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Evaluation of the HER/PI3K/AKT Family Signaling Network as a Predictive Biomarker of Pathologic Complete Response for Patients With Breast Cancer Treated With Neratinib in the I-SPY 2 TRIAL

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abstract **Purpose** In the I-SPY 2 TRIAL (Investigation of Serial Studies to Predict Your Therapeutic Response With Imaging and Molecular Analysis 2), the pan-erythroblastic oncogene B inhibitor neratinib was available to all hormone receptor (HR)/human epidermal growth factor receptor 2 (HER2) subtypes and graduated in the HR-negative/HER2-positive signature. We hypothesized that neratinib response may be predicted by baseline HER2 epidermal growth factor receptor (EGFR) signaling activation/phosphorylation levels independent of total levels of HER2 or EGFR proteins.

Materials and Methods Complete experimental and response data were available for between 130 and 193 patients. In qualifying analyses, which used logistic regression and treatment interaction analysis, 18 protein/phosphoprotein, 10 mRNA, and 12 DNA biomarkers that related to *HER* family signaling were evaluated. Exploratory analyses used Wilcoxon rank sum and *t* tests without multiple comparison correction.

Results *HER* pathway DNA biomarkers were either low prevalence or nonpredictive. In expression biomarker analysis, only one gene (*STMN1*) was specifically associated with response to neratinib in the HER2-negative subset. In qualifying protein/phosphoprotein analyses that used reverse phase protein microarrays, six *HER* family markers were associated with neratinib response. After analysis was adjusted for HR/HER2 status, EGFR Y1173 (pEGFR) showed a significant biomarker-by-treatment interaction ($P = .049$). Exploratory analysis of *HER* family signaling in patients with triple-negative (TN) disease found that activation of EGFR Y1173 ($P = .005$) and HER2 Y1248 (pHER2) ($P = .019$) were positively associated with pathologic complete response. Exploratory analysis in this pEGFR/pHER2-activated TN subgroup identified elevated levels of estrogen receptor α ($P < .006$) in these patients.

Conclusion Activation of *HER* family phosphoproteins associates with response to neratinib, but only EGFR Y1173 and *STMN1* appear to add value to the graduating signature. Activation of HER2 and EGFR in TN tumors may identify patients whose diseases respond to neratinib and implies that there is a subset of patients with TN disease who paradoxically exhibit *HER* family signaling activation and may achieve clinical benefit with neratinib; this concept must be validated in future studies.

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INTRODUCTION

The I-SPY 2 TRIAL (Investigation of Serial Studies to Predict Your Therapeutic Response With Imaging and Molecular Analysis 2; [ClinicalTrials.gov](https://clinicaltrials.gov) identifier: NCT01042379) is a phase II, adaptive neoadjuvant therapy trial in which the primary goal was to determine the predictive probabilities of phase III trial success for various targeted therapeutics. Patients with locally advanced, high-risk breast cancer had their diseases assigned to one of eight subtypes on the basis of hormone receptor (HR), human epidermal growth factor receptor 2 (HER2), and Mammaprint-based high1/(ultra)high2 risk statuses.¹⁻³

Neratinib, a pan-erythroblastic oncogene B (ERBB) inhibitor, was available to patients with all tumor subtypes in the I-SPY 2 TRIAL, and the agent availability was graduated in the HR-negative/HER2-positive signature.⁴ Neratinib has shown activity against HER2-positive metastatic breast cancer, and there is also evidence for activity against HER2-negative tumor cells in vitro.^{5,6} Because neratinib was available to all patients in the trial, this study provided an opportunity to test the efficacy of the drug in patients with HER2-negative tumors.

Cell line-based preclinical studies have implicated alterations in *HER/AKT/mTOR* family genes on the DNA, mRNA, or protein level as predictive of neratinib response.⁷⁻¹¹ However, recent HER2 therapy-based clinical trials in which HER family biomarkers at the total protein and/or mRNA level were analyzed for response prediction found that none provided predictive value compared with the conventional, US Food and Drug Administration-approved immunohistochemistry (IHC)/fluorescence in situ hybridization-based HER2/estrogen receptor (ER) testing methods.¹²⁻¹⁴ Previous work with the I-SPY 1 TRIAL cohort revealed that HER2 phosphorylation was highly concordant with HER2 expression.¹⁵ That study identified a subpopulation of patients who had HER2-negative disease by standard testing yet had HER2 phosphorylation levels similar to patients with HER2-positive disease. The HER2 activation seen in these patients in the HER2-negative subtype appeared coincident with epidermal growth factor receptor (EGFR) activation.

On the basis of the mechanism of action of neratinib as a potent HER family kinase inhibitor

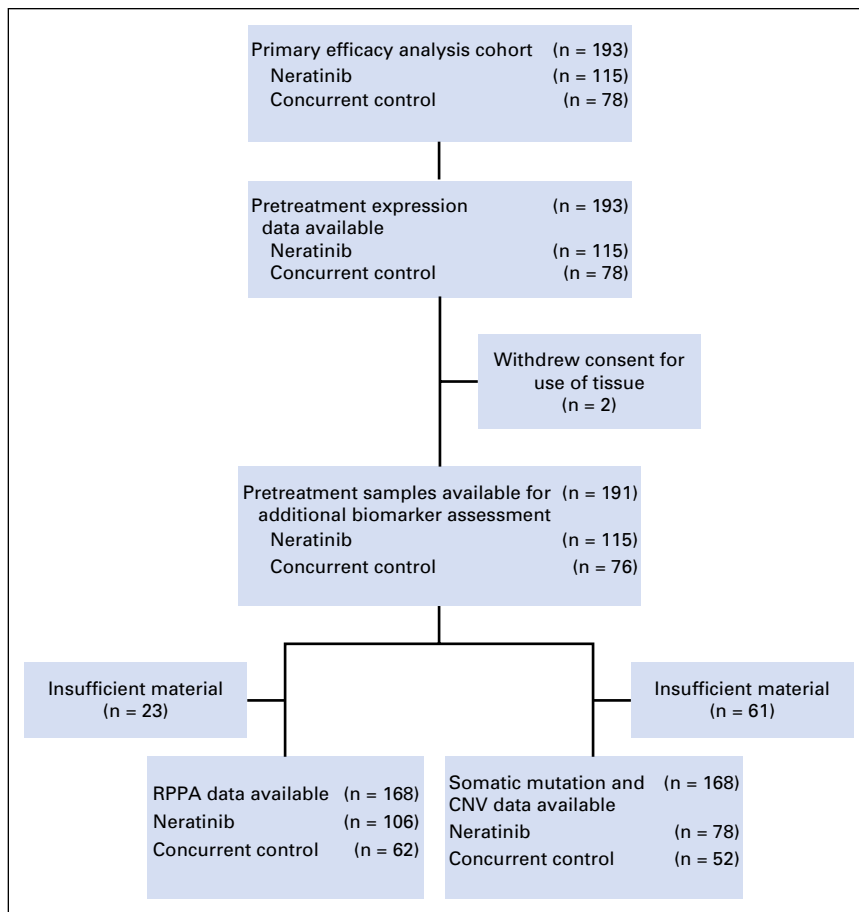
combined with observations of I-SPY 1 TRIAL results, we postulated that HER2 and EGFR activation/phosphorylation may be predictive of neratinib response independent of total HER2 status in the I-SPY 2 TRIAL; this hypothesis was motivation for an exploratory analysis within the HER2-negative and triple-negative (TN) subpopulation. Our analysis of HER/phosphoinositide-3-kinase (PI3K)/AKT signaling family components in pretreatment samples from the neratinib and concurrent control arms of the I-SPY 2 TRIAL was performed at a unique multiomic level. We report results from the following: (1) assessment of selected mutation/copy number alterations by exome sequencing, (2) mRNA expression levels by expression microarrays, and (3) analysis of protein/phosphoprotein levels by reverse phase protein arrays (RPPAs) as specific biomarkers of neratinib response. DNA and mRNA analyses evaluated 10 to 12 key genes in this pathway, including *HER2*, *EGFR*, *mTOR*, *AKT*, and *PIK3CA*. RPPA analysis assessed the ability of 18 protein/phosphoproteins that comprise the known drug targets of neratinib (EGFR, HER2) and downstream effector molecules, such as SHC transforming protein 1 (SHC) and mammalian target of rapamycin (mTOR)/AK-tyrosine (AKT) signaling components to predict complete pathologic response (pCR) to neratinib. To our knowledge, this study is the most comprehensive multiomic investigation of the HER family pathway activation status and its relationship to HER family-targeted neoadjuvant therapy response in patients with early-stage breast cancer to date.

MATERIALS AND METHODS

In the I-SPY 2 TRIAL, all patients received at least standard chemotherapy (paclitaxel followed by doxorubicin/cyclophosphamide [T > AC]). Patients randomly assigned to the experimental arm were treated with neratinib in addition to standard chemotherapy (neratinib + T > AC).⁴ In patients with HER2-positive disease, neratinib was administered in place of trastuzumab (Appendix Fig A1). pCR was the primary end point for analysis. All patients provided institutional review board-approved informed consent before specimens were collected.

Pretreatment biopsy specimens from patients in the neratinib and concurrent control arms of the I-SPY 2 TRIAL were analyzed for mRNA

Fig 1. CONSORT diagram that outlines the number of patients included in the neratinib and control arms of the I-SPY 2 TRIAL (Investigation of Serial Studies to Predict Your Therapeutic Response With Imaging and Molecular Analysis 2) and those included in the subsequent analyses. CNV, copy number variation; RPPA, reverse phase protein array.



expression by using Agilent 44K expression arrays (Agendia, Irvine, CA), signaling protein activation by RPPA, and DNA sequencing (approximately 2,000-gene mini-cancer genome; Utrecht, the Netherlands). A CONSORT diagram with the number of evaluable patients for each molecular profiling analysis is shown in [Figure 1](#). Details of sample preparation and data processing are provided in the Data Supplement.

In our prespecified analysis plan,¹⁶ logistic regression was used to assess association with pCR in the control and neratinib treated populations individually. Relative biomarker performance between arms (biomarker-by-treatment interaction) was assessed with a logistic model (pCR approximately equaled treatment + biomarker + [treatment × biomarker]). Analysis also was performed to adjust for HR/HER2 status (pCR ~ treatment + biomarker + [treatment × biomarker] + HR status + HER2 status). If a continuous biomarker did not follow a normal distribution, we applied a nonparametric method that discretized the score by using a series of cut points, and we applied logistic modeling for the dichotomized biomarker at each cut

point; significance was assessed by permutation testing. This procedure produced an optimal cut point that maximized the significance of the biomarker-by-treatment interaction. Markers with pCR associations that had *P* values < .05 were identified as significant. No multiple comparison adjustments were applied. For significant biomarkers, Bayesian logistic regression analysis was performed, as previously described, with the following model: pCR ~ HR + HER2 + biomarker + treatment + (treatment × HR) + (treatment × HER2) + (treatment × biomarker).¹⁶

In additional analyses, parametric *t* tests (for normally distributed data) or nonparametric Wilcoxon rank sum tests (for non-normally distributed data) were used to assess association of protein end points with pCR in the control and neratinib-treated populations individually. Analytes associated with response only in the neratinib and not the control arm were selected for additional analysis, and *P* values < .05 were identified as significant. Markers were analyzed individually; sample sizes were small, and *P* values were descriptive and not corrected for multiple comparisons. Receiver operating characteristic

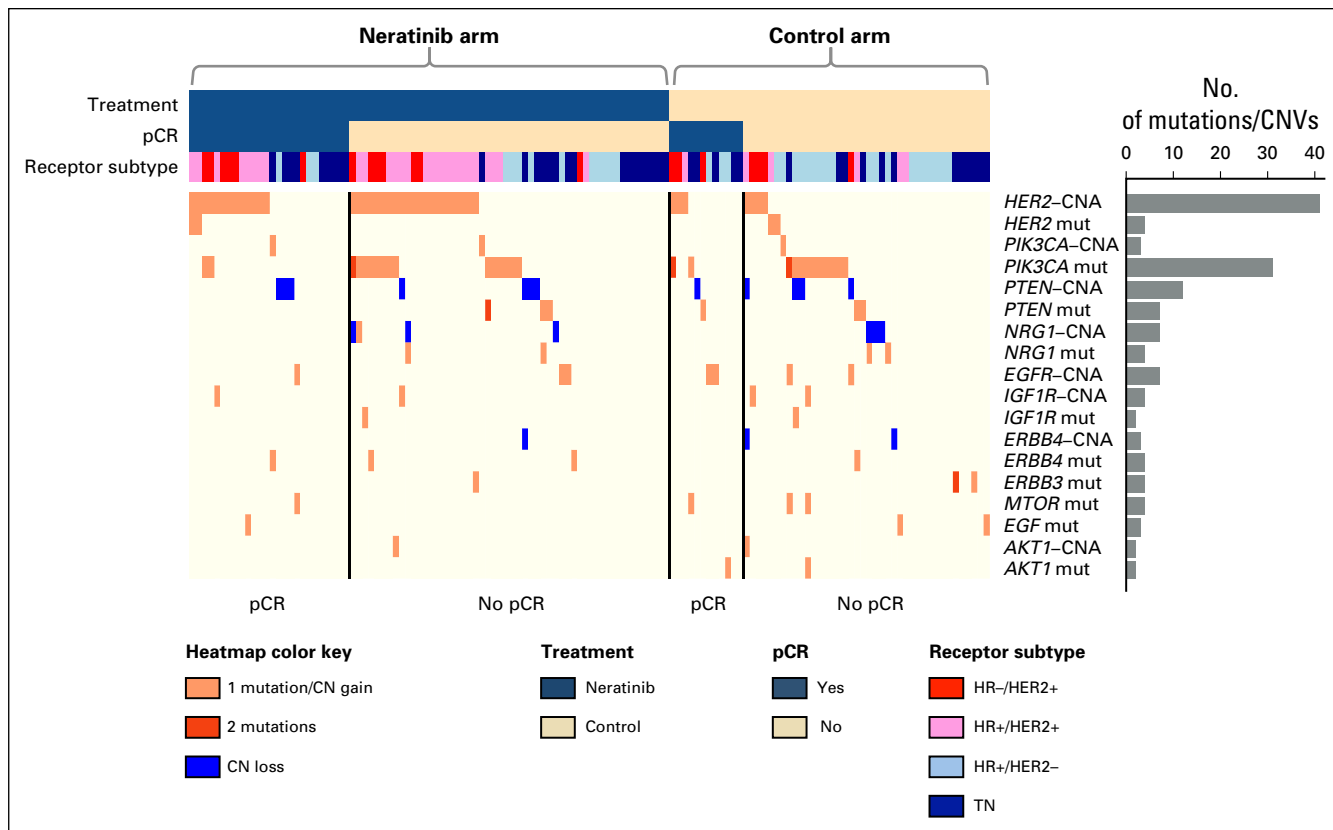


Fig 2. Heatmap of mutations and copy number variations (CNVs) in *HER* family signaling genes. Patient data are arranged first by arm, then by pathologic complete response (pCR) status along columns, and biomarkers are hierarchically clustered along rows. Prevalence of DNA aberrations is represented by the bar plot to the right. Within the heatmap, pink represents a single mutation (mut) or CN gain; red, two mutations; and blue, CN loss. HR, hormone receptor; TN, triple negative.

curves were generated for end points associated with pCR in the neratinib-treated arm of the trial to identify potential cut points for biomarker positivity rates within selected patient subtypes.

RESULTS

Evaluation of HER Family Gene Point Mutations and DNA Amplification/Loss as Predictors of Neratinib Sensitivity

We evaluated somatic mutations and copy number variations (CNVs) in 12 HER family-linked signaling pathway genes—*EGF*, *EGFR*, *HER2*, *NRG1*, *IGF1R*, *PIK3CA*, *AKT1*, *PTEN*, *STMN1*, and *mTOR*—by targeting exome sequencing across all evaluable patients. A total of 50 patients (38%) had at least one mutation, whereas 66 (51%) had at least one CNV in these 12 genes. However, the prevalence of any given alteration was low (Fig 2), which precluded statistical analyses for most of the genes. *PIK3CA* somatic mutations were the most frequent. Consistent with publications that link *PIK3CA* mutation with resistance to neratinib and other HER2-targeted agents,^{14,17-19} only two (13%) of

16 patients with mutated *PIK3CA* achieved pCR in the neratinib arm compared with 24 (39%) of 62 patients with wild-type *PIK3CA*. However, *PIK3CA* mutation status did not show a significant biomarker-by-treatment interaction.

Association of HER Family Gene Expression With Response to Neratinib

We evaluated 10 predefined HER family signaling genes as expression biomarkers of neratinib response in all evaluable patients (Appendix Table A1). *HER2* expression was significantly associated with sensitivity to neratinib combination therapy (and in the HER2-positive subpopulation), and *IGF1R*, to resistance; neither gene was associated with response in the control arm of the trial (Appendix Tables A1 and A2). However, no significant biomarker-by-treatment interactions were observed. Within the HER2-negative subset, *STMN1* was associated with response to neratinib ($P = .0023$) and not control, and this result showed a significant biomarker-by-treatment interaction ($P = .0036$; Fig 3A; Appendix Table A3). At an optimal threshold that maximized the interaction, 27%

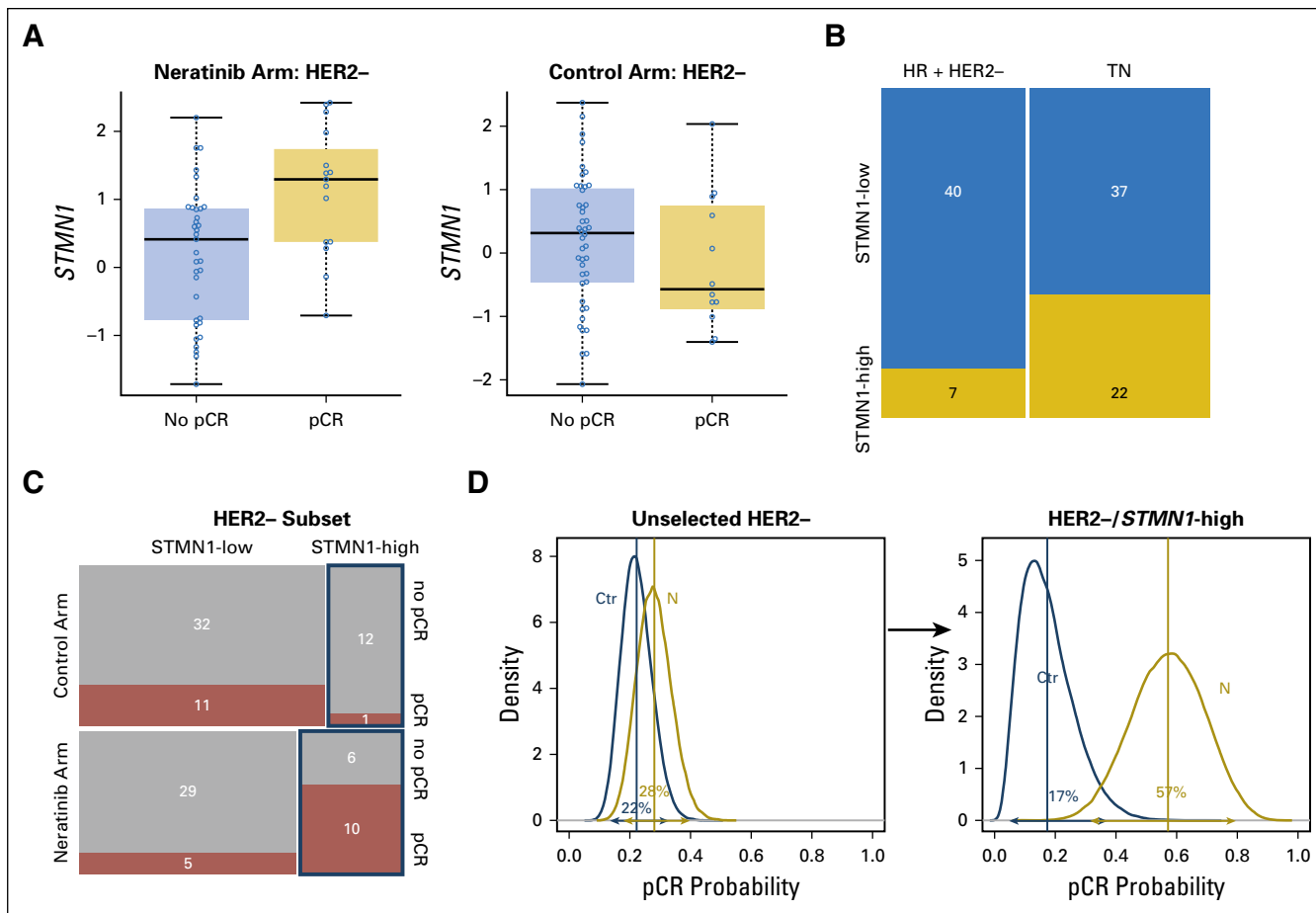


Fig 3. *STMN1* expression level as a biomarker of neratinib response in patients with human epidermal growth factor receptor 2 (HER2)-negative disease. (A) Box plots of *STMN1* expression in the HER2-negative subset by arm and response. (B) Distribution of patients with *STMN1*-high and -low expression levels by hormone receptor (HR) subtype within HER2-negative subset. (C) Mosaic plot of pathologic complete response (pCR) distribution by *STMN1* status within the neratinib and control arms. (D) Bayesian estimated pCR probability distributions in (left) the unselected HER2-negative subset and (right) the HER2-negative/*STMN1*-high subset in the control (blue) and neratinib (gold) arms.

of patients with HER2-negative disease (29 of 106 patients) had *STMN1*-high status (Fig 3B-C). This result held for the continuous variable and as the median for a cut point (data not shown). By using Bayesian modeling, the estimated pCR rate of patients with HER2-negative/*STMN1*-high status in the treatment arm was 57% compared with 17% in the control arm (Fig 3D); the predictive probability of phase III success in this subset was 97%.

HER Family Protein Signaling Activation as Predictors of Neratinib Sensitivity

We evaluated 18 HER family signaling proteins/phosphoproteins as biomarkers of neratinib response by using RPPA data from pretreatment, laser capture microdissection (LCM)-purified tumor epithelia across all evaluable patients in the neratinib and concurrent control arms. Six of the 18 HER family biomarkers tested (EGFR Y1068, EGFR Y1173, EGFR Y992, HER2 total, HER2 Y1248, and SHC Y317) were associated with pCR in neratinib-treated patients but not in concurrent control-arm patients in the trial

(Table 1). The same markers were associated with neratinib response in the HER2-positive population, with the exception of SHC Y317 (Table 1).

The permutation test-based strategy with a logistic regression model that included HER2 and HR status as covariates revealed that EGFR Y1173 showed a significant biomarker-by-treatment interaction ($P = .049$). We dichotomized patients by their EGFR Y1173 intensity values into high and low groups (optimal cut point of 4,501, which maximized the significance of the biomarker-by-treatment interaction term), and we evaluated the distribution of pCR rates (Table 2). The odds ratio (OR) between EGFR Y1173 groups in the neratinib-treated versus the control arm was 10.1. When patients with EGFR Y1173-high status were added to the graduated HR-negative/HER2-positive subset, the OR associated with treatment was 3.2 and was comparable to that of the HR-negative/HER2-positive signature (OR, 2.1), which increased the prevalence of patients with biomarker-positive disease by approximately 50% and increased

Table 1. Assessment of Qualifying Biomarker Association With pCR in Various Populations of Patients in Neratinib-Treated and Control Arms in the I-SPY 2 TRIAL

Analyte Measured	All Patients		HER2-Positive Group		HR-Negative/HER2-Negative Group	
	Control	Neratinib	Control	Neratinib	Control	Neratinib
AKT S473	0.899	0.313	0.941	0.457	0.845	0.839
AKT T308	0.285	0.63	0.84	0.676	0.381	0.855
EGFR total	0.964	0.146	0.536	0.262	0.436	0.156
EGFR Y1068	0.106	0.005	0.194	0.033	0.06	0.059
EGFR Y1148	0.412	0.785*	0.563	0.477	0.006	0.406
EGFR Y1173	0.438	0.0016	0.295	0.026	0.205	0.005
EGFR Y992	0.134	0.047	0.101	0.04	0.311	0.208
HER2 total	0.708	0.009	0.945	0.013*	0.519	0.059
HER2 Y1248	0.363	0.008	0.633	0.019	0.235	0.019
ERBB3 total	0.206	0.875	0.365	0.837	0.533	0.968
ERBB3 Y1289	0.872	0.065	0.448	0.104	0.424	0.152
ERK1/2 T202/Y204	0.879	0.651	0.84	0.949*	0.846	0.641
Heregulin total	0.906	0.781	0.536	0.58	0.207	0.857
mTOR S2448	0.828	0.35*	1	0.799*	0.397	0.264
mTOR total	0.231	0.175	0.295	0.729*	0.78	0.282
PI3K p85 Y458/p55 Y199	0.907	0.257*	0.84	0.179	0.353	0.435
PTEN S380	0.807	0.977*	0.734	0.556*	0.248	0.306
SHC Y317	0.476	0.016	0.233	0.081	0.622	0.346

Abbreviations: AKT, AK-thyoma; EGFR, epidermal growth factor receptor; ERBB3, erythroblastic oncogene B 3; ERK, extracellular signal-related kinase; HER2, human epidermal growth factor receptor 2; HR, hormone receptor; I-SPY 2 TRIAL, Investigation of Serial Studies to Predict Your Therapeutic Response With Imaging and Molecular Analysis 2; mTOR, mammalian target of rapamycin; PI3K, phosphoinositide 3 kinase; PTEN, phosphatase and tensin homolog; SHC, SHC transforming protein.

*Assessed by *t* test. All others assessed by Wilcoxon rank sum test. Bold indicates $P < 0.05$.

the predictive probability of phase III success (Appendix Table A4).

Comparison of *HER* Family Gene Expression, Protein, Phosphoprotein, and Mutation Data

Unsupervised clustering revealed that total protein levels of *HER*-family genes clustered with its gene expression level, whereas phosphoprotein levels were more highly correlated to one another than to their respective gene or total protein expression levels (Fig 4). *PIK3CA* mutations (28 of 130 patients) were not associated with altered levels of *PIK3CA* expression of protein/phosphoprotein; however, they were associated with lower levels of *STMN1* and *EGFR* gene expression and higher levels of the phosphoproteins PTEN S380 and AKT S473 (data not shown).

Exploratory Analysis of *HER* Family Signaling in Patients With TN Disease

Although neratinib graduated in the HR-negative/HER2-positivesignature, this signature

was characterized by IHC/fluorescence in situ hybridization-based analysis of total HER2 expression and did not measure HER2 or EGFR phosphorylation. We were particularly interested in evaluation of HER signaling/phosphorylation in the subgroup of patients with TN disease as a predictor of response to neratinib. Among the 18 HER family qualifying protein biomarkers, increased EGFR Y1173 ($P = .005$) and HER2 Y1248 ($P = .019$) phosphorylation were significantly associated with pCR in the neratinib-treated patients with TN disease but not in the control arm, and this could not be explained by mutations or copy number alterations in *EGFR*, *HER2*, or other genes related to HER family signaling (Table 1; Fig 4).

We used optimal cut points determined by receiver operating characteristic analysis for EGFR Y1173 and HER2 Y1248, which were nearly identical to those obtained by the permutation testing described in HER Family Protein Signaling Activation as Predictors of Neratinib Sensitivity, to assess their correlation with pCR

Table 2. pCR Rates for EGFR Y1173 and HER2 Y1248 Biomarker High/Low Groups by HR/HER2 Subtype in Neratinib-Treated and Control Groups

Subtype	No. With pCR/Total No. (%) of Patients			
	Neratinib (n = 106)		Control (n = 62)	
EGFR Y1173 (RIU > 4501 cutoff)	EGFR Y1173 High	EGFR Y1173 Low	EGFR Y1173 High	EGFR Y1173 Low
HR negative/HER2 positive (n = 28)	12/18 (67)	0/4 (0)	1/5 (20)	1/1 (100)
HR positive/HER2 positive (n = 45)	12/33 (36)	0/3 (0)	1/4 (25)	0/5 (0)
HR positive/HER2 negative (n = 43)	2/9 (22)	1/8 (13)	0/13 (0)	3/13 (23)
HR negative/HER2 negative (n = 52)	10/15 (67)	2/16 (13)	5/12 (42)	1/9 (11)
HER2 Y1248 (RIU > 3100 cutoff)	HER2 Y1248 High	HER2 Y1248 Low	HER2 Y1248 High	HER2 Y1248 Low
HR negative/HER2 positive (n = 28)	12/21 (57)	0/1 (0)	1/5 (20)	1/1 (100)
HR positive/HER2 positive (n = 45)	12/35 (34)	0/1 (0)	1/7 (14)	0/2 (0)
HR positive/HER2 negative (n = 43)	1/9 (11)	2/8 (25)	2/15 (13)	1/11 (9)
HR negative/HER2 negative (n = 52)	10/16 (63)	2/15 (13)	5/15 (33)	1/6 (17)
Dual biomarker	EGFR Y1173 High/ HER2 Y1248 High	All Others	EGFR Y1173 High/ HER2 Y1248 High	All Others
HR negative/HER2 positive (n = 28)	12/18 (67)	0/4 (0)	1/5 (20)	1/1 (100)
HR positive/HER2 positive (n = 45)	12/32 (38)	0/4 (0)	1/4 (25)	0/5 (0)
HR positive/HER2 negative (n = 43)	1/7 (14)	2/10 (20)	0/11 (0)	3/15 (20)
HR negative/HER2 negative (n = 52)	9/11 (82)	3/20 (15)	4/11 (36)	2/10 (20)

Abbreviations: EGFR, epidermal growth factor receptor; HER2, human epidermal growth factor receptor 2; HR, hormone receptor; pCR, pathologic complete response.

in patients with TN disease. Two-way plots of EGFR Y1173 and HER2 Y1248 for neratinib-treated and concurrent controls demonstrated that nine (82%) of 11 patients with TN disease who exhibited elevated phospho-EGFR (pEGFR) and phospho-HER2 (pHER2) levels experienced a pCR in response to neratinib treatment compared with four (36%) of 11 with TN disease among the concurrent controls (Table 2; Fig 5A-B). Bayesian evaluation of EGFR Y1173 and HER2 Y1248 as biomarkers for neratinib response in the TN population revealed a comparable probability of success in a phase III trial to that of the graduated HR-negative/HER2-positive population (Fig 5C; Appendix Tables A4 and A5). The combination of the two biomarkers (TN and EGFR Y1173-high/HER2 Y1248-high) proved to have the highest probability of success (95%) compared with EGFR Y1173 or HER2 Y1248 modeled individually (72% and 82%, respectively; Appendix Table A5).

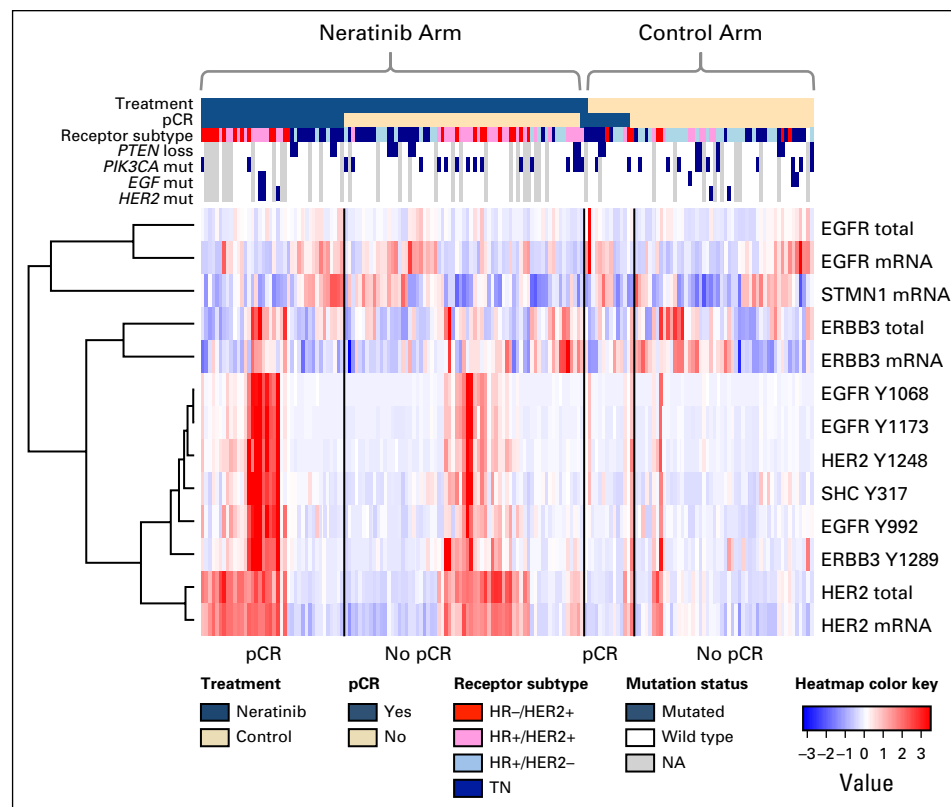
ERs have been shown to be able to act as membrane, nongenomic signaling molecules through direct interaction with tyrosine kinases, including EGFR and HER2, even when present at

low levels, and to activate receptor tyrosine kinases.²⁰⁻²² Across all patients with TN disease in the neratinib arms of the I-SPY 2 TRIAL, levels of total ER- α were higher in pEGFR/pHER2-high TN tumors ($P < .001$). In this pEGFR/pHER2-high group of TN tumors, 16 (76%) of 21 patients had ER- α levels greater than the median value for the TN population compared with 11 (37%) of 30 patients in the rest of the TN population (Fig 5D). In the neratinib-treated population alone, 11 (35%) of 31 patients had pEGFR/pHER2-high status, and nine (82%) of 11 patients had total ER- α levels greater than the TN population median.

DISCUSSION

As precision cancer medicine evolves into concomitant processes of discovery, validation, and development of biomarkers predictive of therapeutic response alongside the clinical assessment of drug efficacy, there is increasing emphasis on identification of predictive biomarker candidates as early as possible. In the I-SPY 2 TRIAL, neratinib was available to all HR/HER2 tumor subtypes, and genomic, transcriptomic, and

Fig 4. Integrated heat-map showing the relationships between expression and protein/phosphoprotein biomarkers. Patient data are arranged first by arm, then by pathologic complete response (pCR) status, along columns, and biomarkers are hierarchically clustered along rows. The subset of biomarkers represented include phosphoproteins associated with response to neratinib, and their associated mRNA and total protein levels (scaled to a median of 0 and standard deviation of 1). The most prevalent DNA alterations are included in the annotation track (blue, mutated; white, wild type; gray, not available). EGFR, epidermal growth factor receptor; ERBB, erythroblastic oncogene B; HER2, human epidermal growth factor receptor 2; HR, hormone receptor; NA, not available; SHC, SHC transforming protein; TN, triple negative.



proteomic/phosphoproteomic biomarkers were measured in all pretreatment biopsy samples. This created a unique opportunity to explore a multiomic view to identify predictive markers that could transcend classic subtypes. Given that HER2 total protein levels do not strictly correlate with HER2 phosphorylation in the HER2-negative setting,¹⁵ and given that the phosphorylation status of HER2 protein (compared with total HER2) provides significant information on breast cancer survival,²³⁻²⁷ we postulated that phosphorylation levels of the neratinib drug targets HER2 and EGFR in pretreatment biopsy specimens would correlate with pCR in both HER2-positive and HER2-negative tumors.

Our data revealed that coactivation of HER2 and EGFR correlated with pCR in both HER2-positive and TN tumors. The pCR rate in the pEGFR/pHER2-high TN population, according to an optimal cut point for both markers, was 82% (nine of 11 patients) in the treatment arm, compared with 36% (four of 11 patients) with pCR in the control arm. Although these data are exploratory, this pCR rate was higher than the 55% of patients who had a complete response observed in the graduated HR-

negative/HER2-positive arm for neratinib.⁴ In keeping with these results, in which phosphorylated EGFR levels rather than total EGFR were predictive for pCR, phosphorylated EGFR has been shown to be a potential predictive marker for anti-EGFR therapies in other tumors, such as non-small-cell lung cancer.^{28,29} Also, our expression analysis produced only one predictive biomarker for neratinib sensitivity, *STMN1*, a gene implicated in HER2 pathway signaling and chemotherapy sensitivity in non-*HER2* amplified cell lines.³⁰⁻³³

Although our data indicate that phosphorylation levels of HER2 and EGFR correlate with pCR in neratinib-treated patients with breast cancer, a number of caveats in the study limit generalizability. The adaptive design of the trial allowed for efficient and quick graduation (or termination) of agent/marker combinations on the basis of their estimated likelihood of phase III success. However, this format tends to produce unbalanced groups with low patient numbers in each arm, which poses clear challenges from a biomarker discovery point of view. We found that phosphorylation of EGFR and HER2 were independently predictive of pCR in patients with either HER2-positive or HER2-negative

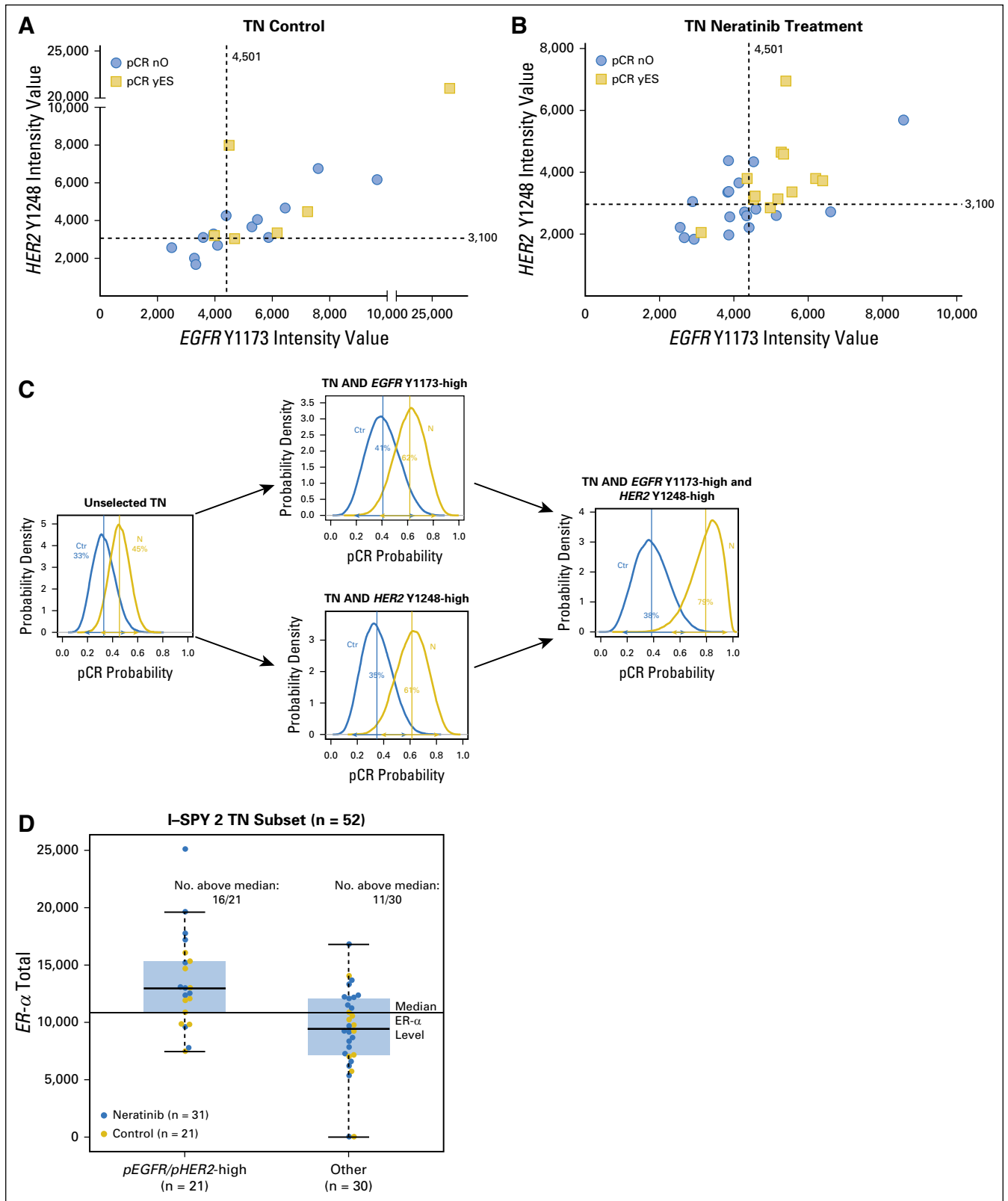


Fig 5. Analysis of epidermal growth factor receptor (EGFR) Y1173 and human epidermal growth factor receptor 2 (HER2) Y1248 as biomarkers for neratinib response in patients with triple-negative (TN) disease enrolled in the I-SPY 2 TRIAL (Investigation of Serial Studies to Predict Your Therapeutic Response With Imaging and Molecular Analysis 2). (A) and (B) Two-way plots of EGFR Y1173 and HER2 Y1248 intensities for TN (A) control group and (B) neratinib-treated patients. Pathologic complete response (pCR): no, blue circles; yes, gold squares. Dashed lines indicate

disease. Furthermore, EGFR and HER2 were coactivated, which suggests a biochemical basis for pCR prediction. Analyses of the National Surgical Breast and Bowel Project B-31 trial and the North Central Cancer Treatment Group N9831 trial found that HER2-negative breast cancers received benefit from trastuzumab in the adjuvant setting,^{34,35} but recent results from the NSABP B-47 adjuvant trial reported no survival improvement with the addition of trastuzumab to standard chemotherapy in patients with HER2-low status.³⁶ However, none of these studies assessed HER family phosphorylation or pathway activation status in HER2-negative subsets of patients who experienced disease response. Other studies with HER family inhibitors have shown that neratinib, in combination with insulin-like growth factor receptor (IGFR) inhibitors had antiproliferative effects in HER2 non-overexpressing breast cancer cell lines, and pilot clinical studies of lapatinib treatment in various metastatic cancers showed a correlation of pHER2 with lapatinib response.^{37,38}

What is the basis for HER2 and EGFR activation in the TN subpopulation? The frequency of *HER2* and *EGFR* mutations in the neratinib cohort is too small ($n = 4$ and 3 , respectively) to be a contributing factor. Indeed, even the most prevalent genomic alterations did not statistically correlate with neratinib pCR, although *PIK3CA* mutation trended as a negative predictor in the I-SPY 2 TRIAL ($P = .07$). In the absence of genomic alterations related to HER family signaling when HER activation is seen, a logical postulate is that the process is mediated by ligand-driven events. Recently, it has been shown that neuregulin 1 (NRG1 or heregulin) and HER2 phosphorylation coincidentally occurred in a subset of HER2-negative tumors and that inhibition of EGFR or HER2 or both receptors reduced breast cancer stem cell survival and self-renewal.³⁹ Although we evaluated NRG1 in our study and did not find any correlation with pCR or HER2 activation status (Table 1), we did explore the possibilities of other ligand-driven events underpinning the HER2 and EGFR activation observed in TN tumors, namely estrogen signaling. Estrogen can exert

nongenomic activity called membrane-initiated steroid signaling through binding with ER at low concentrations⁴⁰; ER may exist in a signal-some complex with a variety of receptor tyrosine kinases, such as IGFR,⁴¹ EGFR,^{42,43} or HER2,^{44,45} and lead to activation of EGFR, HER2, and IGFR¹²¹ in the absence of gene transcription. We found that ER- α levels in the TN cohort were higher in pEGFR/pHER2-high tumors than in tumors that were not HER2/EGFR activated (Fig 5). This result could provide a potential explanation for the paradoxical finding of HER2/EGFR activation in TN cancers in the absence of HER2 genomic alterations and must be confirmed in independent study sets.

The LCM-RPPA workflow used in this study provides a powerful and unique approach to quantitatively measure the activated signaling architecture of a large number of cancer-related pathways, including the HER family, from microscopic quantities of tissue. This technology and workflow is especially well suited for clinical sample assessment.^{46,47} Past studies have revealed the need for LCM to accurately assess phosphorylated and total protein levels, and to facilitate biomarker evaluation in the context of high and low tumor cell content.⁴⁸ Moreover, unlike IHC-based approaches that can be adversely effected by choice of antigen retrieval method, the RPPA technique utilizes fully denatured protein in which phospho-epitopes are fully linearized and recognized by the cognate primary antibody.

Patients with TN breast cancer have a paucity of targeted therapeutic options available. Given the pCR rate observed and biomarker positive prevalence we found in the TN setting, we believe these data provide a strong molecular rationale to consider prospective validation of the findings in patients with TN disease who received neratinib. To our knowledge, this study is the first of its kind to quantitatively assess activated HER family signaling in the context of clinical response to HER-directed therapies in a TN population. The biomarker findings, although prespecified, ultimately are based on small numbers of patients and must be confirmed with larger patient populations in independent clinical studies to validate

Fig 5. (Continued).

cut points for biomarker positivity. (C) Probability distributions in patients with TN disease that incorporate EGFR Y1173 and HER2 Y1248 as biomarkers of response. Blue curves represent concurrent control patients and gold curves represent the neratinib-treated TN population. The mean of each distribution is the estimated rate of pCR. (D) Box plot of estrogen receptor alpha (ER- α) levels in the phospho-EGFR/phospho-HER2 biomarker-high and -low populations.

the findings and establish predictive cut points for pEGFR and pHER2 for prospective clinical use.

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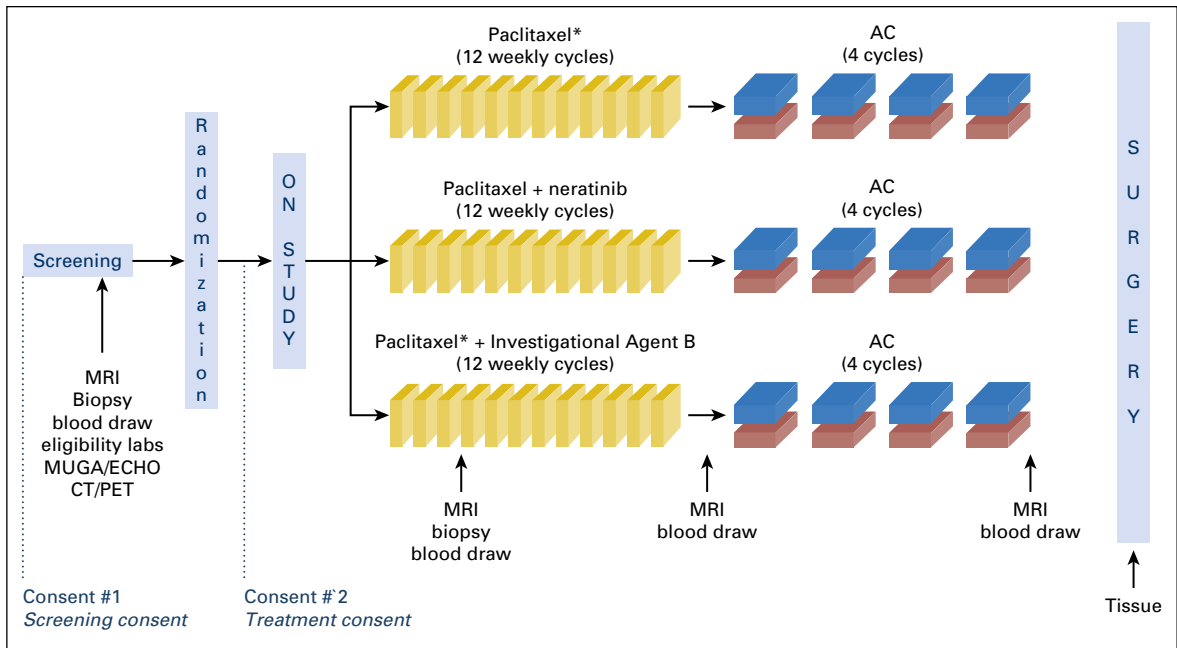


Fig A1. I-SPY2 TRIAL

(Investigation of Serial Studies to Predict Your Therapeutic Response With Imaging and Molecular Analysis 2) schema for patients in the control and experimental therapy arms. (*) HER2-positive patients also received trastuzumab. AC, doxorubicin/cyclophosphamide; CT/PET, computed tomography/positron emission tomography; ECHO, echocardiogram; HER2, human epidermal growth factor receptor 2; MRI, magnetic resonance imaging; MUGA, multigated acquisition.

Table A1. Gene Expression Associations With pCR Population as a Whole

Gene	Neratinib Arm (n = 115)		Control Arm (n = 78)		LR P for Biomarker ×Treatment	LR P for Biomarker ×Treatment (Adjusted for HR and HER2)
	OR/Unit Increase	LR P	OR/Unit Increase	LR P		
<i>EGFR</i>	1.17	.534	1.17	.413	.992	.766
<i>HER2</i>	1.65	.00582	0.974	.946	.209	.153
<i>ERBB3</i>	0.724	.112	0.7	.19	.922	.941
<i>NRG1</i>	0.807	.35	0.979	.937	.599	.798
<i>IGF1R</i>	0.511	.0397	0.563	.173	.888	.848
<i>PIK3CA</i>	0.898	.626	0.74	.227	.566	.518
<i>AKT1</i>	0.875	.511	1.13	.652	.452	.528
<i>PTEN</i>	0.916	.651	0.948	.845	.92	.474
<i>STMN1</i>	1.37	.113	0.791	.39	.102	.0987
<i>FRAP1</i>	0.93	.721	1.22	.458	.42	.479

NOTE: Bold indicates P < .05.

Abbreviations: HER2, human epidermal growth factor receptor 2; HR, hormone receptor; LR, logistic regression; OR, odds ratio; pCR, pathologic complete response.

Table A2. Gene Expression Associations With pCR in HER2-Positive Subset

Gene Expression End Point	Neratinib Arm (n = 65)		Control Arm (n = 22)		LR P for Biomarker ×Treatment
	OR/Unit Increase	LR P	OR/Unit Increase	LR P	
<i>EGFR</i>	1.05	.902	0.7	.62	.627
<i>HER2</i>	3.77	.000979	1.3	.674	.159
<i>ERBB3</i>	0.706	.199	0.336	.163	.389
<i>NRG1</i>	0.811	.613	0.0653	.0729	.127
<i>IGF1R</i>	0.491	.101	0.116	.122	.4
<i>PIK3CA</i>	0.546	.0951	1.68	.413	.129
<i>AKT1</i>	1	.992	0.161	.03	.0428
<i>PTEN</i>	0.689	.273	0.896	.876	.741
<i>STMN1</i>	1.07	.847	1.16	.812	.909
<i>FRAP1</i>	0.851	.567	1.91	.277	.219

NOTE. Bold cells indicate P < .05.

Abbreviations: HR, hormone receptor; HER2, human epidermal growth factor receptor 2; LR, logistic regression; OR, odds ratio; pCR, pathologic complete response.

Table A3. Gene Expression Associations With pCR in HER2-Negative Subset

Gene Expression End Point	Neratinib Arm (n = 50)		Control Arm (n = 56)		LR <i>P</i> for Biomarker × Treatment Interaction
	OR/Unit Increase	LR <i>P</i>	OR/Unit Increase	LR <i>P</i>	
<i>EGFR</i>	2.17	.0715	1.24	.301	.243
<i>HER2</i>	3.19	.27	0.48	.379	.158
<i>ERBB3</i>	0.575	.117	0.762	.371	.557
<i>NRG1</i>	0.833	.52	1.09	.73	.485
<i>IGF1R</i>	0.495	.181	0.697	.426	.686
<i>PIK3CA</i>	1.36	.311	0.632	.0989	.0634
<i>AKT1</i>	0.606	.147	1.75	.106	.0302
<i>PTEN</i>	0.89	.681	0.962	.898	.852
<i>STMN1</i>	2.82	.00233	0.707	.28	.00355
<i>FRAP1</i>	1.05	.876	1.09	.78	.929

NOTE. Bold cells indicate $P < .05$.

Abbreviations: HER2, human epidermal growth factor receptor 2; LR, logistic regression; OR, odds ratio; pCR, pathologic complete response.

Table A4. Bayesian Probabilities of EGFR Y1173 as a Biomarker of Neratinib Response

Subgroup	Probability > Control	Probability of Phase III Success
HR negative/HER2 positive	0.97	0.87
EGFR Y1173-high	0.99	0.93
HR negative/HER2 positive or EGFR Y1173-high	0.99	0.9
HR negative/HER2 positive and EGFR Y1173-high	0.99	0.95

Abbreviations: EGFR, epidermal growth factor receptor; HER2, human epidermal growth factor receptor 2; HR, hormone receptor.

Table A5. Bayesian Probabilities and Biomarker Prevalence for TN Population

Patient Subset	Probability, Neratinib > Control	Predictive Probability of Phase III success (N = 300)	TN Prevalence (%)
Unselected TN (n = 49)	0.76	0.42	100
TN/EGFR Y1173-high (n = 27)	0.88	0.72	55
TN/HER2 Y1248-high (n = 30)	0.95	0.82	61
TN/EGFR Y1173-high and HER2 Y1248-high (n = 21)	0.99	0.95	43

Abbreviations: EGFR, epidermal growth factor receptor; HER2, human epidermal growth factor receptor 2; TN, triple negative.