup>+</sup> (1; range, 1-40;  $P = .05$ ) tissues (Figure 3A). Post-treatment HR<sup>-</sup>/HER2<sup>+</sup> tissues revealed increased median TILs and FoxP3 cells in the specimens with residual disease (5; range, 1-40; OD, 165,436; range, 45,357-348,979) compared with cancers achieving a pCR (5; range, 1-10;  $P = .05$ ; OD, 52,091; range, 11,267-298,464;  $P = .07$ ; Figure 3B). The pretreatment to post-treatment change in median TILs within HR<sup>-</sup>/HER2<sup>+</sup> tissues were significantly decreased in specimens with residual disease (5; range, -30 to -20) compared with cancer tissues attaining a pCR (-25; range, -65 to 0;  $P = .05$ ). We observed no differences in pretreatment, post-treatment, or change from pre- to post-treatment TILs, CD4, CD8, FoxP3, or PD-L1 between the non-pCR and pCR cohorts within the HR<sup>+</sup>/HER2<sup>+</sup> subtype.

There were 4 HER2<sup>+</sup> cancer tissues with a 40% increase in TILs in the residual disease cohort. Compared with pretreatment tissue specimens, these cancers had elevated CD4, FoxP3, CD8, and PD-L1 cells in their post-treatment tissue specimens (Figure 4A).

**Figure 1** Tumor-Infiltrating Lymphocytes Were Identified Using Hematoxylin and Eosin Analysis Before (Pre-) and After (Post-) TCHP Treatment of HER2<sup>+</sup> Early Stage Breast Cancer Patients Pretreatment TILs Did Not Associate With Response



Abbreviations: pCR = pathologic complete remission; TCHP = docetaxel carboplatin, trastuzumab, and pertuzumab; TILs = tumor infiltrating lymphocytes.

There were no significant changes in the immune phenotype from cancers with a 40% decrease in TILs (non-pCR group,  $n = 7$ ; pCR group,  $n = 10$ ; Figure 4B).

### T-Cell Receptor $\beta$ Sequencing Measurement of Immune Cells in Early Stage HER2<sup>+</sup> Breast Cancer Specimens

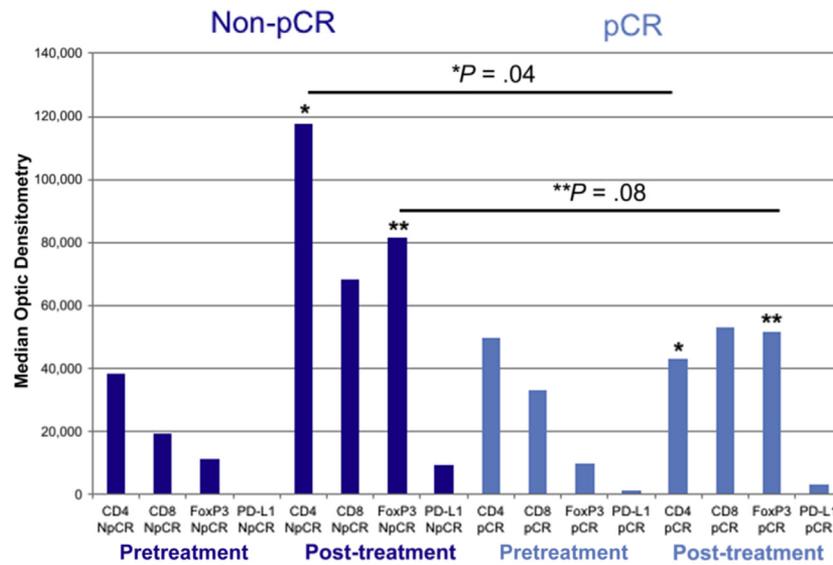
To further understand the adaptive immune milieu, we investigated productive TCR $\beta$  rearrangements and TCR $\beta$  clonality in pre- and post-treatment samples. The productive TCR $\beta$  rearrangements represent T cells with the potential to generate an immune response. TCR $\beta$  clonality ranges from 0 to 1, with 0 representing a polyclonal population and 1 representing a purely clonal population. Our pretreatment TILs correlated positively with pretreatment TCR $\beta$  clonality (correlation coefficient, 0.55;  $P = .003$ ).

Neither pretreatment nor post-treatment productive TCR $\beta$  rearrangements or TCR $\beta$  clonality predicted early-stage HER2<sup>+</sup> breast cancer's response to TCH with or without P ( $P = .84$  and  $P = .4$ , respectively; Figure 5). Changes in productive TCR $\beta$  rearrangements did not significantly vary between pCR and residual disease cohorts ( $P = .11$ ). Also, changes in TCR $\beta$  clonality did not differ in the pCR or residual disease groups ( $P = .32$ ). Last, no significant differences in TCR $\beta$  rearrangements or TCR $\beta$  clonality were associated with cancers having a 40% change in TILs (Figure 4). The sample size was too small to determine if clonal expansion occurred between pre- and post-treatment samples in the pCR or residual disease cohorts.

## Discussion

Our data suggest that early-stage HER2<sup>+</sup> breast cancers treated with neoadjuvant combination TCH with or without P demonstrate tumor immune microenvironment phenotype changes. This was an exploratory, hypothesis-generating study that furthers our understanding of tumor microenvironment immune modulation after treatment with trastuzumab and P in early-stage HER2<sup>+</sup> breast

**Figure 2** Analysis of T-Cell Subsets and PD-L1 Expression in Tumor Tissue. T Regulatory (CD4<sup>+</sup>/FoxP3<sup>+</sup>) Immunosuppressive Phenotype Was Observed in Tumor Tissue Taken From Patients With Early Stage HER2<sup>+</sup> Breast Cancer Treated With TCH With or Without Pertuzumab Who Did Not Fully Achieve a pCR



Abbreviations: CD = cluster of differentiation; NpCR = no pathologic complete response; pCR = pathologic complete response; TCH = doctaxel, carboplatin, and trastuzumab.

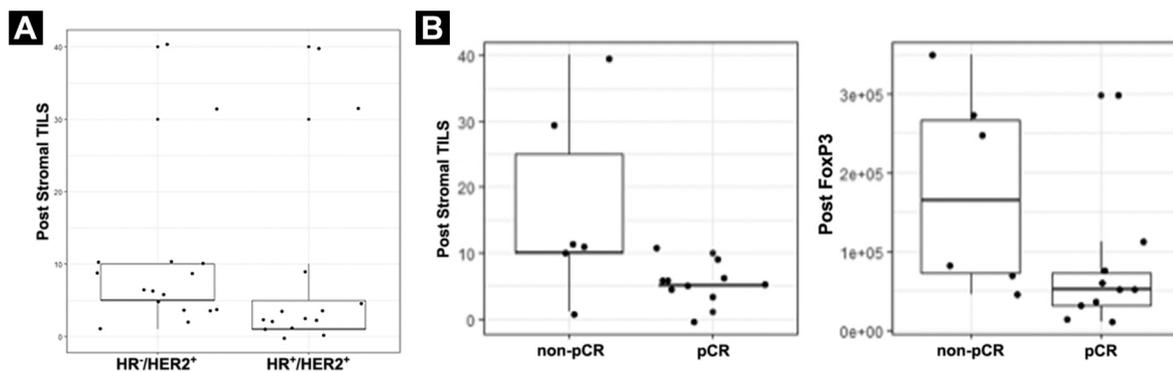
cancers with residual disease, however the study sample was small and needs to be confirmed in a larger cohort.

We sought to identify immune biomarkers in early-stage HER2<sup>+</sup> breast cancers with residual disease. We first investigated TILs as a predictive biomarker using standardized hematoxylin and eosin (H & E) methods developed by the TIL working group to assess TILs.<sup>21</sup> Identifying TILs using H & E is simple and might have clinical utility. However, although the H & E TIL score provides semiquantitative results, it does not recognize differences between

T-cell subsets. Therefore, we sought to better define immune cell subsets and determine if they could predict residual disease. Our study suggests that pretreatment TILs are not predictive of response in early-stage breast cancer patients with HER2<sup>+</sup> disease receiving neoadjuvant TCH with or without P.

An immune analysis from the NeoSphere study evaluated trastuzumab and P-induced treatment modulation of TILs in early-stage HER2<sup>+</sup> breast cancers with residual disease. This study elucidated the immune modulation of CD8, ICOS, 4-1BB,

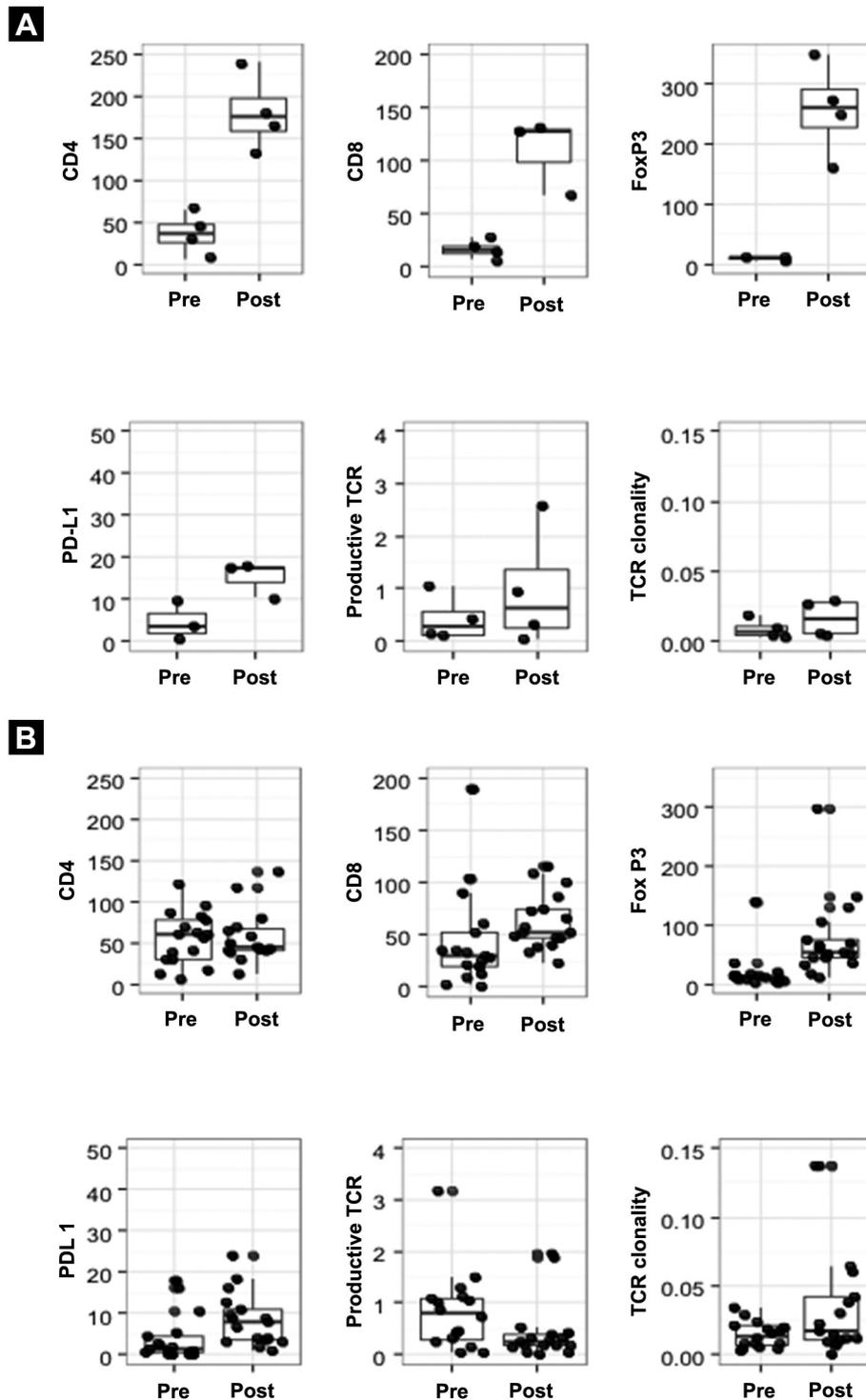
**Figure 3** Post-Treatment Immune Subset Analysis According to Receptor Status. Tumor Tissue Was Analyzed to Examine Stromal TILs Using Hematoxylin and Eosin and FoxP3 Using Immunohistochemistry. (A) Post-Treatment TILs in Cancer Tissues With HR<sup>+</sup>/HER2<sup>+</sup> Disease. (B) Post-Treatment TILs and FoxP3 Cells in HR<sup>+</sup>/HER2<sup>+</sup> Cancer Tissues With Residual Diseases (Non-pCR) or Without Residual Disease (pCR)



Abbreviations: HR = hormone receptor; pCR = pathologic complete response; TILs = tumor infiltrating lymphocytes.

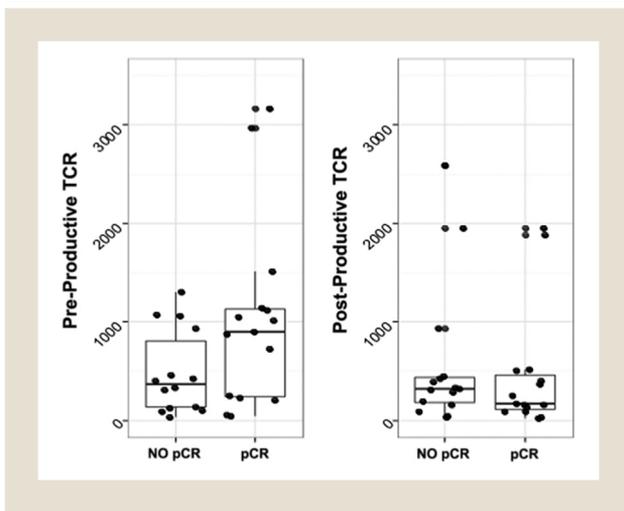
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**Figure 4** Analysis of T-Cell Subsets in Tumor Tissue. Tumor Tissue Was Analyzed to Examine T-Cell Subsets Using Immunohistochemistry and TCR $\beta$  Deep Sequencing in Patient With a 40% Change in Stromal TILs. (A) T-Cell Subset Analysis in Patients With a 40% Increase in TILs and (B) a 40% Decrease in TILs



Abbreviations: CD = cluster of differentiation; PD-L1 = programmed death ligand 1; TCR = T-cell receptor; TILs = tumor infiltrating lymphocytes.

**Figure 5** Productive TCR $\beta$  Immune Modulation in All Early Stage HER2<sup>+</sup> Breast Cancer Patients Receiving Neoadjuvant TCHP. No Significant TCR $\beta$  Differences Were Shown Between Response Subgroups. Four Patients With Elevated Pretreatment Productive TCR $\beta$  Were Observed in the pCR Cohort



Abbreviations: pCR = pathologic complete remission; TCHP = doctaxel, carboplatin trastuzumab, and pertuzumab; TCR = T-cell receptor.

CD40L, PD-L1, and CTLA4 mRNA expression in association with change in TILs, however, CD4 and FoxP3 were not analyzed.<sup>25</sup> No difference between pretreatment and post-treatment TILs in early-stage HER2<sup>+</sup> breast cancers with residual disease was shown. Change in TILs was not associated with distant event-free survival (DEFS), but a positive change in TILs was associated with an improved DEFS in a multivariate analysis. Also, cancers with a 40% change in TILs significantly correlated with changes in tumor immune modulation.<sup>25</sup> Our data complement these results because we similarly did not identify a difference across our entire study between pre- and post-treatment TILs. However, we did observe a decrease in the pre- to post-treatment change in TILs within HR<sup>-</sup>/HER2<sup>+</sup> tissues with residual disease and identified increased regulatory T-cells in the HR<sup>-</sup>/HER2<sup>+</sup> and HR<sup>+</sup>/HER2<sup>+</sup> post-treatment specimens with residual disease. This tumor immunosuppressive phenotype was further demonstrated in HER2<sup>+</sup> residual disease tissues and a 40% increase in TILs.

Using IHC to quantify T-cell phenotypes is attractive because IHC can be easily adapted into clinical practice when validated. Deep sequencing of TCR $\beta$  is a novel alternative because it quantifies T cells and characterizes T-cell clonality. Our data suggest an immunosuppressive tumor immune microenvironment with regulatory T cells in some early-stage HER2<sup>+</sup> breast cancers with residual disease. However, we did not find predictive biomarkers of this immunosuppressive phenotype in the pretreatment samples.

There are potential biological reasons that an immunosuppressive phenotype was not observed at baseline, but might have resulted in the post-treatment specimens from cancers with residual disease. Tumor heterogeneity within the tumor microenvironment is a known feature of breast cancer and any immune infiltrates might have been missed on initial biopsy.<sup>36</sup> A post-treatment

immunosuppressive microenvironment might have developed either because of treatment effects or as a mechanism of resistance to treatment. The innate immune system and ADCC might not have been fully activated by neoadjuvant TCH with or without P or certain aspects of Fc gamma might have been defective on natural killer (NK) cells, monocytes, or macrophages.<sup>37</sup> Antigen presentation might have been muted by hypermethylation of MHC complexes not allowing for adequate adaptive immune response.<sup>38</sup> Additionally, the tumor might have been primed for an immunosuppressive phenotype with a small number of immunosuppressive cognate T cells, but our limited immune analysis did not detect subtle immunologic differences.<sup>39</sup> However, we would expect to see clonal expansion shifting toward a monoclonal phenotype if those immunosuppressive cognate T cells were to expand. The lack of clonal expansion and consistent polyclonal nature of all our pre- and post-treatment samples suggests against this, but does not completely exclude this as an explanation.

Although we did not show productive TCR $\beta$  or TCR $\beta$  clonality to be predictive or prognostic of response in this analysis, we found interesting trends that merit further study. There were 4 patients with HER2<sup>+</sup> disease in the pCR subgroup with elevated pretreatment productive TCR $\beta$  rearrangements significantly increased over the average in the subgroup (Figure 5). This might indicate productive TCR $\beta$  rearrangements could predict response in some early-stage breast cancer patients with HER2<sup>+</sup> disease. Additionally, there were polyclonal variations throughout TILs suggesting tumor immune heterogeneity across HER2<sup>+</sup> early-stage breast cancers. Both of these findings warrant further investigation in a larger cohort of patients.

There are several limitations to our study. In addition to the small sample size, the IHC immune phenotype analysis assessed only 4 biomarkers and did not have direct overlay of the IHC stains to determine if multiple markers were expressed on the same cell. Also, immune functionality was not addressed with our methodology. NK cells, macrophages, monocytes, neutrophils, and eosinophils mediate ADCC, but our study did not address these immunologic phenotypes. Therefore, future clinical research studies should focus on validation of our findings with incorporation of robust immune-based RNA gene expression profiling, ELISpot technology, and novel ADCC assays.

## Conclusion

Approximately 30% to 40% of early-stage HER2<sup>+</sup> breast cancers continue to not fully achieve a pCR with neoadjuvant TCHP. In this exploratory analysis, we detected an immunosuppressive phenotype in early-stage HER2<sup>+</sup> breast cancers with residual disease after receiving neoadjuvant TCH with or without P. This immunosuppressive tumor microenvironment involves CD4<sup>+</sup> and FoxP3 cells that represent a regulatory T-cell population. Further investigation into what triggers immunosuppressive phenotypes in early-stage HER2<sup>+</sup> breast cancers with residual disease is warranted.

## Clinical Practice Points

- Even with significant improvements in the treatment of early-stage HER2<sup>+</sup> breast cancers many patients do not fully achieve a pCR.

## HER2<sup>+</sup> Immunosuppressive Phenotype

- Tumor-infiltrating lymphocytes might predict treatment outcomes in HER2<sup>+</sup> breast cancers despite a lack of detailed information about the tumor immune microenvironment.
- Combined analysis of TILs with CD4 and FoxP3 on pre- and post-treatment early-stage HER2<sup>+</sup> breast cancer samples that received neoadjuvant therapy with trastuzumab and P-based regimens might help identify patients with an immunosuppressive tumor immune microenvironment.

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