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# Early on-treatment transcriptional profiling as a tool for improving pathological response prediction in HER2-positive inflammatory breast cancer

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***Abstract***

**Background:** Inflammatory breast cancer (IBC) is a rare and understudied disease, with 40% of cases presenting with human epidermal growth factor receptor 2 (HER2)-positive subtype. The goals of this study were to (i) assess the pathologic complete response (pCR) rate of short-term neoadjuvant dual-HER2-blockade and paclitaxel, (ii) contrast baseline and on-treatment transcriptional profiles of IBC tumor biopsies associated with pCR, and (iii) identify biological pathways that may explain the effect of neoadjuvant therapy on tumor response.

**Patients and Methods:** A single-arm phase II trial of neoadjuvant trastuzumab (H), pertuzumab (P), and paclitaxel for 16 weeks was completed among patients with newly diagnosed HER2-positive IBC. Fresh-frozen tumor biopsies were obtained pretreatment (D1) and 8 days later (D8), following a single dose of HP, prior to adding paclitaxel. We performed RNA-sequencing on D1 and D8 tumor biopsies, identified genes associated with pCR using differential gene expression analysis, identified pathways associated with pCR using gene set enrichment and gene expression deconvolution methods, and compared the pCR predictive value of principal components derived from gene expression profiles by calculating and area under the curve for D1 and D8 subsets.

**Results:** Twenty-three participants were enrolled, of whom 21 completed surgery following neoadjuvant therapy. Paired longitudinal fresh-frozen tumor samples (D1 and D8) were obtained from all patients. Among the 21 patients who underwent surgery, the pCR and the 4-year disease-free survival were 48% (90% CI 0.29–0.67) and 90% (95% CI 66–97%), respectively. The transcriptional profile of D8 biopsies was found to be more predictive of pCR (AUC = 0.91, 95% CI: 0.7993–1) than the D1 biopsies (AUC = 0.79, 95% CI: 0.5905–0.9822).

**Conclusions:** In patients with HER2-positive IBC treated with neoadjuvant HP and paclitaxel for 16 weeks, gene expression patterns of tumor biopsies measured 1 week after treatment initiation not only offered different biological information but importantly served as a better predictor of pCR than baseline transcriptional analysis.

**Trial Registration:** ClinicalTrials.gov identifier: NCT01796197 (https://clinicaltrials.gov/ct2/show/NCT01796197); registered on February 21, 2013.

***Keywords:*** Inflammatory breast cancer, HER2-positive, on-treatment biopsy, treatment de-escalation, gene expression, immune response

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## Introduction

Inflammatory breast cancer (IBC) is a unique and aggressive form of locally advanced breast cancer which remains relatively understudied. IBC accounts for 2–5% of all invasive breast cancers and is associated with a worse prognosis compared with non-IBC.[1] Trimodality therapy, consisting of neoadjuvant systemic therapy (and anti-human epidermal growth factor receptor 2 (HER2) agents for HER2-positive disease) followed by total mastectomy and radiotherapy, is the standard treatment approach for patients with newly diagnosed nonmetastatic disease, followed by endocrine therapy when indicated.[2] However, despite the aggressive treatment, survival outcomes remain poor, with a 5-year overall survival of less than 50%.[1]

The incidence of HER2-positive disease among patients with IBC is two- to four-fold greater than the incidence observed in non-IBC, and novel therapies targeting HER2 have resulted in more favorable outcomes among this subtype of IBC.[2–5] In the neoadjuvant setting, dual-HER2-blockade significantly enhances the rate of achieving a pathologic complete response (pCR), a potential marker for improved survival in individual patients,[6] with ranges between 46% and 62%, depending upon treatment regimens and duration of neoadjuvant therapy.[7,8] The prevalence of HER2-positive disease among patients with IBC and the recent availability of effective agents targeting HER2 support investigation into the optimal preoperative regimen for the treatment of IBC and the identification of new biomarkers for treatment benefit.

The search for predictive biomarkers in breast cancer has benefited from the development and application of next-generation sequencing technologies, including RNA-sequencing (RNA-seq).[9] In clinical trials of early-stage HER2-positive breast cancer, baseline gene expression patterns distinguished tumors more likely to respond to neoadjuvant therapy from those that do not.[10–13] Importantly, these studies highlighting biomarkers which may identify tumors susceptible to specific neoadjuvant therapy have not focused on IBC. Another limitation of these studies is that the analysis of tumor transcriptomes was only performed on the pretreatment biopsy and do not take into account biologic changes which occur in the tumor and tumor microenvironment soon after the treatment has begun. Neoadjuvant therapy in IBC allows for easy access to on-treatment tumor samples (i.e., in the affected breast), thus providing a 'window of opportunity' to observe both molecular heterogeneity and subsequent changes in response to therapy, which can aid in identifying new biomarkers of disease response and resistance.

We conducted a single-arm phase II clinical trial (NCT01796197) of neoadjuvant trastuzumab, pertuzumab, and paclitaxel for 16 weeks in patients with newly diagnosed HER2-positive IBC (see the Protocol in the Supplemental File). Translational analyses identifying changes in the IBC transcriptome were performed on paired longitudinal fresh-frozen tumor samples prospectively obtained from the affected breast in each patient. The primary objective of the study was to estimate the pCR rate and the residual cancer burden (RCB) using a regimen consisting of optimal anti-HER2 therapy with a tolerable chemotherapy backbone. Secondary objectives included assessing the impact of short-term neoadjuvant dual HER2-blockade on the IBC transcriptome and to determine whether these changes could be used to distinguish patients who achieved a subsequent pCR from those that had residual tumor in the breast following completion of neoadjuvant therapy.

## Methods

### *Study design and patient population*

We conducted a multi-institutional, single-arm prospective phase II clinical trial in patients with newly diagnosed HER2-positive IBC. This included granting of consent for preplanned correlative studies to be performed on two sequential biopsies of the affected breast.

Eligible participants were 18 years of age or older with newly diagnosed invasive breast carcinoma and clinically confirmed IBC without evidence of metastatic disease in viscera or bone (T4d, stage III); distant nodal involvement only (stage IV) was allowed. HER2 status was assessed by immunohistochemistry (IHC) or fluorescence *in situ* hybridization, according to the 2013 American Society of Clinical Oncology/College of American Pathologists guidelines.[14] Good performance status (Eastern Cooperative Oncology Group performance status), left ventricular ejection fraction (LVEF), assessed by

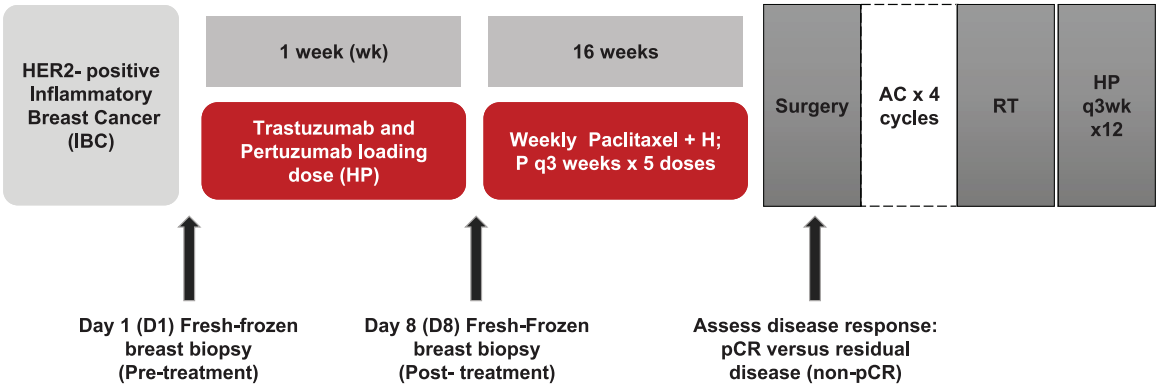


**Figure 1.** Scheme of prospective single-arm phase II clinical trial for newly diagnosed HER2-positive inflammatory breast cancer.
AC, doxorubicin, cyclophosphamide; H, trastuzumab; pCR, pathologic complete response; RT, radiotherapy; wk, week.

either multigated acquisition scan or echocardiogram, greater than or equal to 50%, and adequate organ function were also required.

The primary objective was to estimate pCR rate, defined as the absence of invasive disease in the breast and axillary lymph nodes (ypT0/is, ypN0), following neoadjuvant therapy with trastuzumab (H) and pertuzumab (P) in combination with 16 weeks of paclitaxel (T). RCB was also assessed.[15] Secondary objectives included toxicity assessment of protocol therapy, clinical outcomes, and correlative tissue analysis. Clinical outcomes included event-free survival (EFS), defined among all patients as the time from treatment initiation until disease progression, recurrence, or death; disease-free survival (DFS) defined among patients who underwent surgery as the duration from surgery until disease recurrence or death; or censored at the last time of follow-up.

### *Treatment plan*

Participants received a loading dose of neoadjuvant trastuzumab (4 mg/kg IV) and pertuzumab (840 mg IV, both drugs supplied by Genentech, San Francisco, CA, USA) on day 1 (D1), followed by the initiation of chemotherapy with weekly paclitaxel (80 mg/m$^2$ IV) on day 8 (D8). Paclitaxel was given weekly for a total of 16 weeks and was administered with weekly trastuzumab (2 mg/kg IV). Pertuzumab (640 mg IV) continued every 21 days for five cycles beginning on D1. The treatment scheme is outlined in Figure 1. Following the completion of neoadjuvant paclitaxel, trastuzumab, and pertuzumab (THP), participants whose disease was deemed operable proceeded to total mastectomy and complete axillary lymph node dissection. Following surgery, participants with residual disease found at the time of surgery were treated with doxorubicin (60 mg/m$^2$ IV) and cyclophosphamide (600 mg/m$^2$ IV) (AC) chemotherapy every 2–3 weeks for four cycles. Those participants who achieved a pCR following THP could elect to omit AC per physician and participant preference. Postmastectomy maintenance trastuzumab (6 mg/kg IV) and pertuzumab (640 mg IV) were administered every 21 days for an additional 12 cycles to complete a total of 12 months of anti-HER2 therapy. Postmastectomy radiation therapy to the chest wall and regional lymph nodes and endocrine therapy [in participants with estrogen receptor (ER) and/or progesterone receptor (PR) positive disease] were given per standard of care.

### *Sample collection and analyses*

All participants were required to undergo two research tumor biopsies of the affected breast using a 14-gauge core needle, obtaining four to six core specimens. Biopsies were obtained prior to starting neoadjuvant therapy (D1), and again 8 days following the loading dose of HP (D8). Fresh tumor tissue was immediately embedded in optimal cutting temperature solution to ensure tissue integrity and stored at −80°C at the DF/HCC Core Blood and Tissue Bank. Tumor specimens from D1 and D8 were assessed for tumor cellularity and scored for the proportion of stromal tumor-infiltrating lymphocytes (TILs) using H&E-stained slides from frozen sections. The proportion of TILs was scored as low (0–10%), intermediate

(11–59%), and high (⩾60%) based upon the International TILs Working Group Guidelines.[16] CelTIL score, which considers tumor cellularity in addition to TILs, was also assessed in paired breast biopsies from D1 and D8.[17]

### RNA-seq library preparation and sequencing

RNA-seq was performed on tumor tissue obtained from both the pretreatment breast biopsy (D1) and 8 days following the single loading dose of HP (D8). To optimize the quality of RNA, a ribo-depletion library preparation method was used to remove rRNA. Gene expression profiles were generated by mRNA sequencing using Illumina NextSeq 500 Paired-End 75bp (PE75). Briefly, mRNAseq libraries were made from total RNA using the Illumina Stranded total RNA preparation kit and sequenced on an Illumina NextSeq 500 Paired-End using a $2 \times 75$ pb configuration with an average of 56 million (M) reads per sample (range 15–91 M).

### RNA-seq analysis

Gene-level counts were generated using bulk RNA-seq pipeline in bcibo-nextgen framework.[18] Specifically, reads were aligned with STAR[19] to the hg38 reference genome, and the alignments were used to create quality control (QC) reports using metrics from samtools,[20] qualimap (http://qualimap.bioinfo.cipf.es/), fastqc (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/), multiqc,[21] (https://multiqc.info/), and STAR. Then, salmon[22] was used to pseudo-align the raw reads and to quantify transcript-level expression counts against hg38/ensembl v94 transcriptome reference. Transcript-level counts were combined into gene-level counts with tximport.[23] Quality control and differential expression analysis were performed using R [R Core Team (2021)], tidyverse,[24] DESeq2,[25] DEGreport (http://lpantano.github.io/DEGreport/), and ggrepel. For ROC analysis, the R package pROC was used.[26] ggplot2[27] (https://ggplot2.tidyverse.org), ggplotify (https://github.com/GuangchuangYu/ggplotify), and pheatmap (https://cran.r-project.org/web/packages/pheatmap/index.html) were utilized for visualization. For functional analysis GSEA,[28,29] FARDEEP,[30] and clusterprofiler[31] were utilized. The source code for all analyses and QC reports are available as a GitHub repository (https://github.com/hbc/overmoyer2021_RNAseq_analysis_of_inflammatory_breast_cancer_hbc04141).

Comprehensive quality control of RNA-seq data was performed exploring the following metrics: total read number, mapped reads, number of genes detected, gene detection saturation, exonic/intronic mapping rate, rRNA content, 5′–3′ bias, counts per gene, and counts per protein coding gene. Intercorrelation between all samples was also performed, including covariates analysis, mean variants QC plots, and size factor QS. All but one sample were suitable for a robust downstream differential expression analysis.

### Differential gene expression and pathway enrichment

Differential gene expression scores were computed using DESeq2 1.30.1,[25] with the exact parameter settings specified in the GitHub repository (https://github.com/hbc/overmoyer2021_RNAseq_analysis_of_inflammatory_breast_cancer_hbc04141). Differential expression models included the biopsy timepoint (D1/D8), response (pCR/non-pCR), ER status, tumor purity of the samples (high purity *versus* low purity), and date of library preparation factors. We identified differentially expressed (DE) genes for every factor and compared the strength of DE signal associated with response (pCR *versus* non-pCR) compared to all others. We used standalone GSEA 4.1.0 with MSigDB.v7.4 for the pathway enrichment analysis. Specifically, we examined the list of 50 cancer hallmarks (https://www.gsea-msigdb.org/gsea/msigdb/collections.jsp#H), 6290 curated pathways (https://www.gsea-msigdb.org/gsea/msigdb/collections.jsp#C2), and 14,998 gene sets covering the space of Gene Ontology (GO) terms (https://www.gsea-msigdb.org/gsea/msigdb/collections.jsp#C5).

### Statistical considerations

The primary endpoint was pCR rate after neoadjuvant therapy with TPH for 16 weeks in patients with newly diagnosed HER2-positive IBC and to assess the RCB following neoadjuvant therapy. Participants were enrolled using a single-arm, two-stage Simon minimax design. This regimen would be declared worthy of further study if $>7/27$ pCR were observed ($\leqslant 15\%$ *versus* $\geqslant 40\%$; target one-sided $\alpha = 0.05$, power $= 0.90$). In consideration of the one-sided $\alpha = 0.05$ design, the pCR rate with two-sided 90% confidence interval (CI) that accounted for the two-stage design was reported[32]; two-sided exact binomial CI was reported for the RCB rate. Up to 30 participants were planned to be enrolled, allowing up to three

**Table 1.** Baseline patient and tumor characteristics and distribution of pathologic disease response at surgery, according to RCB.[15]

| Characteristic | Total *N* = 23 | |
|---|---|---|
| Age, median (range) | 48 years (32–74) | |
| Hormone receptor status | | |
| Negative | 11 (48%) | |
| Positive | 12 (52%) | |
| Clinical stage (cT4d)[33] | | |
| IIIB | 16 (70%) | |
| IIIC | 6 (26%) | |
| IV | 1 (4%) | |
| Pathological response | Rate (#/23 pts) % | Rate (#/21 pts)* % |
| RCB 0 (pCR) | 10 (43%; 90% CI 26–62) | 10 (48%; 90% CI 29–67) |
| RCB-I | 7 (30%; 90% CI 15–50) | 7 (33%; 90% CI 17–54) |
| RCB-II | 1 (4%; 90% CI 0.2–19) | 1 (5%; 90% CI 0.2–21) |
| RCB-III | 3 (13%; 90% CI 4–30) | 3 (14%; 90% CI 4–33) |
| NA | 2 (9%) | |

Hormone receptor-negative – estrogen and progesterone receptor-negative; Hormone receptor-positive – estrogen and/or progesterone receptor-positive.
*Evaluable patients who underwent surgery.
NA, not assessed; pCR, pathologic complete response; pts, participants; RCB, residual cancer burden.

participants not evaluable for the pCR endpoint. Enrollment was stopped after 23 participants because >7 pCRs had been observed (the null hypothesis of 15% pCR rate could be rejected) and because during the enrollment period, the efficacy of the addition of neoadjuvant pertuzumab for HER2-positive breast cancer was established. All participants who initiated the preoperative treatment were considered as evaluable for the treatment endpoints. Kaplan–Meier estimates of 4-year EFS and DFS were reported.

## Results

### *Clinical outcomes*

Between August 2013 and June 2018, a total of 23 eligible women with newly diagnosed HER2-positive IBC were enrolled in a multi-institutional trial (Dana-Farber Cancer Institute, University of Michigan). Thirty-one patients were originally screened and eight were found to be ineligible due to the presence of visceral metastatic disease. The median age was 48 years (range, 32–74 years) and 48% of participants had hormone receptor (HR)-negative (ER-negative and PR-negative) disease (Table 1). All participants presented with stage III disease, except one patient with de novo metastatic disease (stage IV) by virtue of distant nodal involvement only. Matched tumor biopsies of the affected breast (D1 and D8) were obtained in all 23 participants. Twenty-one participants underwent total mastectomy and axillary lymph node dissection following completion of neoadjuvant THP; one participant received 12 weeks of THP, then declined further therapy because of travel distance and one developed central nervous system (CNS) metastasis after completing 16 weeks of treatment and did not proceed to surgery.

The majority of the 23 participants (96%) completed 15–16 weeks of preplanned neoadjuvant treatment with THP. There were four grade 3 adverse events, three of which occurred in one participant (hyperglycemia and acute kidney

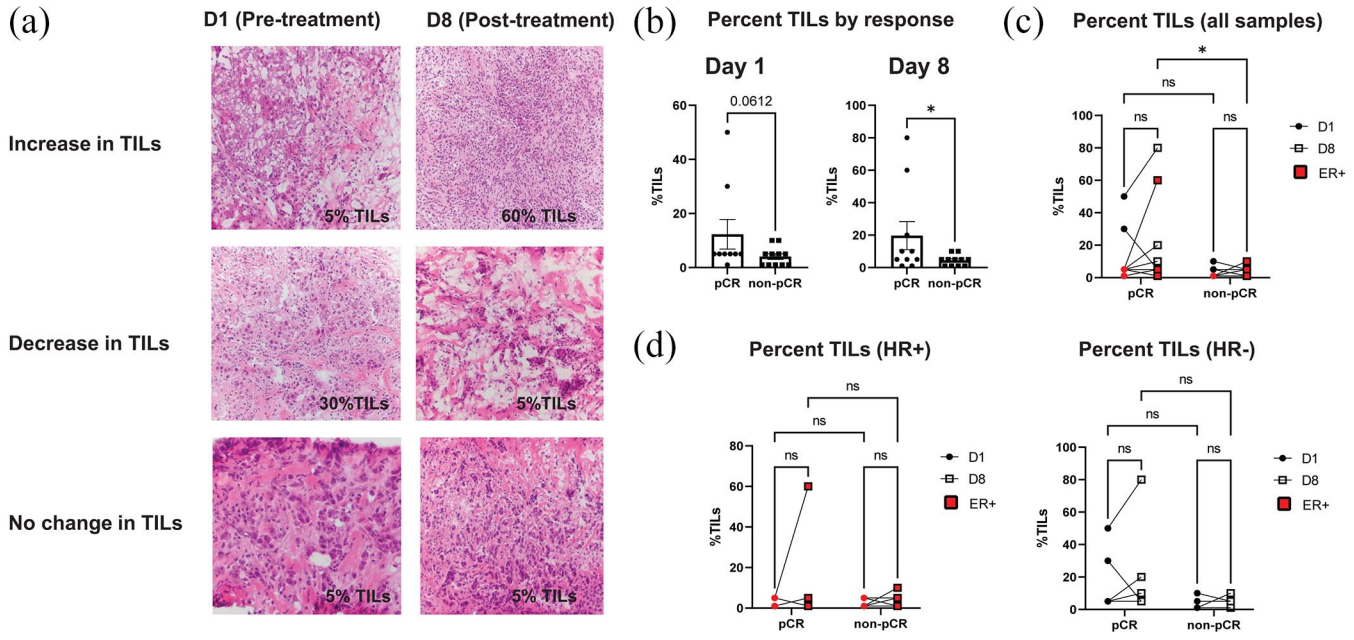


**Figure 2.** Changes in TILs in tumor biopsy samples obtained pretreatment (D1) and post-dual HER2-blockade (D8). (a) Representative H&E images from frozen sections of selected cases with an increase, decrease, or no changes in TILs levels. (b) Percent of TILs by response at baseline (pretreatment, D1) and post-dual HER2 blockade (D8); *$p = 0.0415$. Unpaired one-tailed *t*-test. (c) Individual changes in TIL infiltration between baseline (pretreatment, D1) and post-dual HER2 blockade (D8) *$p = 0.0333$ and (d) according to hormonal receptor status and pathologic response by RCB ($n = 21$ independent paired patient samples). Two-way analysis of variance with Fisher's Least Significant Difference (LSD) test.
ER, estrogen receptor; pCR, pathologic complete response; TIL, tumor infiltrating lymphocyte.

injury following diarrhea) and one event (diarrhea) occurred in another participant. Two participants developed asymptomatic reductions in LVEF of greater than 10% during the maintenance phase of HP: after 10 cycles and 11 cycles, respectively. There were no grade 3 or higher episodes of neuropathy or cardiac toxicity and no grade 4–5 adverse events were observed.

Of the 23 participants who initiated therapy, 10/23 (43%; 90% CI 0.26–0.62) achieved a pCR (RCB-0) and 7/23 (30%; 90% CI 0.15–0.50) had RCB-I residual disease. No significant differences were observed between the HR-positive (ER and/or PR-positive)/HER2-positive and HR-negative/HER2-positive populations. Among the 21 participants who underwent surgery, 48% (90% CI 0.29–0.67) achieved a pCR, 6 of whom did not receive AC post-surgery (Table 1). After a median follow-up of 5.2 years, there were three deaths (13%), two related to disease progression. Among all 23 participants, there were four events: one developed disease progression in the CNS prior to surgery, two experienced IBC recurrence (at 3.7 and 1.1 years since study entry; neither participant achieved a pCR and both received postoperative AC chemotherapy), and one died of renal failure unrelated to IBC. The 4-year EFS was 86% (95% CI, 63–95%). Of the 21 participants who underwent surgery, the 4-year DFS was 90% (95% CI, 66–97%).

### *TILs analysis*

TILs were assessed at baseline (D1) and at D8 in 22 (96%) and 23 (100%) participants, respectively. Among the D1 biopsy specimens, 20 (91%) had low levels, 2 (9%) had intermediate levels, and none had high levels of TILs. Following the single loading dose of HP, 10 biopsy specimens (45%) had an increase in TILs, 6 (27%) had no change, and 6 (27%) had a decrease in the level of TILs [Figure 2(a)]. TIL level at baseline was not associated with pCR. However, increased TIL levels at D8 were significantly associated with pCR ($p = 0.0415$) [Figure 2(b) and (c)]. No significant differences were observed in pre- and posttreatment TILs levels when stratifying by HR status and pathologic response [Figure 2(d)].

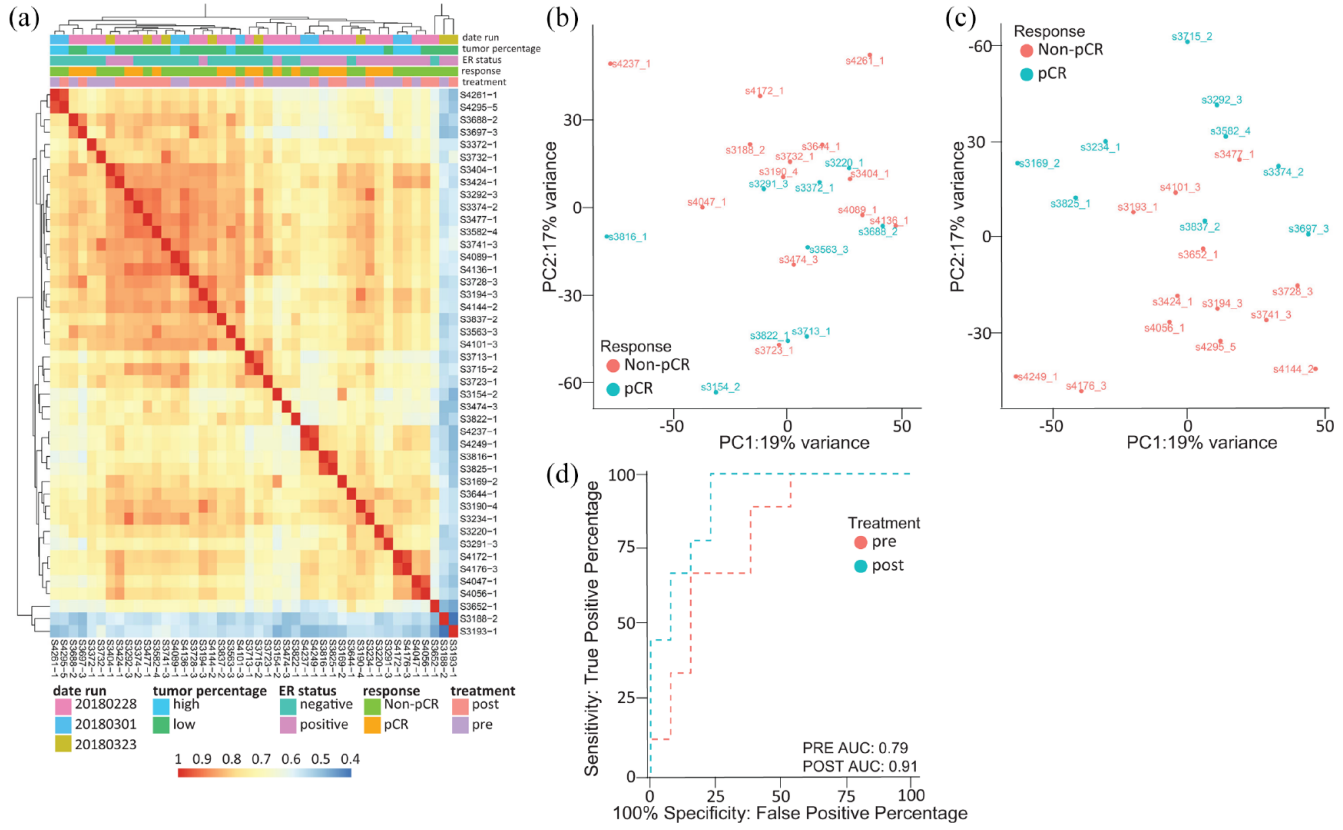


**Figure 3.** The signal distinguishing responders and nonresponders is stronger in post-dual-HER2 blockade (D8) data. (a) Pairwise similarities between all 23 patients, computed using the top 1000 most variable genes. Individual cells of the heatmap show Pearson correlation values. Rows and columns are annotated with patient IDs. The columns are augmented with metadata describing the date of sequencing, tumor percentage, ER status, whether a patient achieved a pCR, and whether pretreatment (D1) or post-dual HER2 blockade (D8) data was used. (b) D1 and (c) D8 gene expression data projected onto the first two principal components, computed on the corresponding data slices. Individual points are colored by their response status [pCR *versus* residual disease (non-pCR)]. (d) ROC curves associated with the ability of the second principal component to distinguish pCR and non-pCR.
ER, estrogen receptor; pCR, pathologic complete response.

### *Gene expression profiles between pCR and non-pCR*

When considering D1 and D8 gene expression data together, we observed no apparent batch effects associated with the date of sequencing, tumor percentage, HR status, and whether the biopsy was collected on D1 or D8 [Figure 3(a)]. We also saw no clear separation between patients who achieved a pCR and those with residual disease (non-pCR) in the top 1000 most-varying genes of the joint dataset, suggesting that different mechanisms may be involved in distinguishing pCR and non-pCR in pre- and post-dual HER2 blockade. We therefore examined mRNA profiles of D1 and D8 biopsies separately, by performing principal component analysis (PCA) on each set of samples [Figure 3(b) and (c)). Importantly, PCA computes directions of most variance in the data while being blinded to whether individual samples are pCR or non-pCR. We observed that in both cases, the second highest direction of variance (principal component) aligns with the signal that distinguishes the two groups of patients (y-axis in Figure 3(b) and (c)). We ranked patients along the second principal component and constructed receiver operator curves (ROC) that report the trade-off between true and false positives [Figure 3(d)]. Area under the ROC curves (AUC) can then be interpreted as the probability that a pCR patient would be correctly ranked above a non-pCR by the corresponding ranking. We observed that the ranking of patients based on D8 data produced a more accurate distinction between pCR and non-pCR patients [AUC = 0.91, 95% CI: 0.7993–1 (DeLong)] than the ranking based on D1 data [AUC = 0.79, 95% CI: 0.5905–0.9822 (DeLong)], ROC test $p$-value = 0.23.

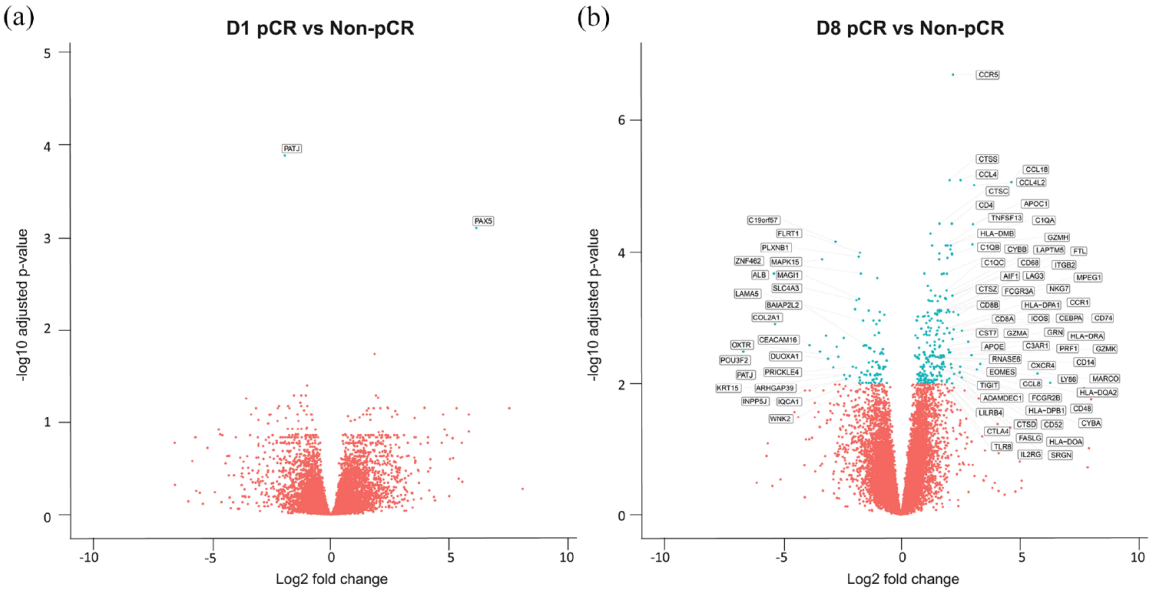


**Figure 4.** Genes with the strongest signal distinguishing pCR from non-pCR. Differentially expressed genes in patients with pCR *versus* pCR in pretreatment (D1) data (a) and post-dual HER2 blockade (D8) data (b). Positive log2 fold change indicates higher expression in pCR samples. Genes corresponding to the False Discovery Rate below 0.01 are colored in cyan.
pCR, pathologic complete response.

To gain further insight into molecular mechanisms that distinguish patients with a pCR from those with residual disease, that is, non-pCR, we performed differential gene expression on D1 and D8 data separately. Only two genes in the D1 data were DE under a false discovery rate threshold of 0.01 [Figure 4(a)]. Consistent with the above result, we observed many more genes that were DE in mRNA profiles of D8 biopsies [Figure 4(b)], indicating again that the signal distinguishing pCR from non-pCR is stronger post-dual HER2 blockade. Among the top genes were markers of adaptive immunity (T cells, CD4 and CD8), innate immunity (macrophages, CD68), and antigen presentation (HLA-DPA1, HLA-DRA), suggesting that the immune cell composition of the tumor microenvironment could play a role in whether a patient achieves pCR.[34–36]

We applied Gene Set Enrichment Analysis to D8 transcriptional profiles using gene sets associated with cancer hallmarks, curated pathways, and GO terms from the Molecular Signatures Database (See Methods section). The analysis revealed 13 hallmarks, largely related to immune signaling and apoptosis, that were significantly (adjusted $p$ value $< 0.05$) upregulated in pCR tumors [Figure 5(a)]. Among curated pathways and GO terms, we observed that a number of pathways related to ERK and PI3K signaling were increased in those who achieved pCR [Figure 5(b)]. The most striking differences between pCR and non-pCR were related to the tumor microenvironment, with increased activity in pathways related to both adaptive and innate immunity, as well as antigen presentation in patients with pCR [Figure 5(b)]. No gene sets were found to be significantly (adjusted $p$ value $< 0.05$) upregulated in non-PCR tumors. Assessment of individual genes revealed increased expression related to adaptive immunity, including CD8A, CD8B, GZMA, GZMH, GZMK, PRF1, CD4, FOXP3, LAG3, TNFα, and IFNα [Figure 5(c)]. Similarly, pCR tumors presented a robust increase in tumor-associated macrophages (CD68 and CD163), as well as chemokines, cytokines, and associated receptors related to innate immunity [Figure 5(b) and (d)]. The expression of PD-L1 (*cd274*) and genes associated with antigen presentation were also increased in tumors associated with pCR [Figure 5(d) and (e)]. While dendritic cells may be involved in antigen presentation, we observed no difference in expression for genes associated with this cell type between pCR and non-pCR tumors at D8 [Figure 5(d)]. Taken together, analysis of D8 transcriptional profiles suggests activation of an antitumor immune response, including both innate and adaptive immunity.

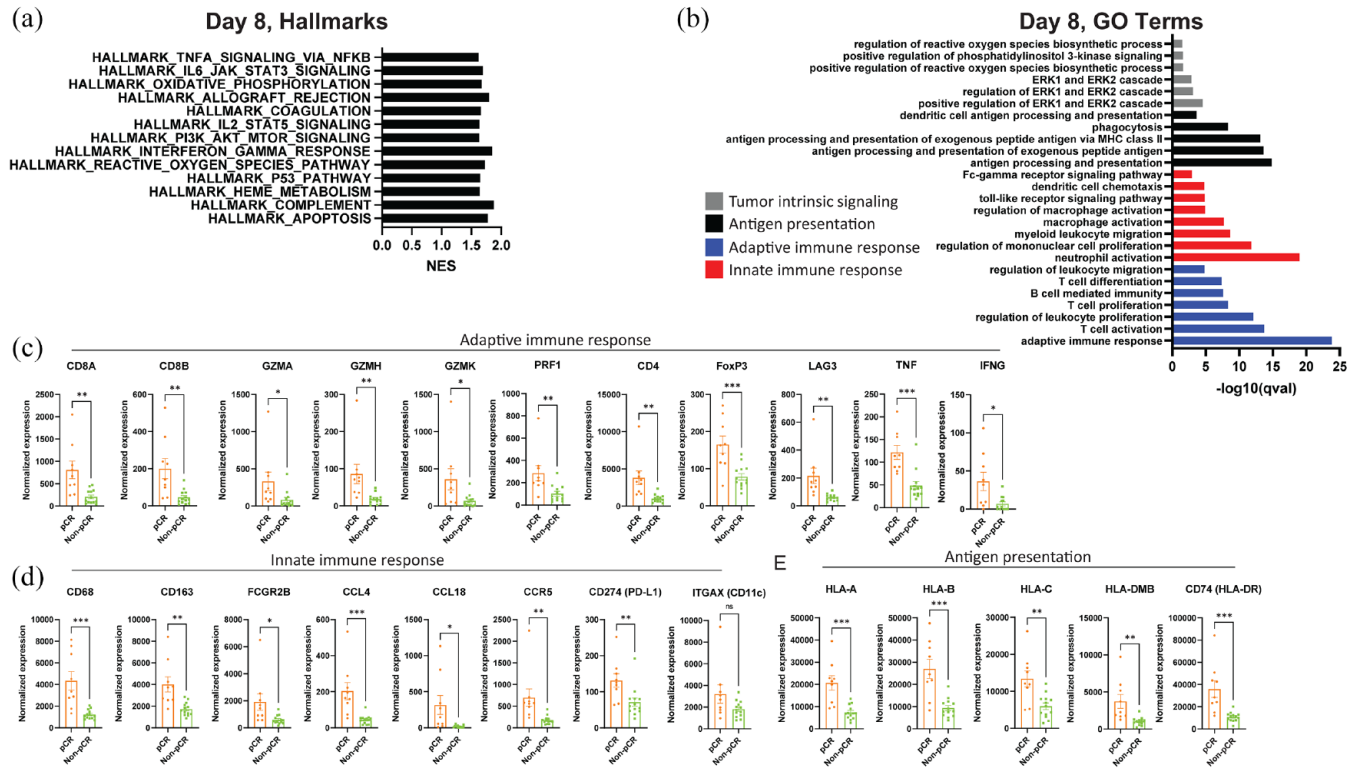


**Figure 5.** Evaluation of tumor cell intrinsic and immune related genes and signatures. (a) Hallmark gene sets enriched in tumors with pCR compared to non-pCR at D8 (post-dual HER2 blockade), with an adjusted $p$-value $< 0.05$. (b) Curated pathways and GO terms related to tumor cell intrinsic signaling, adaptative and innate immune response, and antigen presentation that are enriched in tumors with pCR compared to non-pCR at D8. All $q$ values are below 0.05. (c–e) Raw expression of individual genes. Statistical analyses were performed using two-tailed unpaired $t$-test. Error bars represent ±SEM.
$^{*}p < 0.05$. $^{**}p < 0.01$. $^{***}p < 0.001$. $^{****}p < 0.0001$.
pCR, pathologic complete response.

To further investigate the immune cell composition in the tumor microenvironment, we applied FARDEEP[30] to enumerate immune cell subsets from whole tumor tissue samples, based on the LM22 signature matrix characterizing 22 immune cell types. The choice of FARDEEP over other deconvolution methods was motivated by its robust performance in a recent benchmarking study.[37] Deconvolution of immune cell types revealed significant differences in the presence of CD8+ T cells, T regulatory cells, and macrophages between pCR and non-pCR tumors [Figure 6(a) and (b)].

## Discussion

Our study shows that a single loading dose of dual HER2-blockade with pertuzumab and trastuzumab in patients with newly diagnosed HER2-positive IBC induces significant changes in gene expression patterns which can be used to distinguish tumors that will achieve a pCR after neoadjuvant systemic therapy. A pCR following neoadjuvant therapy has been shown to be a predictor of favorable clinical outcomes among HER2-positive breast cancer in general[6] and, more specifically, among patients with IBC.[38] The results presented here suggest that an assessment of the tumor transcriptome shortly after treatment initiation (i.e., 1 week following dual HER2-blockade) captures pharmacodynamic changes that occur early with neoadjuvant therapy and can serve as a mechanism of identifying patients with responsive tumors likely to achieve a pCR.

The current study focused on a unique patient population, generally excluded from clinical trials, and in whom neoadjuvant systemic therapy is extrapolated from clinical trials primarily involving patients with non-IBC. Both the NeoSphere[7,39] and TRYPHAENA[40] trials which evaluated neoadjuvant dual HER2 blockade with pertuzumab, trastuzumab, and chemotherapy enrolled only 6–7% of patients with IBC. Our study exploited

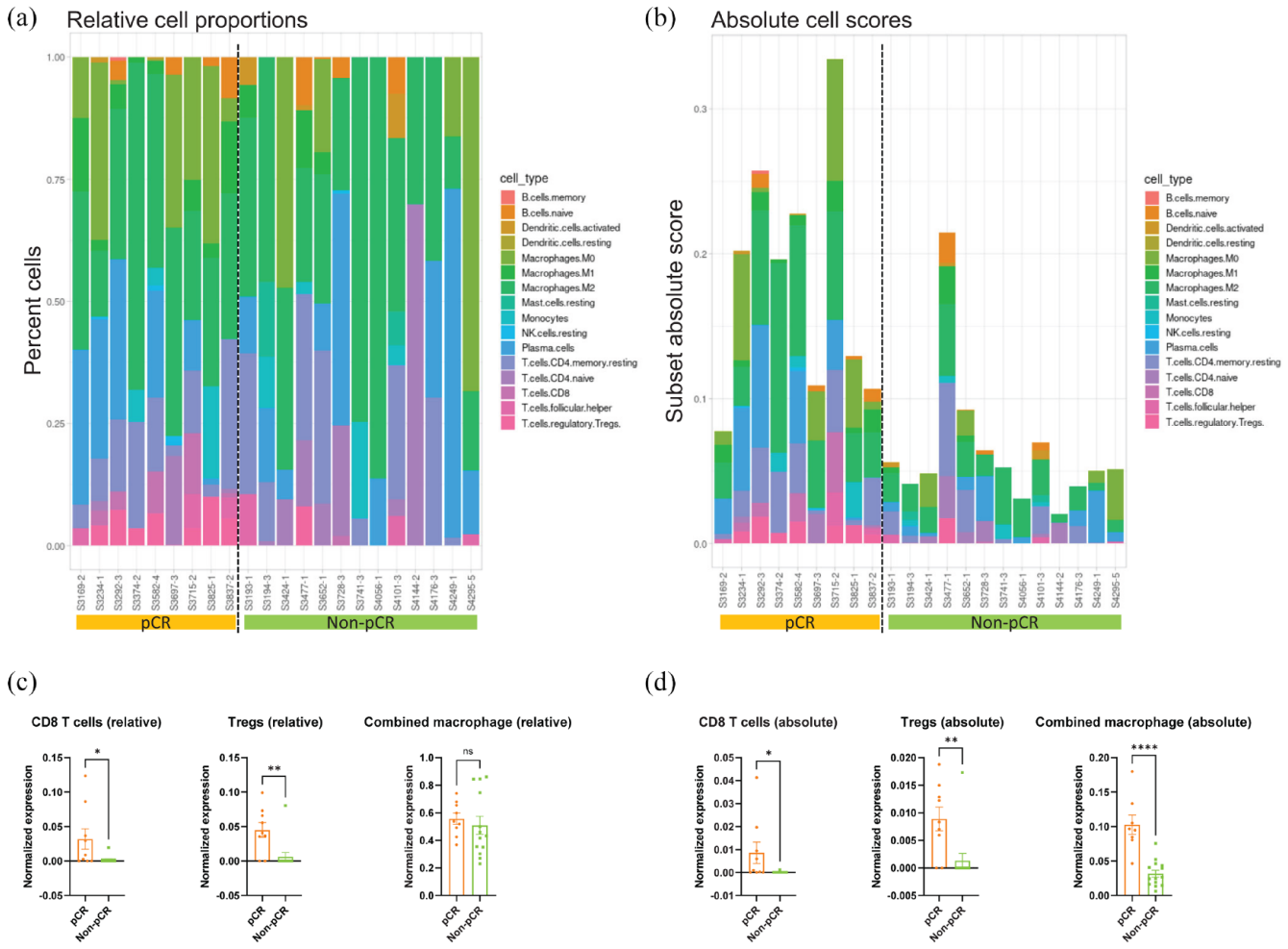


**Figure 6.** Deconvolution of tumor infiltrating immune cells. FARDEEP was used to enumerate immune cell subsets from whole tumor tissue samples using the LM22 signature matrix. (a) Relative cell proportions and (b) absolute cell scores are shown. Distributions of several selected cell types are plotted by relative (c) and absolute (d) values. Statistical analyses were performed using two-tailed unpaired *t*-test. Error bars represent ±SEM.
*$p < 0.05$. **$p < 0.01$. ****$p < 0.0001$.

the exquisite sensitivity[5] of HER2-positive IBC to anti-HER2 therapy and designed a highly tolerable neoadjuvant therapy with single-agent weekly paclitaxel in combination with dual HER2-blockade provided by trastuzumab and pertuzumab. Our pCR rate of 48% among those participants who had surgery is higher than the pCR rate obtained in the chemo-intensive (±trastuzumab) phase III NOAH trial (38%); the only neoadjuvant trial for HER2-positive breast cancer that included enough patients with IBC to enable a subset analysis.[41] In our study, no significant differences were observed in terms of pCR between the HR-positive/HER2-positive and HR-negative/HER2-positive populations, most likely due to our small sample size. Of note, the relatively high pCR and DFS observed in our study may be somewhat surprising given the traditionally poor prognosis of this population. However, the overall survival of HER2-positive IBC has improved over the past decades compared with other subtypes of IBC, driven by the advent of novel anti-HER2 therapies.[5,42] For example, the addition of trastuzumab to neoadjuvant chemotherapy in the NOAH trial resulted in a 30% increase in the 5-year OS in the IBC subset.[41] The favorable clinical outcomes seen in our study add support for the application of neoadjuvant dual-anti-HER2 therapy for HER2-positive IBC.

On-treatment tumor information has been shown to be useful for endocrine therapy response in HR-positive, HER2-negative non-IBC. Breast cancers with high Ki67 levels (>10%) at 2 or

4 weeks after initiating neoadjuvant endocrine therapy exhibit resistance to endocrine treatment.[43] Additionally, changes in gene-expression signatures between pretreatment and on-treatment tumor biopsies of HR-positive, HER2-negative disease were better indicators of response to endocrine therapy than gene expression signatures of pretreatment tumor biopsies alone.[43,44] Several studies have also evaluated gene expression changes in non-IBC during the administration of neoadjuvant chemotherapy.[45–47] Within the IBC population, baseline gene expression profiles, mainly involving genes related to T cell cytotoxic immune response, have been correlated with achieving pCR to neoadjuvant chemotherapy.[48] In HER2-postive breast cancer, studies evaluating neoadjuvant chemotherapy in combination with dual HER2-blockade have demonstrated an association of specific baseline tumor characteristics or pretreatment gene expression signatures, such as HER2-enriched molecular subtype and immune signatures, with subsequent pCR.[11–13,49] However, very few studies have included patients with HER2-positive IBC. Our study is unique in this setting, in that it assesses early on-treatment changes using paired fresh-frozen tumor biopsies collected at baseline (pretreatment) and following the initial loading dose of dual-HER2-blockade (8 days) . Our study demonstrates that gene expression patterns measured approximately 1 week after treatment initiation not only offer different biological information but, importantly, serves as a superior predictor of pCR compared to baseline transcriptional analysis. Demonstration of upregulation of antitumor immunity following one dose of dual HER2-blockade, spanning both innate and adaptive immunity, including increased expression of genes related to antigen presentation, cytokines, and chemokines, may be a strong determinant of pCR for this underrepresented subtype of breast cancer. CD8+ T cells that express effector molecules granzyme and perforin, as well as TNFα, and IFNα are suggestive of enhanced antitumor activity. The increase in FoxP3, PD-L1, and LAG3 is generally associated with immune suppression in the context of cancer therapy and may be representative of both increased immune infiltrates and potentially a new avenue for immune modulatory combinations to reverse the nature of the suggested suppressed, but actionable tumor microenvironment of IBC.[34,50] Likewise, a robust increase in the presence of tumor associated macrophages was identified in pCR tumors. While tumor-associated macrophages can either function as pro-tumor or antitumor, the observed robust increase in antigen presentation signaling and pathways suggests activation of both CD8+ and CD4+ T cells in the context of antitumor immunity. A higher TIL count and a higher expression of immune signatures have been associated with higher rates of pCR following neoadjuvant anti-HER2 therapy in both IBC and non-IBC.[17,51,52] We could not confirm an association between TIL levels nor CelTIL score determined at baseline (D1) and pCR; however, increased infiltration of TILs at D8 was significantly associated with pCR ($p = 0.0415$).

This study has several limitations. A single-arm study does not have the benefit of a comparison control group; therefore, it cannot specifically answer the question of whether our findings are specific for IBC or associated with the administration of dual anti-HER2-blockade (HP) or the complete treatment regimen (THP). The small sample size restricts overinterpretation of the identification of specific transcriptome profiles associated with subsequent pCR, especially with regard to differentiating between the HR-positive and HR-negative subsets. Additionally, an analysis of protein expression by IHC of the gene sets enriched in the tumors achieving a pCR would have strengthened the findings of our study. Unfortunately, the underlying pathology of IBC, that is, the predominance of tumor cells localized within breast tissue lymphatic channels, is very challenging and limits the amount of adequate neoplastic tissue for scientific assessment. We chose gene expression over IHC in our study, utilizing the tissue samples with substantial tumor cells present. Thus, there were very few samples with enough tumor to ensure a valuable assessment of both analyses, IHC and RNA-seq. We intend to focus on a confirmatory assessment using IHC in our future IBC studies. Despite these limitations, this was a prospective, phase II clinical trial specifically designed for patients with a rare form of breast cancer, underrepresented in clinical trials, yet possessing a unique biology that renders it more aggressive than non-IBC. Moreover, the results presented here covered preplanned analyses of paired frozen tumor biopsies [pretreatment (D1)] and post-dual anti-HER2 therapy (D8) prospectively obtained in all 23 patients with 95.6% of the collected samples being evaluable for the correlative studies.

The results of our study are hypothesis generating, rather than definitive, but further support the potential value of early on-treatment tumor sampling to identify dynamic changes in gene

expression that may correlate with disease response and may allow earlier modifications to a more effective therapy. Additionally, these results elucidate the role of the immune microenvironment in response to HER2-targeted therapy which may have implications for considering immune modulatory interventions to improve clinical outcomes for this unique patient population.

## Declarations

### *Ethics approval and consent to participate*

The trial was approved by the IRB within each institution, and all participants signed a protocol-specific informed consent in accordance with the principles described in the Declaration of Helsinki.

### *Consent for publication*

Not applicable.

### *Author contributions*

**Sonia Pernas:** Conceptualization; Data curation; Investigation; Project administration; Resources; Visualization; Writing – original draft; Writing – review & editing.

**Jennifer L. Guerriero:** Data curation; Formal analysis; Visualization; Writing – original draft; Writing – review & editing.

**Sergey Naumenko:** Data curation; Formal analysis; Methodology; Validation; Visualization; Writing – original draft; Writing – review & editing.

**Shom Goel:** Conceptualization; Investigation; Project administration; Resources; Supervision; Writing – original draft; Writing – review & editing.

**Meredith M. Regan:** Conceptualization; Data curation; Formal analysis; Methodology; Supervision; Validation; Visualization; Writing – original draft; Writing – review & editing.

**Jiani Hu:** Data curation; Formal analysis; Methodology; Project administration; Resources; Software; Supervision; Visualization; Writing – original draft; Writing – review & editing.

**Beth T. Harrison:** Data curation; Investigation; Resources; Visualization; Writing – original draft; Writing – review & editing.

**Filipa Lynce:** Resources; Writing – review & editing.

**Nancy U. Lin:** Investigation; Writing – review & editing.

**Ann Partridge:** Investigation; Writing – review & editing.

**Aki Morikawa:** Investigation; Writing – review & editing.

**John Hutchinson:** Data curation; Formal analysis; Methodology; Project administration; Resources; Software; Supervision; Visualization; Writing – original draft; Writing – review & editing.

**Elizabeth A. Mittendorf:** Supervision; Writing – review & editing.

**Artem Sokolov:** Data curation; Formal analysis; Software; Visualization; Writing – original draft; Writing – review & editing.

**Beth Overmoyer:** Conceptualization; Funding acquisition; Investigation; Project administration; Visualization; Writing – original draft; Writing – review & editing.

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### *Competing Interests*

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### *Availability of data and materials*

Computational framework made use of the O2 High Performance Computer Cluster, supported by the Research Computing Group, at Harvard Medical School (http://rc.hms.harvard.edu).

Source code is available at: https://github.com/hbc/overmoyer2021_RNAseq_analysis_of_inflammatory_breast_cancer_hbc04141

The repository also contains raw and TPM counts at: https://github.com/hbc/overmoyer2021_RNAseq_analysis_of_inflammatory_breast_cancer_hbc04141/blob/master/tables/counts.raw.csv.gz

All other data supporting the findings of this study are available from the corresponding authors on request.

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## Supplemental material

Supplemental material for this article is available online.

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