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

Sonia Pernas1\*a#,\*, Jennifer L. Guerriero6,7, Sergey Naumenko3,Shom Goel1,4b, Meredith M. Regan5, Jiani Hu5, Beth T. Harrison8, Nancy Lin1, Ann Partridge1, Eric Winer1, Aki Morikawa9, John Hutchinson3, Elizabeth A. Mittendorf1,6,7, Artem Sokolov2\*, Beth Overmoyer1

1Susan F. Smith Center for Women’s Cancers, Inflammatory Breast Cancer Program, Dana-Farber Cancer Institute, Boston, MA, USA

2Laboratory of Systems Pharmacology, Harvard Medical School, Boston, MA, USA

3Department of Biostatistics, Harvard Chan School of Public Health, Boston, MA, USA

4Department of Cancer Biology, Dana-Farber Cancer Institute, Boston, MA, USA

5Division of Biostatistics, Dana-Farber Cancer Institute, Boston, MA, USA

6Breast Tumor Immunology Laboratory, Dana-Farber Cancer Institute; Boston, MA, USA

7Division of Breast Surgery, Department of Surgery, Brigham and Women’s Hospital, Boston, MA, USA

8Department of Pathology, Brigham and Women’s Hospital, Boston, MA, USA

9Medical Oncology, University of Michigan Health, Ann Arbor, MA, USA

**aCurrent address:** Department of Medical Oncology, Institut Catala d’Oncologia-IDIBELL, Barcelona, Spain

**bCurrent address:** The Sir Peter MacCallum Department of Medical Oncology, University of Melbourne, Australia

**\*Co-Corresponding authors:**

Sonia Pernas (corresponding author for submission)

Department of Medical Oncology

Institut Catala d’Oncologia-IDIBELL

Barcelona, Spain

Artem Sokolov

Harvard Medical School

200 Longwood Ave.

Armenise Building Rm. 137

Boston, MA 02115

**Short title:** On-treatment transcriptional profiling in HER2+ inflammatory breast cancer

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

**Author contributions:**

Conceptualization: BO, MMR, SP, SG

Data Curation: MMR, JH, SP, AR, SN, BTH, JH, JLG

Formal Analysis: MMR, JH, AR, JLG, SN

Funding Acquisition: BO

Investigation: SP, SG, BTH, NL, AP, EW, , AM, BO

Methodology: MMR, JH, SN

Project Administration: BO, SP, SG, JH

Resources: BO, SG, SP, JH, BTH

Software: SN, AS, JH

Supervision: BO, JH, EAM, EW, SG, MMR

Validation: SN, MMR

Visualization: SP, SN, AS, JLG, BTH, BO, MMR, JH

Writing – Original Draft: SP, AS, JLG, BO, SN, MMR, JH, SG

Writing – Review & Editing: All authors

**Funding:** **B.O.** obtained funding for this project through the following sources: Genentech Inc.(https://www.gene.com), The IBC Network Foundation (www.theibcnetwork.org), and the Dana-Farber Inflammatory Breast Cancer Research Fund (https://www.dana-farber.org/inflammatory-breast-cancer-program/). **S.P.** was supported by grants from the Fundación Asociación Española Contra el Cáncer (AECC), the Spanish Society of Medical Oncology (SEOM), and Ayudas Para la Movilidad del Personal Investigador (M-BAE) del Instituto de Salud Carlos III. SG is supported by a Career Catalyst Award from the Susan G. Komen Foundation and a Young Investigator Grant from the Breast Cancer Alliance. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. The academic investigators participated in the design and oversight of the project. They had full access to all the data and had final responsibility for the decision to submit for publication. All authors gave approval to submit for publication.

**Competing Interests**: **S.P.** has received travel and accommodation grants from Novartis outside of the submitted work and Consulting/Advisory boards for Polyphor, Roche, Daiichi Sankyo, AstraZeneca, Novartis, Pierre-Fabre, Eisai, and SeattleGenetics. **S.G**. has received laboratory research funding from Eli Lilly and performs clinical research sponsored by Novartis and Eli Lilly. S.G. has served as a paid advisor to Eli Lilly, G1 therapeutics, and Novartis. **J.L.G** is a consultant for Glaxo-Smith Kline (GSK), Array BioPharma, Codagenix, Verseau Therapeutics, Kymera, Carisma, Kowa, Duke Street Bio and MPM Capital, and receives sponsored research support from GSK, Eli Lilly and Array BioPharma. **M.R.** reports research funding (and/or provision of drug supply for clinical trials) from Novartis, Pfizer, Ipsen, TerSera, Merck, Ferring, Pierre Fabre, Roche, AstraZeneca, Bayer, Bristol-Myers Squibb; consulting or advisory role for Ipsen, Bristol-Myers Squibb, Tolmar Pharmaceuticals. **B.O**. has received clinical trial support from Genentech, Incyte and Eisai. **A.S.** is a paid statistical reviewer for PLoS Medicine.

**Part of the data was presented as abstracts at the following meetings:**

* 41st Annual San Antonio Breast Cancer Symposium: December 4-8, 2018. Pernas S. *et al.* Assessment of the tumor immune microenvironment in inflammatory breast cancer treated with neoadjuvant dual-HER2 blockade. Abstract PD3-08.
* ESMO Congress 2019, Barcelona: 27 Sep-01 Oct 2019. Pernas S. *et al*. Early on-treatment vs pre-treatment tumor transcriptomes as predictors of response to neoadjuvant therapy for HER2-positive inflammatory breast cancer. Abstract 120P.
* ISMB/ECCB 2019, Basel Switzerland: 21 July - 25 July 2019. Johnson N. T. et al. Transcriptomic profile after a single-dose of neoadjuvant dual-HER2 blockade better predicts pathologic response than at baseline in HER2-positive inflammatory breast cancer. Abstract G-43

**Abstract**

**Background:** Inflammatory breast cancer (IBC) is a rare and understudied disease, with 40% of cases presenting with HER2-positive IBC. Within a cohort of newly diagnosed patients with HER2-positive IBC, we sought to (i) assess the pathologic complete response (pCR) rate of short-term neoadjuvant dual-HER2-blockade and paclitaxel, (ii) define baseline and on-treatment transcriptional profiles of tumor biopsies that are associated with pCR, and (iii) identify biologic pathways that may explain the effect of neoadjuvant therapy on pathologic tumor response.

**Methods and Findings:** We conducted a single-arm phase II trial of neoadjuvant trastuzumab (H), pertuzumab (P) and paclitaxel for 16 weeks as neoadjuvant therapy for patients with newly diagnosed HER2-positive IBC (NCT01796197). Fresh-frozen tumor biopsies of the breast were taken pre-treatment (D1) and 8 days later, following a single-dose of HP (D8), prior to adding paclitaxel. The primary endpoint was pCR, defined as no invasive tumor in the breast or lymph nodes (LN). We performed RNA-seq on D1 and D8 tumor biopsies, compared the predictive value of the two transcriptomes as biomarkers for subsequent pCR using Principal Component Analysis (PCA), and identified genes and pathways associated with pCR using standard differential gene expression and pathway enrichment methods. Twenty-three participants were enrolled, of whom 21 completed mastectomy following neoadjuvant therapy. Paired tumor biopsies (D1, D8) were obtained from all 23 patients. The rate of pCR among the 23 treated participants was 43% (10/2390% CI 26-62%There were 4 episodes of grade 3 toxicity, which included diarrhea, hyperglycemia and acute kidney injury. With a median follow-up of 5.2 years, the 4-year disease-free survival was 90% (95% CI 66-97%), We found that the transcriptional state of D8 biopsies was more predictive of pCR (AUC=0.91) than the equivalent in D1 biopsies (AUC=0.79). Limitations included those associated with a single arm study lacking a control group and involving a small cohort of participants.

**Conclusions:** Neoadjuvant HP and paclitaxel for 16 weeks led to a pCR rate of 43% in patients with HER2-positive IBC. Gene-expression profiling of tumor biopsies following a single-dose of dual-HER2 blockade was able to better distinguish tumors that achieved a pCR than pretreatment biopsies. These results warrant further study of how neoadjuvant therapy affects the molecular state of IBC.

**Introduction**

Inflammatory breast cancer (IBC) is a unique and aggressive form of locally advanced breast cancer and remains relatively understudied. IBC accounts for 2-5% of all invasive breast cancers and is associated with worse survival outcomes compared with non-IBC (1). Trimodality therapy, consisting of neoadjuvant or preoperative systemic therapy followed by mastectomy and radiation, is the standard treatment approach for patients with newly diagnosed disease (2).

The incidence of HER2-positive disease among IBC is 2-4-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 (3-6). 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 (7), with ranges between 46-62%, depending upon treatment regimens and duration of neoadjuvant therapy (8, 9). 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 (10). In large 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 (11-14). 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 pre-treatment biopsy, prior to the initiation of neoadjuvant therapy, but does not take into account biologic changes which occur soon after treatment has begun. The primary use of 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 prospective single-arm phase II clinical trial (NCT01796197) of neoadjuvant trastuzumab, pertuzumab and paclitaxel for 16 weeks in patients with newly diagnosed HER2-positive IBC. 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 differentiate those 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. The trial was approved by IRB within each institution, and all participants signed a protocol-specific informed consent in accordance with the principles described in the Declaration of Helsinki. This included granting of consent for pre-planned 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 (ASCO/CAP) guidelines (15). Good performance status (Eastern Cooperative Oncology Group performance status [ECOG PS] 0-1), left ventricular ejection fraction (LVEF), assessed by either multigated acquisition (MUGA) 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 (16). 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) on day one (D1), followed by the initiation of chemotherapy with weekly paclitaxel (80 mg/m2 IV) on day eight (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 **Fig 1**.

[place figure 1 here]

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

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/m2 IV) and cyclophosphamide (600 mg/m2 IV) (AC) chemotherapy every 2-3 weeks for four cycles. Those participants who achieved a pCR following THP could avoid AC per physician preference. Post-mastectomy maintenance trastuzumab (6 mg/kg IV) and pertuzumab (640 mg IV) was administered every 21 days for an additional 12 cycles to complete a total of 12 months of anti-HER2 therapy. Post-mastectomy 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) was administered 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 4-6 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 (OCT) solution to ensure tissue integrity and stored at -80oC 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. The proportion of TILs was scored as low (0-10%), intermediate (11-59%), and high (≥60%) based upon the International TILs Working Group Guidelines (17). CelTIL score, which considers tumor cellularity in addition to TILs, was also assessed in paired breast biopsies from D1 and D8 (18).

**RNA-Seq Library Preparation and Sequencing**

RNA-sequencing was performed on tumor tissue obtained from both the pre-treatment 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 2x75 pb configuration with an average of 56 million (M) reads per sample (range 15-91M).

**RNA-Seq Analysis**

Gene-level counts were generated using bulk RNA-seq pipeline in bcibo-nextgen framework (17). Specifically, reads were aligned with STAR (18) to the hg38 reference genome, and the alignments were used to create QC reports using metrics from samtools (19), qualimap (<http://qualimap.bioinfo.cipf.es/>), fastqc (<https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>), multiqc (20) <https://multiqc.info/>, and STAR. Then, we used salmon (21) to pseudo-align the raw reads and to quantify transcript-level expression counts against hg38/ensembl v94 transcriptome reference. We combined transcript-level counts into gene-level counts with tximport (22). We performed quality control and differential expression analysis using R (R Core Team (2021),, tidyverse (23), DESeq2 (24), DEGreport (<http://lpantano.github.io/DEGreport/>), ggrepel, and pROC (25). We used ggplot2 (26),<https://ggplot2.tidyverse.org>), ggplotify (https://github.com/GuangchuangYu/ggplotify), and pheatmap (https://cran.r-project.org/web/packages/pheatmap/index.html) for visualization. For functional analysis we used GSEA (27, 28), FARDEEP (29), and clusterprofiler (30). The source code for all analyses is available as a GitHub repository (<https://github.com/hbc/overmoyer2021_RNAseq_analysis_of_inflammatory_breast_cancer_hbc04141>).

We performed a comprehensive quality control of RNA-seq data, 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. We also explored inter-correlation between all samples, performed covariates analysis, mean variants QC plots and size factor QS. We confirmed that all samples but one (Study 6, Sample 3373-3) were suitable for a robust downstream differential expression analysis. QC reports are available in the GitHub repository.

**Differential Gene Expression and Pathway Enrichment**

Differential gene expression scores were computed using DESeq2 1.30.1 (24), with the exact protocol available in the GitHub repository. Differential expression models included the biopsy timepoint (D1/D8), response (pCR/non-pCR), ER status, tumor purity of the samples (high purity vs low purity), 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 vs. 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), 6,290 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 objective of this phase II trial was to estimate pCR rate after neoadjuvant therapy with TPH for 16 weeks in 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 (≤15% vs ≥40%; target α=0.05 power=0.90). All participants who initiated the preoperative treatment were considered as evaluable for the treatment endpoints. The pCR rate with two-sided 90% confidence interval that accounted for the two-stage design was reported (31); two-sided exact binomial confidence interval was reported for the RCB rate. Up to 30 participants were planned to be enrolled, allowing up to 3 participants not evaluable for the pCR endpoint; enrollment was stopped after 23 participants because >7 pCRs had been observed.

**Results**

**Clinical Outcome**

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 institution (Dana-Farber Cancer Institute, University of Michigan Health). Thirty-one patients were screened and 8 were found to be ineligible due to the presence of visceral metastatic disease. The median age was 48 years (range, 32 to 74 years) and 48% of participants had hormone receptor-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, D8) were obtained in all 23 participants. Twenty-one participants underwent 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.

**Table 1**: **Baseline characteristics of participants and distribution of pathologic disease response at surgery, according to residual cancer burden (RCB) (16)**

|  |  |  |
| --- | --- | --- |
| **Characteristic** | **Total N=23** | |
| **Age, median (range)** | 48 years (32 - 74) | |
| **Hormone receptor status** |  | |
| Negative | 11 (48%) | |
| Positive | 12 (52%) | |
| **Clinical stage (cT4d) (32)** |  | |
| 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%) |  |

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

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 which occurred in one participant (hyperglycemia and acute kidney 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 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. 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 entry) 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%).

**Tumor Infiltrating Lymphocyte (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, two (9%) had intermediate levels, and none had high levels of TILs (33). There was an increase in the level of TILs following the single loading dose of HP in the majority of tumor specimens: ten (45%) had an increase in TILs (**Fig 2A**), six (27%) had no change, and six (27%) had a decrease in the level of TILs. Because of the small sample size, a correlation between IHC-based measurement of TILs or CelTIL score could not be assessed (**Fig 2B**). TIL levels appeared to be similar regardless of hormone receptor status.

[place figure 2 here]

**Figure 2. Changes in tumor infiltrating lymphocytes (TILs) in tumor biopsy samples obtained pre-treatment (D1) and post-dual HER2 blockade (D8).** **A)** Representative H&E images of one selected case with an increase in TIL levels from 5% (pre-treatment, D1) to 60% following administration of dual HER2 blockade (D8). **B)** Individual changes in TIL infiltration between baseline (pre-treatment, D1) and post-dual HER2 blockade (D8) according to hormonal receptor status and pathologic response by RCB (n=22 paired patient samples). D-day; RCB-residual cancer burden

**Gene Expression Profiles Between pCR and Non-pCR**

When considering D1 and D8 data together, we observed no apparent batch effects associated with the date of sequencing, tumor percentage, hormone receptor status, and whether the biopsy was collected on D1 or D8 (**Fig 3A**). We also saw no clear separation between patients that achieved a pCR and those with residual disease (non-pCR) in the top 1,000 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 on each set of samples (**Fig 3B,C**). Projection onto the first two principal components (PCs) revealed that in both cases the second PC aligns with the signal that distinguishes pCR and non-pCR patients, and that the signal is stronger in D8 data. We quantified this observation by constructing receiver operator curves (ROC) associated with using the coordinate along the second PC to predict whether a patient will achieve a pCR (**Fig 3D**). Area under the ROC curve (AUC) was then used as a metric of success, with predictions based on D8 data observed to be more accurate (AUC=0.91) than those made from D1 data (AUC=0.79).

[figure 3 here]

**Figure 3. The signal distinguishing responders and non-responders is stronger in Day 8 data. A)** Pairwise similarities between all 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 responded to therapy, and whether Day 1 or Day 8 data was used. **B, C)** Day 1 **(B)** and Day 8 **(C)** data projected onto the first two principal components, computed on the corresponding data slices. Individual points are color red by their response status. **D)** ROC curves associated with the ability of the second principal component to distinguish pCR and non-pCR.

To gain further insight into molecular mechanisms that distinguish patients with a pCR from those with residual disease, i.e., non-pCR, we performed differential gene expression on D1 and D8 data separately. Only two genes in D1 data were differentially expressed under a false discovery rate threshold of 0.01 (**Fig 4A**). Consistent with the above result, we observed many more genes that were differentially expressed in mRNA profiles of D8 biopsies (**Fig 4B**), 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 innate immunity (macrophages, CD68), suggesting that the immune cell composition of the tumor microenvironment could play a role in whether a patient achieves pCR (34-36).

[place figure 4 here]

**Figure 4. Genes with the strongest signal distinguishing pCR from non-pCR. A, B)** Differentially expressed genes in responders vs. non-responders in D1 data **(A)** and 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.

We applied Gene Set Enrichment Analysis using gene sets associated with cancer hallmarks, curated pathways and Gene Ontology (GO) terms from the Molecular Signatures Database (See Methods). The analysis revealed 13 hallmarks that were significantly (adjusted p value < 0.05) associated with pCR and largely related to immune signaling and apoptosis. (**Fig 5A**). 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 (**Fig 5B**). 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 (**Fig 5B**). Direct inspection of individual genes revealed increases in gene expression of CD8A, CD8B, GZMA, GZMH, GZMK, PRF1, CD4, FOXP3 and LAG3 (**Fig 5C**). Similarly, a robust increase in tumor associated macrophages (CD68, CD163) was identified, (**Fig 5 B,E**). Dendritic cells may be involved in antigen presentation but did not change between pCR and non-pCR at D8 (**Fig 5D**).

[insert figure 5 here]

**Figure 5. Evaluation of tumor cell intrinsic and immune related genes and signatures.**  **A)** Hallmark gene sets enriched in the pCR compared to non-pCR at Day 8, 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 pCR compared to non-pCR at Day 8. 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 ±S.E.M. \*, P < 0.05; \*\*, P < 0.01; \*\*\*, P < 0.001; \*\*\*\*, P < 0.0001.

To further investigate the immune cell composition in the tumor microenvironment, we applied FARDEEP (29) to enumerate immune cell subsets from whole tumor tissue samples, based on the LM22 signature matrix characterizing 22 immune cell types. Deconvolution of immune cell types revealed significant changes in CD8+ T cells, T regulatory cells and macrophages between pCR and non-pCR patients (**Fig 6A, B**).

[insert figure 6 here]

**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 ±S.E.M. \*, P < 0.05; \*\*, P < 0.01; \*\*\*\*, P < 0.0001.

**Discussion**

Our study shows that a single-loading dose of dual HER2-blockade with pertuzumab and trastuzumab in newly diagnosed patients with HER2-positive IBC induces significant changes in gene-expression patterns which can be used to distinguish those tumors which 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 (7), and, more specifically, among patients with IBC (37). The results presented here suggest that an assessment of the tumor transcriptome shortly after treatment initiation (i.e., one 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 identification of those IBCs that are unlikely to have a complete pathologic response to neoadjuvant therapy may allow earlier modifications to a more effective therapy.

On-treatment tumor information has already been shown to be useful for endocrine therapy response in hormone receptor-positive, HER2-negative non-IBC. Breast cancers with high Ki67 levels (>10%) at two or four weeks after initiating neoadjuvant endocrine therapy exhibit resistance to endocrine treatment (38). Additionally, changes in gene-expression signatures between pre-treatment and on-treatment tumor biopsies of hormone receptor-positive, HER2-negative disease were better indicators of response to endocrine therapy than the evaluation of gene-expression signatures in pre-treatment tumor biopsies alone (38, 39).

Several studies have also evaluated gene-expression changes in non-IBC during the administration of neoadjuvant chemotherapy (40-42). Bownes et al (42) performed differential gene expression analysis in sequential tumor samples from a cohort of 50 patients with operable breast cancer treated with neoadjuvant chemotherapy. Tumor biopsies were taken at baseline, two weeks on treatment, mid-chemotherapy, and at the time of breast surgery. In this cohort, very few genes were found to be consistently different between tumors from patients achieving a pCR compared with non-pCR. Only one potential biomarker (AAGAB) detected in the on-treatment biopsies was found to be associated with pCR, though its overall role in breast cancer is unknown.

To date, gene-expression based studies of neoadjuvant chemotherapy in HER2-positive breast cancer have been primarily limited to evaluating the association of characteristics in pre-treatment tumor samples with subsequent pCR. Several studies evaluating dual HER2-blockade have demonstrated an association of specific baseline gene expression signatures with pCR, such as HER2-enriched molecular subtype and immune signatures, however, very few studies included patients with IBC (12, 14, 43, 44).

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 (8, 45-47). Unfortunately, because of our small sample size, we could not confirm an association between TIL levels nor CelTIL determined at D1 and D8 and pCR. However, our study suggests that upregulation of anti-tumor immunity, in both adaptive and innate immunity, as well as antigen presentation, following one dose of dual HER2-blockade may be a stronger determinant of pCR for an under-represented subtype of breast cancer. CD8+ T cells that express effector molecules granzyme and perforin are suggestive of enhanced anti-tumor activity in both baseline and on-treatment tumor samples. The increase in FoxP3 and LAG3, generally associated with immune suppression in the context of cancer therapy, may be both representative of increased immune infiltrates and potentially a new avenue for combination therapy.

Likewise, a robust increase in tumor associated macrophages was identified in our study, which can either function as pro-tumor or anti-tumor, but given the robust increase in antigen presentation signaling and pathways, they may be involved in activation of both CD8+ and CD4+ T cells, leading to anti-tumor immunity. These results elucidate the role of the immune microenvironment in response to HER2-targeted therapy and may have implications for considering immune interventions in this unique patient population.

The current study focused on a unique patient population who present with inoperable disease that requires treatment with neoadjuvant systemic therapies which are extrapolated from clinical trials primarily involving patients with non-IBC. Both the NeoSphere (44) and TRYPAENA (48) trials enrolled only 6-7% of patients with IBC, and both regimens are associated with significant toxicity. Our study exploited the exquisite sensitivity (6) 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 43% among the entire treated population and 48% among those participants who had surgery , is comparable to the pCR rate obtained in the chemo-intensive NOAH trial (49); the only neoadjuvant trial for HER2-positive breast cancer that included a large enough number of patients with IBC to perform a subset analysis.

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 over-interpretation of the identification of specific transcriptome profiles associated with subsequent pCR. Despite these limitations, this was a prospective, multi-institutional phase II clinical trial specifically designed for patients with HER2-positive IBC, a subpopulation underrepresented in clinical trials, yet possessing a unique biology that renders it more aggressive than non-IBC. Moreover, the data presented here included the results of a preplanned analyses of paired frozen tumor biopsies (pre-treatment [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 in order to identify dynamic changes in gene expression that may correlate with disease response and impact on subsequent treatment modifications in order to improve clinical outcomes for those patients diagnosed with IBC.

**Acknowledgments**

We thank all the patients for participating in this study. We thank M. Claire Remolano for her assistance in data management and Grace Winship for her assistance in clinical trial administration. We also thank Kaitlyn T. Bifolck, BA, for her assistance in manuscript preparation and submission.

**Data and Code Availability**

Computational framework made use of theO2 High Performance Computer Cluster, supported by the Research Computing Group, at Harvard Medical School ([http://rc.hms.harvard.edu](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>

<https://github.com/hbc/overmoyer2021_RNAseq_analysis_of_inflammatory_breast_cancer_hbc04141/blob/master/tables/counts.tpm.csv.gz>. All other data supporting the findings of this study are available from the corresponding authors on request.

**References:**

1. Dawood S, Lei X, Dent R, Gupta S, Sirohi B, Cortes J, et al. Survival of women with inflammatory breast cancer: a large population-based study. Ann Oncol. 2014;25(6):1143-51.

2. Ueno NT, Espinosa Fernandez JR, Cristofanilli M, Overmoyer B, Rea D, Berdichevski F, et al. International Consensus on the Clinical Management of Inflammatory Breast Cancer from the Morgan Welch Inflammatory Breast Cancer Research Program 10th Anniversary Conference. J Cancer. 2018;9(8):1437-47.

3. Dawood S, Broglio K, Gong Y, Yang WT, Cristofanilli M, Kau SW, et al. Prognostic significance of HER-2 status in women with inflammatory breast cancer. Cancer. 2008;112(9):1905-11.

4. Curigliano G. Inflammatory breast cancer and chest wall disease: The oncologist perspective. Eur J Surg Oncol. 2018;44(8):1142-7.

5. Gianni L, Eiermann W, Semiglazov V, Manikhas A, Lluch A, Tjulandin S, et al. Neoadjuvant chemotherapy with trastuzumab followed by adjuvant trastuzumab versus neoadjuvant chemotherapy alone, in patients with HER2-positive locally advanced breast cancer (the NOAH trial): a randomised controlled superiority trial with a parallel HER2-negative cohort. Lancet. 2010;375(9712):377-84.

6. Li J, Xia Y, Wu Q, Zhu S, Chen C, Yang W, et al. Outcomes of patients with inflammatory breast cancer by hormone receptor- and HER2-defined molecular subtypes: A population-based study from the SEER program. Oncotarget. 2017;8(30):49370-9.

7. Cortazar P, Zhang L, Untch M, Mehta K, Costantino JP, Wolmark N, et al. Pathological complete response and long-term clinical benefit in breast cancer: the CTNeoBC pooled analysis. Lancet. 2014;384(9938):164-72.

8. Gianni L, Pienkowski T, Im YH, Tseng LM, Liu MC, Lluch A, et al. 5-year analysis of neoadjuvant pertuzumab and trastuzumab in patients with locally advanced, inflammatory, or early-stage HER2-positive breast cancer (NeoSphere): a multicentre, open-label, phase 2 randomised trial. The lancet oncology. 2016;17(6):791-800.

9. Pernas S, Barroso-Sousa R, Tolaney SM. Optimal treatment of early stage HER2-positive breast cancer. Cancer. 2018;124(23):4455-66.

10. Byron SA, Van Keuren-Jensen KR, Engelthaler DM, Carpten JD, Craig DW. Translating RNA sequencing into clinical diagnostics: opportunities and challenges. Nat Rev Genet. 2016;17(5):257-71.

11. Pernas S, Petit A, Climent F, Pare L, Perez-Martin J, Ventura L, et al. PAM50 Subtypes in Baseline and Residual Tumors Following Neoadjuvant Trastuzumab-Based Chemotherapy in HER2-Positive Breast Cancer: A Consecutive-Series From a Single Institution. Front Oncol. 2019;9:707.

12. Fumagalli D, Venet D, Ignatiadis M, Azim HA, Jr., Maetens M, Rothe F, et al. RNA Sequencing to Predict Response to Neoadjuvant Anti-HER2 Therapy: A Secondary Analysis of the NeoALTTO Randomized Clinical Trial. JAMA Oncol. 2017;3(2):227-34.

13. Carey LA, Berry DA, Cirrincione CT, Barry WT, Pitcher BN, Harris LN, et al. Molecular Heterogeneity and Response to Neoadjuvant Human Epidermal Growth Factor Receptor 2 Targeting in CALGB 40601, a Randomized Phase III Trial of Paclitaxel Plus Trastuzumab With or Without Lapatinib. J Clin Oncol. 2016;34(6):542-9.

14. Llombart-Cussac A, Cortes J, Pare L, Galvan P, Bermejo B, Martinez N, et al. HER2-enriched subtype as a predictor of pathological complete response following trastuzumab and lapatinib without chemotherapy in early-stage HER2-positive breast cancer (PAMELA): an open-label, single-group, multicentre, phase 2 trial. Lancet Oncol. 2017;18(4):545-54.

15. Wolff AC, Hammond MEH, Hicks DG, Dowsett M, McShane LM, Allison KH, et al. Recommendations for Human Epidermal Growth Factor Receptor 2 Testing in Breast Cancer: American Society of Clinical Oncology/College of American Pathologists Clinical Practice Guideline Update. Journal of Clinical Oncology. 2013;31(31):3997-4013.

16. Symmans WF, Peintinger F, Hatzis C, Rajan R, Kuerer H, Valero V, et al. Measurement of residual breast cancer burden to predict survival after neoadjuvant chemotherapy. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2007;25(28):4414-22.

17. Chapman B. ea. Validated, scalable, community developed variant calling, RNA-seq and small RNA analysis. <https://doi.org/10.5281/zenodo.3564938>. 2021.

18. Dobin A, Davis CA, Schlesinger F, Drenkow J, Zaleski C, Jha S, et al. STAR: ultrafast universal RNA-seq aligner. Bioinformatics. 2013;29(1):15-21.

19. Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, et al. The Sequence Alignment/Map format and SAMtools. Bioinformatics. 2009;25(16):2078-9.

20. Ewels P, Magnusson M, Lundin S, Kaller M. MultiQC: summarize analysis results for multiple tools and samples in a single report. Bioinformatics. 2016;32(19):3047-8.

21. Patro R, Duggal G, Love MI, Irizarry RA, Kingsford C. Salmon provides fast and bias-aware quantification of transcript expression. Nat Methods. 2017;14(4):417-9.

22. Soneson C, Love MI, Robinson MD. Differential analyses for RNA-seq: transcript-level estimates improve gene-level inferences. F1000Res. 2015;4:1521.

23. Wickham H, Averick M, Bryan J, Chang W, McGowan L, Francois R, et al. Welcome to the Tidyverse. the Journal of Open Source Software. 2019;4(43).

24. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 2014;15(12):550.

25. Robin X, Turck N, Hainard A, Tiberti N, Lisacek F, Sanchez JC, et al. pROC: an open-source package for R and S+ to analyze and compare ROC curves. BMC Bioinformatics. 2011;12:77.

26. Wickham H. ggplot2: Elegant Graphics for Data Analysis. Second Edition ed: Springer International Publishing; 2016.

27. Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proc Natl Acad Sci U S A. 2005;102(43):15545-50.

28. Mootha VK, Lindgren CM, Eriksson KF, Subramanian A, Sihag S, Lehar J, et al. PGC-1alpha-responsive genes involved in oxidative phosphorylation are coordinately downregulated in human diabetes. Nature genetics. 2003;34(3):267-73.

29. Hao Y, Yan M, Heath BR, Lei YL, Xie Y. Fast and robust deconvolution of tumor infiltrating lymphocyte from expression profiles using least trimmed squares. PLoS Comput Biol. 2019;15(5):e1006976.

30. Yu G, Wang LG, Han Y, He QY. clusterProfiler: an R package for comparing biological themes among gene clusters. OMICS. 2012;16(5):284-7.

31. Atkinson EN, Brown BW. Confidence limits for probability of response in multistage phase II clinical trials. Biometrics. 1985;41(3):741-4.

32. Hortobagyi G, Connolly JL, D'Orsi C, Edge SB, Mittendorf EA, Rugo HS, et al. AJCC Cancer Staging Manual. Eighth Edition ed. Chicago, Illinois: The American College of Surgeons (ACS)

Springer; 2017.

33. Salgado R, Denkert C, Demaria S, Sirtaine N, Klauschen F, Pruneri G, et al. The evaluation of tumor-infiltrating lymphocytes (TILs) in breast cancer: recommendations by an International TILs Working Group 2014. Annals of oncology : official journal of the European Society for Medical Oncology / ESMO. 2015;26(2):259-71.

34. Bertucci F, Boudin L, Finetti P, Van Berckelaer C, Van Dam P, Dirix L, et al. Immune landscape of inflammatory breast cancer suggests vulnerability to immune checkpoint inhibitors. Oncoimmunology. 2021;10(1):1929724.

35. Reddy SM, Reuben A, Barua S, Jiang H, Zhang S, Wang L, et al. Poor Response to Neoadjuvant Chemotherapy Correlates with Mast Cell Infiltration in Inflammatory Breast Cancer. Cancer Immunol Res. 2019;7(6):1025-35.

36. Bertucci F, Finetti P, Colpaert C, Mamessier E, Parizel M, Dirix L, et al. PDL1 expression in inflammatory breast cancer is frequent and predicts for the pathological response to chemotherapy. Oncotarget. 2015;6(15):13506-19.

37. Biswas T, Jindal C, Fitzgerald TL, Efird JT. Pathologic Complete Response (pCR) and Survival of Women with Inflammatory Breast Cancer (IBC): An Analysis Based on Biologic Subtypes and Demographic Characteristics. Int J Environ Res Public Health. 2019;16(1).

38. Dowsett M, Smith IE, Ebbs SR, Dixon JM, Skene A, A'Hern R, et al. Prognostic value of Ki67 expression after short-term presurgical endocrine therapy for primary breast cancer. J Natl Cancer Inst. 2007;99(2):167-70.

39. Ellis MJ, Suman VJ, Hoog J, Goncalves R, Sanati S, Creighton CJ, et al. Ki67 Proliferation Index as a Tool for Chemotherapy Decisions During and After Neoadjuvant Aromatase Inhibitor Treatment of Breast Cancer: Results From the American College of Surgeons Oncology Group Z1031 Trial (Alliance). J Clin Oncol. 2017;35(10):1061-9.

40. Korde LA, Lusa L, McShane L, Lebowitz PF, Lukes L, Camphausen K, et al. Gene expression pathway analysis to predict response to neoadjuvant docetaxel and capecitabine for breast cancer. Breast Cancer Res Treat. 2010;119(3):685-99.

41. Magbanua MJ, Wolf DM, Yau C, Davis SE, Crothers J, Au A, et al. Serial expression analysis of breast tumors during neoadjuvant chemotherapy reveals changes in cell cycle and immune pathways associated with recurrence and response. Breast Cancer Res. 2015;17:73.

42. Bownes RJ, Turnbull AK, Martinez-Perez C, Cameron DA, Sims AH, Oikonomidou O. On-treatment biomarkers can improve prediction of response to neoadjuvant chemotherapy in breast cancer. Breast Cancer Res. 2019;21(1):73.

43. Bianchini G, Pusztai L, Pienkowski T, Im YH, Bianchi GV, Tseng LM, et al. Immune modulation of pathologic complete response after neoadjuvant HER2-directed therapies in the NeoSphere trial. Ann Oncol. 2015;26(12):2429-36.

44. Gianni L, Pienkowski T, Im YH, Roman L, Tseng LM, Liu MC, et al. Efficacy and safety of neoadjuvant pertuzumab and trastuzumab in women with locally advanced, inflammatory, or early HER2-positive breast cancer (NeoSphere): a randomised multicentre, open-label, phase 2 trial. The lancet oncology. 2012;13(1):25-32.

45. Nuciforo P, Pascual T, Cortes J, Llombart-Cussac A, Fasani R, Pare L, et al. A predictive model of pathologic response based on tumor cellularity and tumor-infiltrating lymphocytes (CelTIL) in HER2-positive breast cancer treated with chemo-free dual HER2 blockade. Annals of oncology : official journal of the European Society for Medical Oncology / ESMO. 2018;29(1):170-7.

46. Salgado R, Denkert C, Campbell C, Savas P, Nuciforo P, Aura C, et al. Tumor-Infiltrating Lymphocytes and Associations With Pathological Complete Response and Event-Free Survival in HER2-Positive Early-Stage Breast Cancer Treated With Lapatinib and Trastuzumab: A Secondary Analysis of the NeoALTTO Trial. JAMA Oncol. 2015;1(4):448-54.

47. Denkert C, von Minckwitz G, Darb-Esfahani S, Lederer B, Heppner BI, Weber KE, et al. Tumour-infiltrating lymphocytes and prognosis in different subtypes of breast cancer: a pooled analysis of 3771 patients treated with neoadjuvant therapy. Lancet Oncol. 2018;19(1):40-50.

48. Schneeweiss A, Chia S, Hickish T, Harvey V, Eniu A, Waldron-Lynch M, et al. Long-term efficacy analysis of the randomised, phase II TRYPHAENA cardiac safety study: Evaluating pertuzumab and trastuzumab plus standard neoadjuvant anthracycline-containing and anthracycline-free chemotherapy regimens in patients with HER2-positive early breast cancer. Eur J Cancer. 2018;89:27-35.

49. Gianni L, Eiermann W, Semiglazov V, Lluch A, Tjulandin S, Zambetti M, et al. Neoadjuvant and adjuvant trastuzumab in patients with HER2-positive locally advanced breast cancer (NOAH): follow-up of a randomised controlled superiority trial with a parallel HER2-negative cohort. Lancet Oncol. 2014;15(6):640-7.