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IDENTIFYING PREDICTIVE MARKERS OF RESPONSE AND RESISTANCE TO POTENT MULTI-AGENT HER2-TARGETED THERAPY

Mentors

Sabino De Placido, M.D.

Rachel Schiff, Ph.D.

Candidate

Carmine De Angelis, M.D.

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CHAPTER 1: Introduction and Background

1.1 Breast cancer overview

Breast cancer is the most common cancer, and the second leading cause of cancer death, in women worldwide [1]. Every year, invasive breast cancer is diagnosed in about 240,000 women and 2,600 men in the United States [1]. The etiology of the vast majority of breast cancer is unknown, although several risk factors have been established including family history of breast cancer, increasing patient age, early menarche, older age at first childbirth, prolonged hormone therapy replacement, and genetic mutations (e.g. BRCA1/2 genes) [1].

Proliferative breast abnormalities are limited to the lobular and the ductal epithelium. In both these tissues, a spectrum of proliferative lesions may be seen, including hyperplasia, atypical hyperplasia, *in situ* carcinoma, and invasive carcinoma. Approximately 85% to 90% of invasive carcinomas originate from the ducts (ductal carcinoma) [1].

Disease stage is commonly assessed according to the TNM system, which stages breast cancer based on tumor size (T), extent of spread to nearby lymph nodes (N), and the presence or absence of distant metastases (M) [2]. Stage is expressed as a number on a scale of 0 through IV based on the T, N, and M. Stage 0 describes non-invasive breast cancers (in situ) that remain within their original location (either duct or lobules). The invasive breast cancer staging range from stage I with smaller size tumors with no or microscopic nodal involvement to stage IV describing invasive cancers that have spread beyond the breast and nearby lymph nodes to distant organs [2].

General breast cancer treatments include surgery, radiation therapy, chemotherapy, and targeted therapies [2]. Breast cancer is a molecular and pathological heterogeneous disease. Treatment decisions are based on pathological prognostic variables, such as tumor stage and histological grade, and on the expression of three predictive markers of response to therapy: the estrogen receptor (ER), the progesterone receptor (PR), and the human epidermal growth factor receptor 2 (HER2) [2].

Endocrine therapies, which target ER activity, represent the main therapeutic option for patients with hormone receptor (HR)-positive breast cancers (ER-positive and/or PR-positive). HER2-

targeting agents are administered in patients with HER2-positive breast cancer; whereas, no targeted therapies are currently available for treatment of triple-negative (ER/PR/HER2 negative) breast cancer [3].

Recently, gene expression profiling studies have designated six major breast cancer molecular subtypes (Luminal A, Luminal B, HER2-enriched, Claudin-low, Basal-like, and Normal Breast-like) [4, 5] that overlap with the conventional clinical-pathological classification systems, correlate with clinical behavior, and potentially predict for treatment response [4, 5]. Within HR-positive/HER2-negative early breast cancer, luminal A subtype is associated with better 10-year outcome regardless of systemic therapy and a lower risk of distant recurrence after 5 years of endocrine therapy compared to luminal B subtype [4]. Within clinically HER2-positive disease, patients with HER2-enriched disease seem to benefit the most from neoadjuvant anti-HER2 treatment in combination with chemotherapy, and patients with HER2-positive/Luminal A disease seem to have a relative better outcome compared to the other subtypes [4].

1.2 The Human Epidermal growth factor Receptor (HER) family: structure and function

The human epidermal growth factor (EGF) receptor (HER) family contains four members: EGFR/HER1, HER2, HER3, and HER4 [6]. These structurally related receptors consist of an extracellular ligand-binding region, a single transmembrane segment, and an intracellular tyrosine kinase domain [7, 8]. HER2 is the only member for which a specific ligand has not been identified, whereas HER3 lacks intrinsic kinase activity [7, 8]. Growth factor binding favors homo- and/or heterodimerization of the HER receptors, followed by transphosphorylation of their kinase domains and activation of their downstream signaling through the phosphatidylinositol-3 kinase (PI3K)/Akt and Ras/mitogen-activated protein kinase (MAPK) cascades which regulate cell proliferation, differentiation, invasion, and apoptosis [7, 8].

1.3 HER2-positive breast cancer

Dysregulated expression and activation of HER family members is frequent in several epithelial cancers, including breast cancer. The HER2 (erbB2) gene is amplified and/or overexpressed in about 15-20% of breast cancers, which clinically defines the HER2-positive breast cancer subtype [6]. HER2-status is routinely assessed by immunohistochemistry (IHC) or by measuring

the number of HER2 gene copies using in situ hybridization (ISH) techniques. According to the American Society of Clinical Oncology (ASCO)/College of American Pathologists (CAP), breast cancers are classified as HER2-positive if they are scored as 3+ by an IHC method defined as uniform intense membrane staining of more than 10% of invasive tumor cells, or demonstrate HER2 gene amplification by an ISH method (average *HER2* copy number ≥ 6.0, *HER2*/*chromosome 17 centromeres (CEP17)* ratio ≥ 2.0) [9]. When HER2 is overexpressed, it is constitutively dimerized and active leading to increased proliferation, angiogenesis, and reduced apoptosis [6]. Historically, HER2 positivity has been associated with aggressive disease and poor prognosis. However, the introduction of effective HER2-targeting treatments in the clinic has changed the natural history of HER2-positive breast cancer [6].

1.4 HER2-targeting breast cancer therapies and mechanisms of resistance

Four agents targeting the HER2 receptor are the available treatments for HER2-positive breast cancer, while many others are under clinical development [6].

Trastuzumab, a monoclonal antibody (mAb) that binds to subdomain IV of the HER2 extracellular domain, was the first anti-HER2 targeted drug approved by the US Food and Drug Administration (FDA) [6]. Although the exact antitumor mechanism of trastuzumab is not known, several potential mechanisms of tumor cell stasis and/or death have been identified in the preclinical setting. These include inactivation of intracellular downstream signaling of HER2, activation of antibody-dependent cellular cytotoxicity (ADCC), and inhibition of extracellular domain cleavage, internalization, and degradation of the receptor [6]. In patients with HER2-positive metastatic breast cancer, trastuzumab, in combination with chemotherapy, resulted in significant improvements in progression-free survival (PFS) and overall survival (OS) compared to chemotherapy alone, and has represented the standard first-line treatment for many years [6]. The efficacy of trastuzumab was also confirmed in early–stage HER2-positive disease, where pivotal clinical trials reported a higher rate of pathological complete response and improved long-term outcomes in patients who received (neo)adjuvant treatment with trastuzumab-containing regimes [10].

Pertuzumab is a second HER2-targeting mAb, which binds to the extracellular subdomain II and inhibits the ligand-dependent heterodimerization of HER2 with other HER family members, including EGFR, HER3, and HER4 [11]. The combination of trastuzumab and pertuzumab in addition to chemotherapy demonstrated significantly improved PFS and OS in patients receiving first-line treatment for metastatic breast cancer [12], and resulted in a higher proportion of pathological complete response in the neoadjuvant setting [13] . The addition of pertuzumab to trastuzumab plus chemotherapy is currently being tested in the adjuvant setting (APHINITY trial, NCT01358877).

Trastuzumab-DM1 (T-DM1) is an antibody-drug conjugate of trastuzumab and emtansine (DM1), a highly potent microtubule polymerization inhibitor [6]. T-DM1 maintains the HER2-targeting functions of trastuzumab, allowing selective delivery of DM1 to HER2-positive cells [6]. Approval in clinical practice was based on a phase III clinical trial that showed T-DM1 to be associated with prolonged PFS and OS compared with lapatinib plus capecitabine in patients with trastuzumab-pretreated MBC [14].

Lapatinib, a small-molecule dual tyrosine kinase inhibitor targeting EGFR and HER2, was approved by the FDA in 2007 for the treatment of HER2-positive, trastuzumab-resistant metastatic breast cancer [6]. Based on preclinical studies that demonstrated synergy between lapatinib and trastuzumab [15, 16], as well as phase III data in the metastatic setting showing survival benefit for the combination of lapatinib plus trastuzumab compared with lapatinib alone [17], multiple neoadjuvant trials have been conducted to assess this combination, some trials showing a significantly improved pathological complete response (pCR) with dual inhibition [18, 19] and other trials showing no difference [20]. However, lapatinib did not improve the therapeutic benefit of adjuvant trastuzumab in the adjuvant setting [21]. Second-generation irreversible HER2 inhibitors such as afatinib and neratinib are in clinical development for breast cancer and have shown promising results in both preclinical and clinical models [22-24].

Despite the proven clinical efficacy of anti-HER2 therapies, *de novo* and acquired resistance still occur and constitute a major impediment to successful treatment [6, 11]. Several mechanisms for resistance have been suggested and extensively reviewed elsewhere [6, 11]. These include

reactivation of HER signaling itself through overexpression of ligands/receptors or HER2 alterations (e.g. variants or mutations), or in the case when HER signaling remains inhibited, through activation of alternative survival pathways or hyper-activation of downstream signaling pathways (e.g. phosphoinositol-3 kinase (PI3K) pathway deregulation) [6, 11]. These escape pathways may be derived from genetic aberrations and/or epigenetic compensatory signals; and may preexist predominantly in the primary tumor, or emerge and become enriched by treatment pressure selection [6, 11].

1.5 The TBCRC 006 trial

One mechanism of resistance to trastuzumab or to other single anti-HER2 agents can arise from incomplete blockade of the receptor layer (HER1, 2, and 3) [6, 11]. This type of resistance may be circumvented by multiple anti-HER2 drug regimen that more completely blocks signals generated by homo- and hetero-dimers of the HER family members. We have previously shown in preclinical models that targeted treatment with multi-agent anti-HER2 drug regimen results in complete tumor xenograft disappearance and tumor eradication without the use of cytotoxic chemotherapy. We have also demonstrated that when ER is expressed, targeting it concurrently with HER2 is necessary to avoid treatment resistance mediated by ER. We have confirmed these findings in the phase II neoadjuvant clinical TBCRC 006 (NCT00548184) [25], a multicenter single-arm study conducted in collaboration with the Translational Breast Cancer Research Consortium. The study design and efficacy results related to pathologic complete response (pCR) are shown in Figure 1. Patients with HER2-positive invasive breast carcinomas, assessed by IHC or FISH according to ASCO/CAP guidelines of 2007 [26], that were >3 cm by clinical measurement or >2 cm with a palpable ipsilateral axillary lymph node, were eligible. Study participants were treated for 12 weeks with lapatinib (1000 mg orally every day) and trastuzumab (4 mg/kg loading dose followed by 2 mg/kg per week). If the tumor was ER-positive and/or PR-positive by IHC (according to ASCO/CAP 2010 guidelines [27]), patients were also treated with the aromatase inhibitor letrozole (2.5 mg orally once per day; combined with a LHRH agonist of choice in premenopausal women). Tumor biopsies were collected at baseline (before treatment), and after 2, 8, and 12 weeks of treatment. Collected tissues were placed in formalin for subsequent paraffin embedding (formalin-fixed paraffin embedded tissue, FFPE) or flash frozen on dry ice.

The rate of pCR, defined as disappearance of all invasive carcinoma in the breast, was assessed at the time of surgery according to the guidelines and practice of participating institutions. Sixty-six patients were enrolled, and 64 were eligible and evaluable for response. Median tumor size was 6 cm (range, 1.5 to 30 cm). The overall pCR rate was 27% (36% in ER-negative tumors and 21% in ER-positive tumors), and an additional 22% of patients were down-staged to residual tumor size <1 cm.

1.6 A new de-escalation approach for the treatment of HER2-positive breast cancer

In patients with locally advanced HER2-positive breast cancer, dual HER2 blockade with lapatinib plus trastuzumab, without cytotoxic chemotherapy, resulted in a substantial pCR rate. These results support the hypothesis that selected patients with HER2-positive tumors may be safely treated with targeted therapy alone. Nevertheless, the majority of patients fail to achieve pCR, suggesting the presence of resistance mechanisms and the need for alternative or additive treatments. Thus, the identification and the validation of biomarkers able to predict sensitivity to dual anti-HER2 therapy alone is crucial for the development of more tailored treatment strategies that may translate to improved outcomes and quality of life for patients with HER2-positive breast cancer.

Deregulation of the PI3K pathway and the accumulation and activation of immune effector cells in tumor tissue have been suggested as potential determinants of resistance and response to HER2-targeting therapies [11, 28]. This thesis investigated the predictive value of these two components in patients enrolled on the TCBCR 006 who received a neo-adjuvant dual anti-HER2 treatment without chemotherapy.

CHAPTER 2: The role of low PTEN and activating mutations in phosphoinositol-3 (PI3) kinase (*PIK3CA*) in predicting resistance to neoadjuvant lapatinib and trastuzumab without chemotherapy in patients with HER2-positive breast cancer

2.1 INTRODUCTION

The phosphoinositide 3-kinase (PI3K) pathway is one of the main downstream signaling pathways of HER2 [29]. PI3K heterodimers, which belong to the class IA of PI3Ks, consist of a catalytic subunit (p110α, p110β, p110γ, or p110δ) and a regulatory subunit (p85) [29, 30]. Activated PI3K catalyzes the conversion of phosphatidylinositol bisphosphate to phosphatidylinositol triphosphate, which leads to the phosphorylation of the serine/threonine kinase AKT and to the subsequent activation of several downstream effectors that promote cellular proliferation, survival, metabolism, and motility [29, 30]. The PI3K pathway is negatively regulated by PTEN (phosphatase and tensin homolog), a phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase encoded by the tumor suppressor gene PTEN on chromosome 10 [29, 30].

Dysregulation of the PI3K pathway, due to activating mutations of the *PIK3CA* gene encoding the p110α catalytic subunit of PI3K and to loss-of-function mutations or epigenetic silencing of PTEN, is commonly observed in breast cancer, and has been implicated in resistance to HER2-targeting therapy in preclinical and clinical models [29-40]. Therefore, *PIK3CA* mutation status and PTEN represent two candidate biomarkers with a potential role in the prediction of pCR after anti-HER2 treatment.

In two prior neoadjuvant trials that included chemotherapy we reported that *PIK3CA* mutations or PTEN loss were associated with resistance to trastuzumab but not lapatinib [41]. Similar to our findings, Xia *et al.* reported that lapatinib antitumor activity in HER2-positive breast cancer was not dependent upon PTEN status [42]. In contrast to our findings, however, recent data from two large adjuvant trials of trastuzumab with chemotherapy showed no effect of PTEN loss on disease-free survival [43, 44]. It is possible that simultaneous use of chemotherapy with HER2-targeting therapy might have obscured the mechanisms of resistance to the targeted therapy itself. Furthermore, complete loss of PTEN is not required to activate the PI3K pathway, suggesting the possibility that this arbitrary cut-off might not be correct [45].

In the present analysis we evaluated baseline, pretreatment tumor biopsies from patients on the TBCRC 006 trial that investigated lapatinib and trastuzumab (combined with endocrine therapy if the tumor was also ER-positive), but did not include chemotherapy. We hypothesized that activating *PIK3CA* mutations or decreased expression of PTEN will correlate with *de novo* resistance (or notably decreased response) to this dual anti-HER2 therapy.

2.2 METHODS

PTEN status analysis

PTEN expression was assessed by immunohistochemistry (IHC) on freshly cut 4μm tissue sections of FFPE specimens that were deparaffinized, followed by heat-mediated antigen retrieval using 0.1M Tris-HCl (pH 9.0) buffer. To decrease nonspecific binding, sections were first treated with 3% hydrogen peroxide solution. Tissues were then incubated for 1 hour at room temperature with rabbit polyclonal PTEN (D4.3) antibody (Cell Signaling, Beverly, MA) at 1:100 in SignalStain antibody diluents (Cell Signaling). Detection and expression were done with Envision Labelled Polymer-HRP Anti-Rabbit (Dako, Carpenteria, CA), DAB+ solution (Dako), and DAB Sparkle Enhancer (Biocare, Concord, CA). Staining was evaluated by two pathologists (A.C. and S.H.) who were blinded to outcome. As a specimen's internal control, stromal cells were assessed for positive staining. Each section was scored for percent positivity (0-100%) and level of intensity (0-3). The H-score of each sample was calculated by multiplying the percentage and intensity scores.

PIK3CA mutation analysis

Tumor genomic DNA was extracted from frozen tissue using the AllPrep DNA/RNA Mini Kit (Qiagen, Valencia, CA) or from FFPE tissue using the Maxwell CSC DNA FFPE Kit in combination with the Maxwell CSC Instrument (Promega, Madison, WI) according to the manufacturer's suggested protocols. *PIK3CA* mutations were determined by NGS or Sanger sequencing. NGS utilized the Ion AmpliSeq^{TM} Kit 2.0 and the Ion Ampliseq^{TM} Cancer Hotspot Panel v2 (Life Technologies, Carlsbad, CA) according to the manufacturers' protocols. Sequencing alignment, variant calling, and curation were carried out by using the Ion Torrent Suite (TS) software V3.6 and TS Variant Caller Plugin software V3.4.1 (Life Technologies) and

as previously described [46]. For Sanger sequencing, genomic DNA from tumor samples was used for PCR amplification of exons 10 and 20 of the *PIK3CA* gene (mRNA reference sequence: NM_006218.2). Primers for both exons flank the respective intron/exon junctions. Direct sequence analysis of PCR products was performed in both forward and reverse directions using automated fluorescence dideoxy sequencing methods. Reference sequence NT_005612.16 is used for wild type *PIK3CA* sequence comparison. The output sequences were analyzed using Mutation Surveyor version 3.97 (Soft Genetics). The mutation nomenclature is based on the convention recommended by the Human Genome Variation Society (http://www.hgvs.org/mutnomen/). All mutations were confirmed by a different technology.

Statistical analysis

Data were summarized by descriptive analysis. Chi-square tests (not continuity corrected; used when expected values > 5) or Fisher's Exact tests were performed to assess the associations of pCR with PTEN, *PIK3CA*, or the combination, and of PTEN and *PIK3CA* with ER and with each other. Odds ratios and 95% confidence intervals were calculated to measure the strength of the associations, with adjustment for empty cells as suggested by Agresti [47].

2.3 RESULTS

Patient demographic and clinical characteristics are summarized in Table 1. Sixty-six patients were enrolled of whom 64 patients were evaluable. Seventeen patients had a pCR (27%). The rate of pCR was 21% in the ER-positive subgroup and 36% in the ER-negative subgroup.

Tissue at baseline was available from 59 evaluable patients for PTEN IHC studies and 38 had enough tissue to make DNA for *PIK3CA* mutation analysis (Figure 2). Fourteen out of the 59 patients with baseline PTEN analysis had a pCR (24%) and 7 out of the 38 patients with *PIK3CA* analysis had a pCR (18%). In the 35 patients where both PTEN and *PIK3CA* analysis were performed, five had a pCR (14%).

PTEN immunohistochemistry

Since reduced expression of PTEN can contribute to downstream activation of the PI3K pathway, we measured PTEN by IHC and correlated its expression level with pCR. PTEN IHC

was successfully performed and scored on all 59 available baseline biopsy tumors. PTEN median H-score was 100 (range 0-300). Figure 3 shows examples of results of staining demonstrating negative, weak, and strong staining.

Since enhanced PI3K activation could result from not only complete loss of PTEN but also its low expression, PTEN status was dichotomized by the median H-score of 100 as low (< 100) or high (≥100). When correlated with pCR (Table 2), we found a remarkable difference, where pCR was observed in 12 out of 37 patients (32%) with high PTEN expression and only 2 out of 22 patients (9%) with low PTEN (p=0.04) (Table 2). Complete PTEN loss by IHC was found in only 6 (10%) patients. One of these 6 (17%) patients had a pCR, while there were 13 pCRs (25%) out of 53 patients with any PTEN expression (p=1.0). Thus, the median value proved a better discriminator.

PIK3CA mutation analysis

PIK3CA mutations may result in activation of the PI3K pathway downstream from HER2, negating the impact of receptor inhibition approaches, and resulting in treatment resistance.

In the 38 patient samples with sufficient material to make DNA and sequence for *PIK3CA* mutations, 12 exhibited activating mutations (32% of samples). Table 3 shows details of the observed mutations. Three of them were in the helical and nine in the catalytic domain. As Table 2 shows, seven out of 26 patients with wild-type *PIK3CA* achieved pCR (27%). However, no patient with an activating mutation (0/12) achieved pCR (0%, p=0.07).

Cohort with known *PIK3CA* mutation and PTEN status

We next turned to the cohort in which both *PIK3CA* mutational status and PTEN expression level were known. As these are both surrogates for PI3K pathway activation, we aimed to investigate their correlation with pCR. The cohort with known PTEN expression and mutation analysis included 35 patients (Table 2). Out of the 16 patients whose tumors expressed high PTEN levels and had no *PIK3CA* mutation, 5 patients had a pCR (31%). In contrast, no patient (0/19) achieved pCR whose tumor harbored a mutation or had low PTEN expression (p=0.01) (Table 2).

Moreover, *PIK3CA* mutations and PTEN status were independent of ER status (p=0.76 and 0.72, respectively) and of each other (p=0.71), suggesting these to be independent variables or that a larger sample may be required to detect a correlation.

2.4 DISCUSSION

Potent inhibition of HER2 with combination therapy targeting HER family receptors has shown a clinically significant response and also pCR in the neoadjuvant setting without chemotherapy. We have previously reported the results of a clinical trial showing a pCR rate of 27% using the chemotherapy-sparing regimen of lapatinib and trastuzumab (with endocrine therapy in patients with ER-positive tumors) [25]. The NeoSphere trial showed a similar result using trastuzumab plus pertuzumab [13]. The potential of treating a subset of patients with targeted therapy alone and without chemotherapy is clinically meaningful as it would spare patients the acute and long-term toxicity and cost of chemotherapy.

However, to consider the use of targeted therapy alone in the future, it is mandatory to correctly distinguish patients who would benefit from this approach from those requiring chemotherapy. Results from the recently presented ALTTO trial showed a small and non-significant benefit for adding lapatinib to standard care: chemotherapy and trastuzumab [21]. This highlights the importance of identifying patients who may benefit from a certain treatment as well as those in whom a de-escalation strategy might be pursued. Paclitaxel plus trastuzumab adjuvant therapy in patients with small, node-negative HER2-positive tumors is one de-escalation strategy recently reported. Identification of patients with resistant tumors and elucidation of causal mechanisms of resistance may lead to discovery of novel drugs that block these escape pathways and, therefore, overcome resistance. The PI3K pathway has been implicated in resistance to anti-HER2 therapy, and it is interesting to speculate that patients with PI3K pathway activation on the basis of PTEN down-regulation or *PIK3CA* mutations might benefit from the addition of inhibitors of this pathway [32-36, 38, 39].

Our study is unique in that it allowed us to understand the influence of aberrant activation of this pathway via decreased levels of PTEN and/or the presence of activating *PIK3CA* mutations in

patients treated exclusively with targeted therapy only, without the confounding effects of chemotherapy. Here, we report results of our molecular studies on baseline tumor biopsies obtained from the TBCRC 006 trial, investigating the potential role of PTEN expression and activating mutations in *PIK3CA* in resistance to lapatinib combined with trastuzumab. Our data show that low PTEN expression is associated with significantly lower response to anti-HER2 therapy with a low pCR rate of 9% vs. 32% with high PTEN. Having a significant difference in pCR when data was dichotomized by the median argues that complete loss of PTEN is not necessary, and that a partial loss may be enough to release its regulatory effect on PI3K signaling resulting in its activation, [45, 48] [45, 48] and subsequently in treatment resistance. These results are consistent with other reports suggesting that moderate reductions in PTEN are sufficient to activate the pathway.

Several studies in the neoadjuvant, the adjuvant, and the metastatic settings investigating the role of PTEN levels in response to HER2-targeted therapies have yielded conflicting results [34, 36, 38, 39, 44, 49-55]. Two large adjuvant trials of chemotherapy plus trastuzumab also evaluated PTEN level by IHC [43, 44]. These studies found no effect of PTEN level on response. However, in addition to the use of chemotherapy, which could obscure the effect of PTEN loss on resistance to trastuzumab, these studies used a different primary antibody or required complete loss of PTEN by IHC to dichotomize between low and high. Interestingly, in a recent analysis from a neoadjuvant trial, PTEN levels evaluated by a quantitative immunofluorescent assay were able to predict pCR after chemotherapy plus anti-HER2 treatment [54].

Our data suggest that low but not necessarily absent PTEN as assessed by IHC staining might activate the PI3K pathway. Only 6 patients in our study had absent PTEN staining, and its relationship with pCR was no longer significant. One possible explanation for our findings (in contrast to the larger chemotherapy/trastuzumab intergroup adjuvant trial) [43] is that dichotomization into positive and negative subsets using a cut-point of absent PTEN staining imparts less effect because of dilution of the "positive-PTEN" group of tumors with the group of "low-PTEN" tumors that have hyperactive PI3K pathway. In support of this idea are results from a recent study using a *Pten* hypermorphic mouse model [48]. This study shows that subtle downregulation of PTEN levels results in pleiotropic biological consequences in the breast tissue, including the activation of PI3K downstream signaling, induction of gene expression

signatures associated with cell cycle and tissue hyper-proliferation, and development of breast tumors at the highest frequency among a spectrum of other tumors [48]. Similarly, in a prior study using an inducible siRNA we showed that moderate reductions in PTEN activated the PI3K pathway in breast cancer cells. The fact that PTEN is infrequently inactivated at the genomic level in breast cancer, especially within the HER2-enriched and the luminal subtypes [56], further emphasizes the importance of subtle reductions in its expression for the activation of the PI3K pathway and mediating resistance to HER2-targeting therapy. A clinically useful assay to measure PTEN with appropriate cutoff values remains a challenge and warrants further study.

Our data also show that no tumors with *PIK3CA* activating mutations had a pCR (0% vs. 27% with wild type *PIK3CA*, p=0.07) showing a statistically non-significant trend, likely due to small sample size. However, this highlights the potential role of these activating mutations in resistance to anti-HER2 treatment by downstream activation of the pathway, thereby circumventing potent inhibition of the receptor layer and rendering the treatment ineffective. Similar to our findings, recent data from the large phase III NeoALTTO trial also suggested that tumors with *PIK3CA* mutations are less likely to achieve a pCR from HER2-targeting therapy combined with chemotherapy [57]. Reduced pCR rates were most pronounced in the arm of dual HER2-targeted therapy with lapatinib plus trastuzumab. The lower but still substantial pCR rates observed in tumors with *PIK3CA* mutations in NeoALTTO is likely due to the use of chemotherapy for all patients. Our results are also concordant with recent reports from the Neosphere [58] and the GeparSixto and GeparQuinto studies [59]. Of note, the two Gepar studies also suggested that the largest differences in pCR rate between PIK3CA mutant and wild type were found in patients receiving dual HER2 blockade. Although chemotherapy may partially overcome the resistance to lapatinib plus trastuzumab caused by *PIK3CA* mutations in our study, the favorable effect of HER2 inhibition on chemosensitivity would be lost in such tumors. Overcoming resistance to HER2 inhibition caused by PIK3CA mutations would likely also improve the cytotoxic effects of the chemotherapy itself by effectively blocking the HER2/PI3K/AKT cell survival pathway. The role of *PIK3CA* is different in ER-positive/HER2-negative tumors, where PIK3CA mutations are associated with luminal A tumors with a better prognosis and improved response to endocrine therapy [18].

In our study, we found no patient with either low PTEN or a *PIK3CA* activating mutation in the 35 patients for which both were measured that had a pCR. Equally important, PTEN and *PIK3CA* status were not correlated with each other or with ER expression, arguing they are independent variables that can lead to deregulation of the PI3K pathway.

Our findings also support current interest in targeting the PI3K pathway in combination with HER2-targeted therapy. This strategy is also supported by recent data in experimental models of HER2-positive PI3K-mutant breast cancer [40, 60, 61] as well as clinical evidence from a phase Ib study combining the PI3K inhibitor buparlisib (BKM120) with trastuzumab in patients with HER2+ advanced or metastatic breast cancer resistant to trastuzumab-based therapy [62]. It will be important to select appropriate patients for this approach that if successful might identify another subset of patients in whom a targeted therapy alone might be adequate, sparing them the cost and toxicity of chemotherapy.

Our study has limitations; our cohort of patients is relatively small, and we were not able to obtain PIK3CA mutational status on all tumors. Differences in pCR between *PIK3CA* mutant and wild type tumors did not achieve statistical significance on their own, albeit they did when combined with PTEN. Our results need validation in a larger cohort. Still, we observed differences in response in patients with low PTEN and *PIK3CA* mutations that are statistically significant and, if validated, will be clinically meaningful.

CHAPTER 3: Evaluation of tumor immune infiltrate as a determinant of response to neo-adjuvant lapatinib and trastuzumab without chemotherapy in patients with HER2-positive breast cancer

3.1 INTRODUCTION

The tumor immune microenvironment has recently emerged as a potential modulator of tumor progression and response to treatment [63, 64]. Tumor-infiltrating lymphocytes (TILs) are mononuclear immune cells that infiltrate tumor tissue and have been described in most types of solid tumors, including breast cancer [63, 64] . In HER2-positive breast cancer, a growing body of clinical evidence has shown that high levels of TILs and activation of immune pathways are associated with more favorable prognosis as well as increased rates of pCR with neoadjuvant therapy [65-68]. However, almost all of these data derive from trials that studied chemotherapy in combination with trastuzumab or dual anti-HER2 therapy, so that the predictive value of TILs in patients with HER2-positive disease receiving an anti-HER2 treatment alone without the confounding effect of chemotherapy is unclear.

TILs are usually detected using standard hematoxylin and eosin (H&E)-stained tissue sections under light microscopy and classified as stromal-TILs (s-TILs), which are located into the stroma surrounding tumor cells, or intratumoral-TILs (i-TILs) which are the immune cells that are in direct contact with the tumor epithelial cells [69]. In breast cancer, s-TILs have been demonstrated to be the predominant population and considered a more suitable predictive and prognostic marker compared to i-TILs [69].

Although the evaluation of TILs by standard H&E staining is simple, inexpensive, and reproducible, limitations of this approach are its semi-quantitative nature and the impossibility of discriminating subtypes of immune cells as well as their spatial organization within the immune infiltrate.

Here, we measured s-TILs in baseline tumor samples from patients on the TBCRC 006 trial by standard H&E-based method and investigated their role in predicting response to potent neoadjuvant anti-HER2 treatment without chemotherapy. We also employed an advanced multispectral imaging-based technology in a subset of tumor samples in order to characterize s-TILs and i-TILs as well as TIL subpopulations in a quantitative and spatial manner.

3.2 METHODS

Standard assessment of TILs in H&E sections

Visual assessment of TILs was performed using full-face H&E slides by light microscopy as currently recommended [69]. All mononuclear cells, including lymphocytes and plasma cells and excluding granulocytes and other polymorphonuclear leukocytes, were identified as TILs. TILs in the stromal compartment (s-TILs) were quantified as a percentage of occupied stromal areas (% s-TILs). Of the 64 patients enrolled on the TBCRC 006 trial, slides from baseline core biopsies were available for 59 (92.2%) patients. A threshold of 60% was used to distinguish cases with High s-TILs (≥ 60%) from Low s-TILs (< 60%).

Multispectral fluorescent immunohistochemistry

Multiplexed immune-fluorescent staining and multispectral image analysis [70] were applied on 35 available baseline tumor tissues from patients enrolled in the TBCRC 006 trial. Briefly, 4 μm thick formalin-fixed, paraffin-embedded (FFPE) slides were deparaffinized in a Leica autostainer using the following protocol: xylene 10 minutes, 100% ethanol 10 minutes, 95% ethanol 5 minutes, 70% ethanol 5 minutes, ddH2O briefly, 10% buffered formalin 10-20 minutes, ddH2O briefly. Antigen retrieval was performed using citrate buffer pH 6.0 and microwave treatment (45 seconds 100% power, 15 minutes 20% power). Slides were blocked with Antibody Diluent (Biogenex Technologies, Fremont, CA) for 10 minutes. Primary antibodies were diluted in Antibody Diluent and incubated for 30 minutes at room temperature. Primary antibodies were subsequently removed by vacuum, and slides were washed in TBST. Anti-rabbit or anti-mouse IgG, HRP-linked Antibodies (Life Technologies, Carlsbad, CA) were added drop-wise to slides. Slides were incubated for 10 minutes at room temperature and then washed in TBST. Tyramide (TSA)-conjugated fluorophores (Opal^{TM}, PerkinElmer, Inc., Hopkinton, MA) were added to slides at 1:50 dilution, and incubated for 10 min at room temperature. TSA were vacuumed off, and slides were washed in TBST. This process was repeated recursively for 6 antibodies (Table below). DAPI (Life Technologies, Carlsbad, CA) was diluted 1:500 in TBST and added to slides. Slides were incubated for 10 minutes at room temperature. Slides were then rinsed with ddH2O, cover slipped with VECTASHIELD Hard Mount (Vector Laboratories, Burlingame, CA), and stored at 4°C in a covered slide box. Slides were scanned using a Vectra automated multispectral

microscope and images were analyzed using the inForm analysis software (PerkinElmer, Hopkinton, MA). A tissue segmentation algorithm was applied to define stromal and tumoral areas. Then, single or multiple IF-markers were used to localize, phenotype, and quantitate the density (number of positive cells per mm^2) of specific cell types in the tumoral stroma.

Markers	**Dilution**	**Sources**	**Incubation**	**TSA-dyes**	**Clone**
CD4	1:25	Leica/Mouse	30 minutes	520	4B12
CD8	RTU	Leica/Mouse	30 minutes	570	4B11
CD20	1:100	Leica/Mouse	30 minutes	690	L26
Foxp3	1:500	CST/Rabbit	30 minutes	540	D608R
CD68	1:400	Dako/Mouse	30 minutes	650	PGM-1
CK	1:1000	Dako/Mouse	30 minutes	620	Ae1/Ae3

Statistical analysis

The predictive value of s-TILs was tested by considering s-TILs as a continuous variable, and then according to the high vs. low categorical definition. Spearman correlations and Fisher's exact test was used to examine the associations between s-TILs and other tumor baseline biomarkers. Frequency of s-TILs normalized to measured stromal area (mm^2) was summarized descriptively to characterize diversity of s-TILs patterns among tumors using single or sets of IF-markers. All statistical tests were two-sided and considered significant when $P \leq 0.05$.

3.3 RESULTS

Pretreatment s-TILs evaluation using H&E slides was conducted on 59 available samples out of the 64 patients who were evaluable for pCR. In our cohort, 12 patients (20%) exhibited High s-TILs and 37 (80%) presented with a Low s-TILs profile (Figure 4A, 4B). Patient demographic and clinical characteristics are reported in Table 4. No significant differences were found between High- and Low-s-TILs subgroups regarding age, race, ethnicity, menopausal status, tumor size, expression of hormonal receptors (ER and PR), *PI3KCA* mutational status, or PTEN levels (Table 4). Levels of s-TILs were positively associated with Ki67 (Spearman's correlation

= 0.304, p=0.02), but not with other tumor biomarkers including ER, PR, and HER2 (HER2 H-score, and HER2/CEP17 ratio) (Table 6).

Levels of s-TILs considered either as a categorical or continuous parameter were not significantly associated with pCR (High- vs. Low-, p=0.057; 10% increase OR=1.22; 95% CI 0.97- 1.53, p=0.094); however, a numerically higher pCR rate in patients with High s-TILs compared to patients with Low s-TILs was observed (50% vs. 19%) (Table 6). No significant association of s-TILs with pCR was found in ER-positive or ER-negative subgroups.

Tumor immune infiltrate in baseline tissues from 10 patients enrolled in the TBCRC 006 trial was also analyzed using a multispectral imaging-based technology. Single FFPE slides were co-stained for CD8 (cytotoxic T cells), CD4 (helper T cells), FoxP3 (T-reg cells), CD20 (B cells), CD68 (macrophages), cytokeratin (epithelial tumor cells), and DAPI, and multispectral imaging analysis was performed (Figure 5A, 5B). Multispectral imaging successfully captured multiple immune cell types in all 10 multiplexed-stained samples. Densities of specific immune subpopulations are reported in Figure 6. Notably, the total number of stromal immune cells defined by multispectral fluorescent immunohistochemistry showed a high correlation with H&E-based TILs scoring (Spearman's correlation = 0.85, P=0.003). Interestingly, quantitative assessment of various TILs subtypes in baseline specimens revealed a marked inter-patient heterogeneity in composition of stromal immune infiltrates in both High-s-TILs and Low-s-TILs groups (Figure 7).

3.4 DISCUSSION

In the last few years, the tumor immune microenvironment has become an area of intense research in breast cancer [28, 63, 64]. The tumor-host immune interaction may be particularly significant in determining the therapeutic effect of the monoclonal antibodies trastuzumab and/or pertuzumab, since their therapeutic efficacy depends in part on innate and adaptive immune-mediated mechanisms [28]. This tumor-host immune interaction may be even more important in the context of lapatinib plus trastuzumab therapy, which was used in our study. As such, it has been suggested that the synergistic effect of lapatinib and trastuzumab may stem, at least partly, from an increased trastuzumab-mediated ADCC due to a lapatinib-induced accumulation of HER2 at the cell surface as shown in preclinical models [28]. Previous reports showed that in

patients with HER2-positive breast cancer, high levels of TILs and the activation of immune pathways assessed by immune-related genes and gene signatures have been associated with more favorable prognosis in the adjuvant setting as well as increased rates of pCR with neoadjuvant treatment [28, 63, 64]. However, most published data have come from trials that studied treatment regimens of anti-HER2 therapy in combination with chemotherapy. Therefore the role of the chemotherapy component in the predictive value of the immune infiltrate could not be excluded.

Multiple studies from randomized clinical trials have investigated the role of immune-related components in predicting response to neoadjuvant anti-HER2 treatment. In the NeoALTTO trial, levels of s-TILs greater than 5% were associated with higher pCR rates independent of the anti-HER2 treatment groups [65]. Furthermore, a positive association between the quantity of s-TILs and event-free survival was also observed in this trial [65]. A retrospective analysis from the GeparQuattro and GeparQuinto studies also reported a positive predictive and prognostic value for s-TILs in HER2-positive breast cancer patients [66]. In contrast, the levels of s-TILs were not significantly associated with pCR rates in the NeoSphere trastuzumab plus pertuzumab trial [67]. Likewise, in the CherLob study, while levels of i-TILs and s-TILs were significantly correlated with pCR rates [68], the association between TILs and pCR did not maintain statistical significance in multivariate analysis corrected for molecular subtypes (using the multigene PAM50 assay) [68]. Taken together, these somewhat conflicting results about the predictive value of s-TILs in HER2-positive breast cancer patients may be attributed either to the lack of an appropriate quantitative approach and/or a standard cut-off for TILs, or to the differences in the HER2 and the chemotherapy treatment regimens among the studies.

In our study, s-TILs were evaluated using full-face H&E slides, in accordance with the current recommended methodology. In line with the previous studies [65, 67], high levels of s-TILs were observed in 20% of patients and were not associated with *PIK3CA* genotype or PTEN status. However, in contrast to other previous reports, we did not find a significant association between increased s-TILs and ER-negative status [65, 67, 68], though we did notice a positive correlation of s-TILs with the Ki67 index as also reported by the CherLob study [68]. We further observed a numerically higher pCR rate in patients with high levels of s-TILs as opposed to low s-TILs

(50% vs 19%). However, the P-value did not reach statistical significance. Notably, in our cohort, no patients with levels of stromal TILs lower than 10% achieved a pCR. Similar results have also been reported in the NeoALTTO and the NeoSphere trials, where patients with very low levels of TILs (s-TILs = <5%) showed the lowest pCR rates [65, 67].

In our study, the lack of a statistically significant association of s-TILs with pCR could be ascribed to the small patient cohort in the TBCRC 006 trial or to the lack of biological association between TILs and response to dual HER2-targeted therapy in the absence of chemotherapy. Overall, based on the literature, we believe that the tumor immune component plays a crucial role in determining the efficacy of pure dual anti-HER2 therapy, and therefore we are now extending the H&E-based analysis of s-TILs to two additional neoadjuvant lapatinib plus trastuzumab trials with no chemotherapy recently completed by us and by our collaborators.

As mentioned before, the evaluation of TILs using an H&E-based method is semi-quantitative and provides only limited information about the complexity of the tumor immune microenvironment, which may be crucial for a better understanding of the biology and clinical activity of the immune infiltrates. As such, several studies have investigated the predictive and/or prognostic value of specific immune cell markers, checkpoints, and gene expression signatures using different methodological approaches [20, 63, 64, 67, 69, 71, 72]. A correlative analysis from the Cancer and Leukemia Group B (CALGB) 40601 has identified an immune-cell infiltrate signature (IgG signature) as an independent predictor of pCR [20]. Similarly, in the GeparSixto trial, s-TILs as well as 12 immune-activating and immune-suppressive markers were found to be predictive of pCR in triple-negative and HER2-positive breast cancer patients [72].

Therefore, we attempted to further investigate the composition and spatial distribution of the immune infiltrate by adapting a multiplexed immunofluorescence technology to quantify and appropriately classify the various immune cell subtypes present in the tumor [70]. While our analysis has been conducted only on a small subset of patients so far, we have clearly demonstrated that this approach is feasible using FFPE tumor material. More importantly, the high correlation we observed between the classical visual assessment [69] of TILs and the automated digital image-based quantification of immune cells further validated this new

analytical approach. Moreover, our analysis also showed that the frequencies of various immune cell populations are extremely variable among patients in both the Low s-TILs and the High s-TILs groups. Future studies with larger patient populations are needed to investigate whether the repertoire of immune cells may add predictive and/or prognostic information to the quantification of TILs.

CHAPTER 4: Conclusion and future goals

The development of HER2-targeted therapies has greatly improved the outcome of HER2-positive breast cancer patients. Based on compelling preclinical evidence, large randomized clinical trials have focused on the benefits and toxicity of adding dual HER2 targeting agents, such as lapatinib or pertuzumab plus trastuzumab, to chemotherapy. This therapeutic strategy resulted in significant improvements of response rate and long-term outcomes in the neoadjuvant and the metastatic setting. In the adjuvant setting however, the ALTTO trial failed to demonstrate improved efficacy of dual HER2 blockade with trastuzumab and lapatinib over the standard trastuzumab treatment. A second trial evaluating adjuvant trastuzumab and pertuzumab with chemotherapy is ongoing (APHINITY) and a longer follow-up for the ALTTO trial is needed to determine the potential benefit of adding pertuzumab or lapatinib to trastuzumab [21].

However, the results from the ALTTO trial, even if they remain negative, might not necessarily indicate a failure of the dual inhibition concept but rather may imply a failure of the strategy of continuous treatment escalation. This notion also highlights the challenge of determining which patients need more and which patients need less therapy.

De-escalation of treatment may decrease toxicity and cost in many patients without compromising the clinical outcomes, as demonstrated in a recent adjuvant trial where patients with small, node-negative HER2-positive tumors were treated only with paclitaxel plus trastuzumab [73]. In our neoadjuvant TBCRC 006 trial, HER2-positive breast cancer patients treated with 12 weeks of lapatinib plus trastuzumab achieved a substantial rate of pCR without the use of chemotherapy. Similar results with the same dual anti-HER2 regimen with no chemotherapy have been recently reported by our second trial TBCRC 023 trial (NCT00999804) [74], where the extension of treatment duration from 12 to 24 weeks doubled the pCR rate, as well as by the PAMELA trial (NCT01973660), where the same anti HER2 treatment given for 18 weeks resulted in an overall pCR rate of 30% [75].

The appropriate selection of patients who will benefit from this de-escalation strategy is therefore crucial. In this regard, identifying the major determinants of sensitivity and resistance

to anti-HER2 treatment will help distinguish patients with HER2-positive disease who may be optimally treated with anti-HER2 agents alone from those who may benefit the most from a combination of anti-HER2 and cytotoxic therapies or from the addition of new agents. This is the overall goal of our research, where we hypothesize that oncogenic HER2 addiction, deregulation of the PI3K pathway, and tumor immune infiltrate represent key molecular determinants of response and resistance and that associated biomarker assays of these components would be crucial to build a molecular classifier identifying patients that may benefit from the de-escalated HER2-targeted therapy alone approach. Tumor samples from our two clinical trials (TBCRC 006 and 023) as well as from the PAMELA trial provide a unique opportunity to investigate these mechanisms of response and resistance without the confounding effect of chemotherapy.
In this thesis, studies in our first trial (TBCRC 006) have shown that, i) deregulation of the PI3K/PTEN pathway by PIK3CA mutations or low PTEN and, ii) low levels of s-TILs are associated with decreased response to dual HER2 blockade in the absence of chemotherapy. These findings possess high clinical impact and if validated could change the treatment practice for patients with HER2-positive breast cancer.

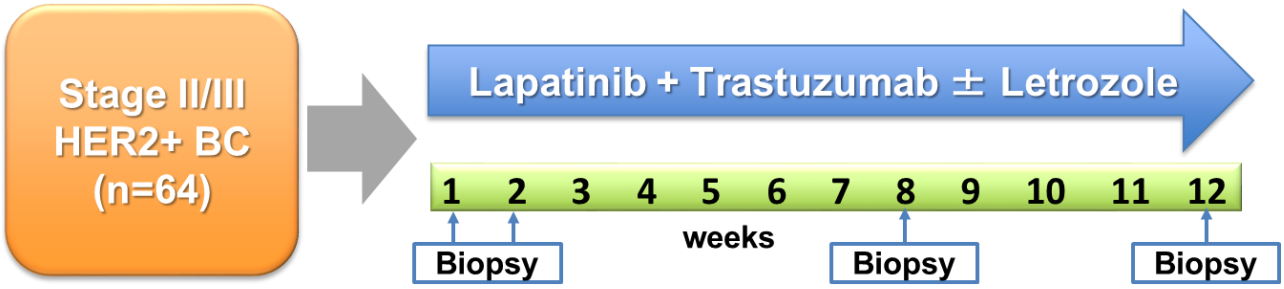
In parallel to the studies of this thesis, studies to confirm our hypothesis that levels of HER gene copy number, HER protein expression, and molecular subtyping (by PAM50 to identify the HER2-enriched subtype) can predict HER2 oncogenic addiction and response to lapatinib plus trastuzumab treatment are ongoing.

Overall we plan to confirm in the TBCRC 023 trial and validate in the PAMELA trial our early results from the TBCRC 006 trial. Through these studies we plan to identify optimal cut-points of the key molecular markers and eventually to develop a multi-parameter molecular classifier to identify patients who can be safely treated by anti-HER2 targeted therapy alone. To extend the study of my thesis, future studies will employ specimens from the TBCRC 023 and the PAMELA trial to confirm and validate our findings that activation of the PI3K pathway by PIK3CA mutations or other mutations affecting this pathway, or by down regulation of PTEN protein expression, cause resistance (lack of pCR). Likewise, parallel studies evaluating s-TILs as well as imagine-based analysis of the tumor immune infiltrate will validate the role of this

component in predicting response to the lapatinib plus tratuzumab treatment without chemotherapy in HER2-positive breast cancer.

In the future, based on the above described studies, we hope to conduct a next-generation clinical trial that will use a multi-parameter molecular classifier to identify patients who can benefit from anti-HER2 therapy alone, without chemotherapy.

FIGURES AND TABLES



	pCR	%
Overall	**17/64**	**27**
ER positive	8/39	21
ER negative	9/25	36

Figure 1. Study schema of TBCRC 006. Patients were treated for 12 weeks with LT (with endocrine therapy in ER-positive cases) followed by surgery. Serial biopsies were obtained, and only baseline samples (pre-treatment) were considered for the current study.

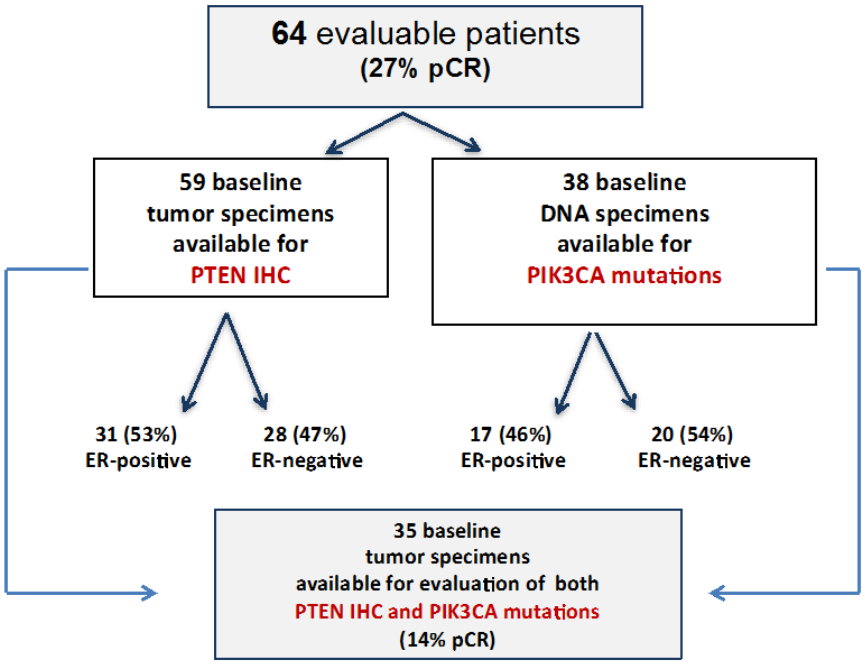


Figure 2. Evaluable material for baseline biomarker analysis and associated pathologic complete response (pCR) rates

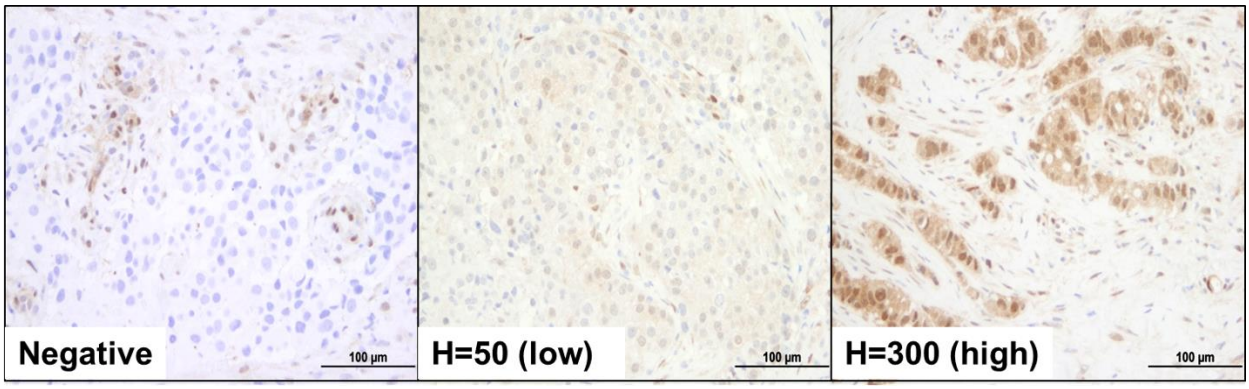


Figure 3. Immunohistochemical staining and associated H scores for PTEN in baseline specimens: Shown (left to right) are representative images for tumors with negative, low, and high PTEN expression.

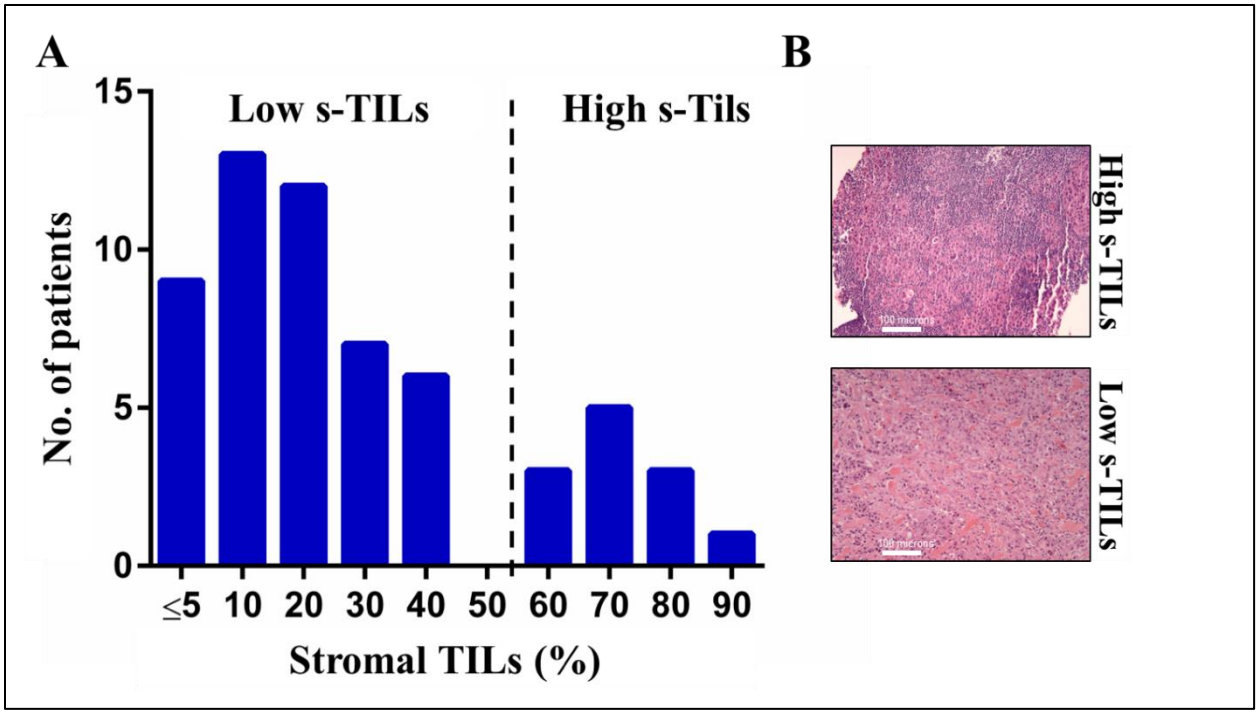


Figure 4. Evaluation of stromal TILs using H&E. A, Bar plot showing the distribution of patients according to percentage of stromal TILs. **B**, Representative images of High and Low-stromal TILs H&E staining.

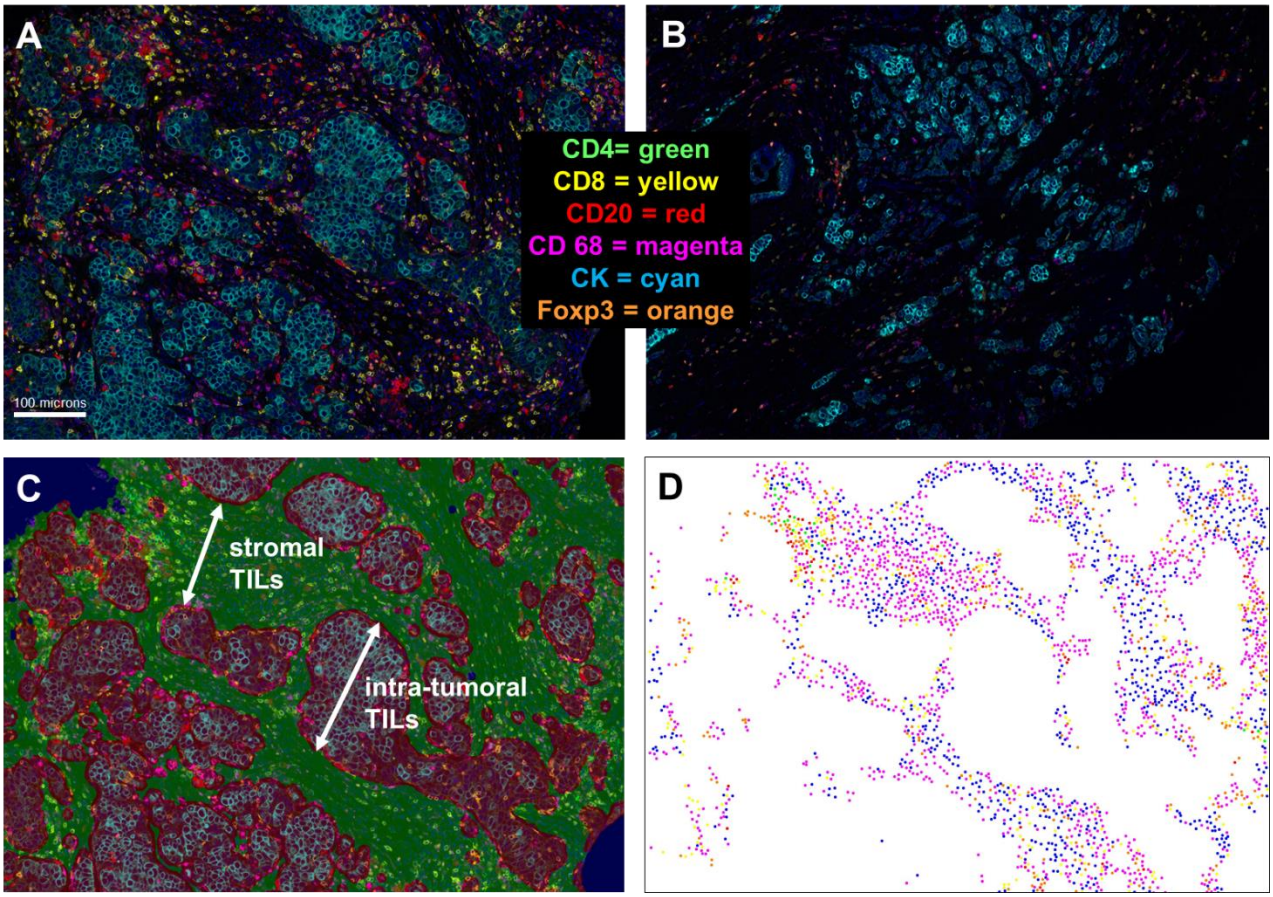


Figure 5. Multiplexed immunofluorescence of tumor infiltrates of TBCRC 006 baseline specimens. A, B, representative examples of high (A) and low (B) TILs. **C,** Automated tissue segmentation: To distinguish stromal TILs from intra-tumoral TILs (immune cells with direct cell-cell contact with tumor cells) the tumor area was expanded by 5 microns. **D,** Cell phenotype map of stromal TILs.

	H&E	Density (positive cells/mm^2)			
Specimen	TILs (%)	CD8	CD4	CD20	CD68
1	75	1513	1087	2238	1226
2	15	567	141	93	255
3	75	628	505	887	1134
4	65	909	722	1151	925
5	40	805	349	857	439
6	70	367	249	252	790
7	80	2367	438	1591	1000
8	80	1036	324	1292	917
9	15	128	310	585	387
10	60	895	424	484	248

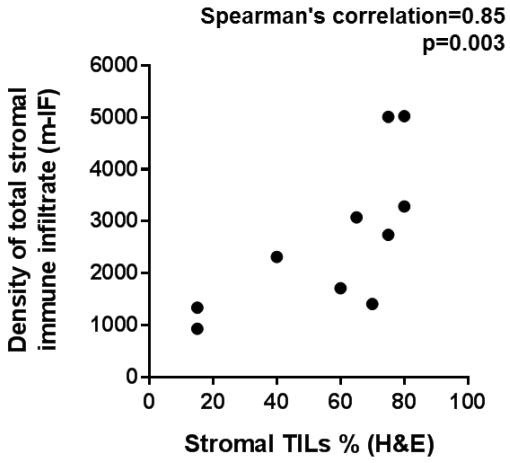


Figure 6. Correlation of density of total stromal immune infiltrate by multispectral immunoflourescent (m-IF) and % with stromal TILs assessed by H&E. The table reports for each sample the % of stromal TILs assessed by H&E slides and the respective density of CD8, CD4, CD20, and CD68 positive immune cells in the stroma (number of positive cells per mm^2 of stroma) assesed by m-IF.

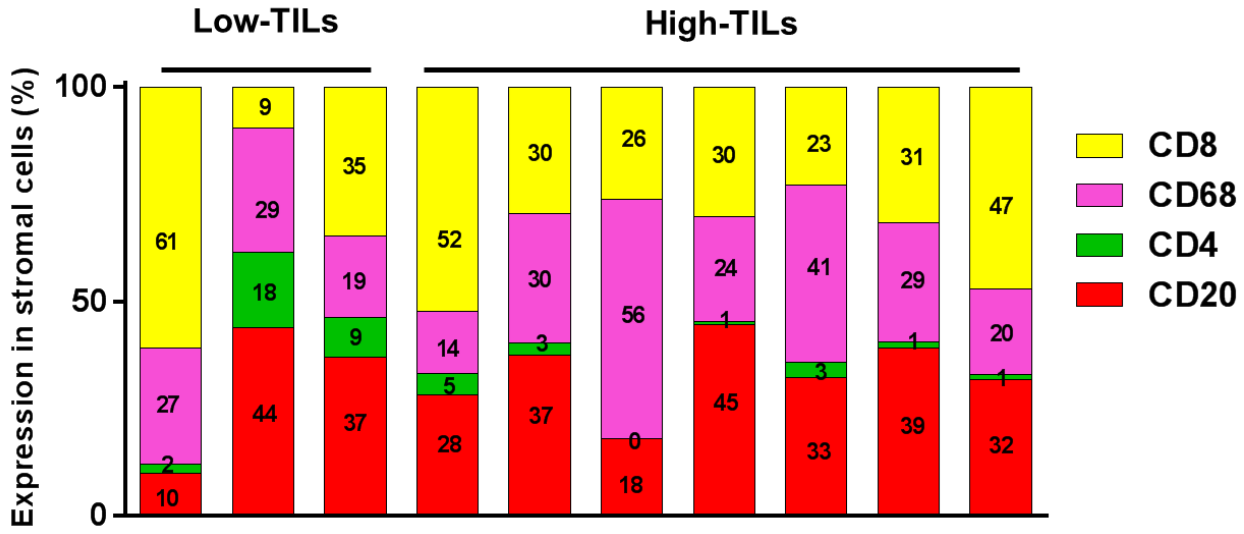


Figure 7. Inter-patient heterogeneity in composition of stromal immune infiltrates in the TBCRC006 trial. Each bar represents a baseline sample. Samples were ordered from low to high % of stromal TILs assessed by H&E. Numbers show the frequencies of the various immune cell types (CD8+ cytotoxic T cells, CD4+ helper T cells, CD20+ B cells, and CD68+ macrophages) within each sample.

Characteristic	Evaluable* Patients (n=64)	PTEN IHC Available (n=59)	*PIK3CA* Mutation Available (n=38)	PTEN and *PIK3CA* Available (n=35)
Age, years				
Median	50	50	51	51
Range	31-74	31-74	31-70	31-70
Tumor size, cm				
Median	6	6	6	6
Range	1·5-30	2-30	1·5-30	3-30
Biomarkers†				
ER positive	31 (51%)	31 (52%)	13 (41%)	13 (42%)
PR positive	22 (36%)	22 (37%)	11 (34%)	11 (36%)
pCR				
Yes	17 (27%)	14 (24%)	6 (16%)	5 (14%)
No	47 (73%)	45 (76%)	32 (84%)	30 (86%)

Abbreviations: ER, estrogen receptor; pCR, pathological complete response; PR, progesterone receptor.
* 64 patients in the study were evaluable for efficacy
† ER, PR were from central review IHC, 61 out of 64 patients' data were available

Table 1. Patient Demographic and Clinical Characteristics

Marker	pCR		No pCR		OR	95% CI	*P**
	n	%	n	%			
PTEN (n=59)							0.04
High (≥100)	12	32	25	68	1	-	
Low (< 100)	2	9	20	91	0.21	0.04 - 1.04	
***PIK3CA* (n=38)**							0.07
WT	7	27	19	73	9.62†	0.50- 186.62	
Mutation	0		12	100	1	-	
PTEN low or *PIK3CA* mutation (n=35)							0.01
No	5	31	11	69	18.65†	0.94 - 369.20	
Yes	0		19	100	1	-	

Abbreviations: PI3K, phosphoinositol-3 kinase; pCR, pathologic complete response; OR, odds ratio; PTEN, phosphatase and tensin homolog; WT, wild type.

* P values were calculated by Chi-square or Fisher's Exact test.

† OR was estimated by adding 0.5 to every cell since table contains zero cell.

Table 2. Activation of PI3K Pathway by Low PTEN and/or *PIK3CA* Mutations as Pretreatment Predictive Markers of Response to the Trastuzumab + Lapatinib regimen

Mutation	*PIK3CA* domain	n	%
p.E542K	Helical	2	6·06
p.E545K	Helical	1	3·03
p.G1049R	Catalytic	1	3·03
p.H1047L	Catalytic	1	3·03
p.H1047R	Catalytic	7	21·21
Wild-type		21	63·64

Table 3. Baseline *PIK3CA* Mutation Frequency

	High TILs (n=12)		Low TILs (n=47)		All (n=59)		Fisher's exact P
	n	%	n	%	n	%	
Age							1.0
<= 50	6	50	25	53.2	31	52.5	
>50	6	50	22	46.8	28	47.5	
Race							0.1982
White	7	58.3	37	78.7	44	74.6	
Black or African American	4	33.3	8	17	12	20.3	
Asian	0		1	2.1	1	1.7	
American Indian or Alaska Native	0		1	2.1	1	1.7	
Unknown	1	8.3	0		1	1.7	
Ethnicity							0.7884
Hispanic or Latino	5	41.7	15	31.9	20	33.9	
Not Hispanic or Latino	7	58.3	31	66	38	64.4	
Unknown	0		1	2.1	1	1.7	
Menopausal Status							0.749
Postmenopausal	6	50	20	42.6	26	44.1	
Pre/Perimenopausal	6	50	27	57.4	33	55.9	
ECOG Status							0.5725
0	12	100	43	91.5	55	93.2	
1	0		4	8.5	4	6.8	
Tumor Size							0.3341
<=5cm	3	25	20	42.6	23	39	
>5cm	9	75	27	57.4	36	61	
Estrogen Receptor							0.1974
Positive	4	33.3	27	57.4	31	52.5	
Negative	8	66.7	20	42.6	28	47.5	
Progesterone Receptor							0.1795
Positive	2	16.7	20	42.6	22	37.3	
Negative	10	83.3	27	57.4	37	62.7	
PTEN							
Low	3	13.84	19	86.36	22	38.59	0.3350
High	9	25.71	26	74.29	35	61.41	
PIK3CA							1.0
Wild type	6	25.0	18	75.0	24	66.66	
Mutation	3	75.0	9	75.0	12	33.34	

Table 4. Patients' baseline clinico-pathological characteristics

Biomarkers	Spearman's correlation	P-value
ER H-score	-0.014	0.9183
PR H-score	0.021	0.8726
Ki67 (%)	0.304	**0.0241**
HER2 H-score	-0.217	0.0988
HER2/CEP17 ratio	-0.1836	0.1838

Table 5. Correlation of s-TILs with tumor biomarkers. ER, PR, HER2 H-score (percent positivity (0-100%) X level of intensity (0-3), Ki67 and HER2/CEP17 ratio were centrally analyzed by IHC and/or FISH in the baseline specimens.

	pCR		non-pCR		P-value*
	No. of patients	**%**	**No. of patients**	**%**	
High-TILs	6	50	6	50	**0.057**
Low-TILs	9	19	38	81	

Table 6. Association between pathological complete response (pCR) and s-TILs. *Fisher's Exact Test two-sided

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