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Breast Cancer

Prospective Biomarker Analysis of the Randomized CHER-LOB Study Evaluating the Dual Anti-HER2 Treatment With Trastuzumab and Lapatinib Plus Chemotherapy as Neoadjuvant Therapy for HER2-Positive Breast Cancer

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Disclosures of potential conflicts of interest may be found at the end of this article.

Key Words. Breast neoplasms • Neoadjuvant therapy • Molecular targeted therapy • Gene expression profiling • PIK3CA • p95-HER2

Abstract

Background. The CHER-LOB randomized phase II study showed that the combination of lapatinib and trastuzumab plus chemotherapy increases the pathologic complete remission (pCR) rate compared with chemotherapy plus either trastuzumab or lapatinib. A biomarker program was prospectively planned to identify potential predictors of sensitivity to different treatments and to evaluate treatment effect on tumor biomarkers.

Materials and Methods. Overall, 121 breast cancer patients positive for human epidermal growth factor 2 (HER2) were randomly assigned to neoadjuvant chemotherapy plus trastuzumab, lapatinib, or both trastuzumab and lapatinib. Pre- and post-treatment samples were centrally evaluated for HER2, p95-HER2, phosphorylated AKT (pAKT), phosphatase and tensin homolog, Ki67, apoptosis, and *PIK3CA* mutations. Fresh-frozen tissue samples were collected for genomic analyses.

Results. A mutation in *PIK3CA* exon 20 or 9 was documented in 20% of cases. Overall, the pCR rates were similar in *PIK3CA* wild-type and *PIK3CA*-mutated patients (33.3% vs. 22.7%; $p = .323$). For patients receiving trastuzumab plus lapatinib, the probability of pCR was higher in *PIK3CA* wild-type tumors (48.4% vs. 12.5%; $p = .06$). Ki67, pAKT, and apoptosis measured on the residual disease were significantly reduced from baseline. The degree of Ki67 inhibition was significantly higher in patients receiving the dual anti-HER2 blockade. The integrated analysis of gene expression and copy number data demonstrated that a 50-gene signature specifically predicted the lapatinib-induced pCR.

Conclusion. *PIK3CA* mutations seem to identify patients who are less likely to benefit from dual anti-HER2 inhibition. p95-HER2 and markers of phosphoinositide 3-kinase pathway deregulation are not confirmed as markers of different sensitivity to trastuzumab or lapatinib. ***The Oncologist*** 2015;20:1001–1010

Implications for Practice: HER2 is currently the only validated marker to select breast cancer patients for anti-HER2 treatment; however, it is becoming evident that HER2-positive breast cancer is a heterogeneous disease. In addition, more and more new anti-HER2 treatments are becoming available. There is a need to identify markers of sensitivity to different treatments to move in the direction of treatment personalization. This study identified *PIK3CA* mutations as a potential predictive marker of resistance to dual anti-HER2 treatment that should be further studied in breast cancer.

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Introduction

Preoperative chemotherapy can increase the chance for breast-conserving surgery in patients who are initially candidates for mastectomy and offers the opportunity for a better definition of prognosis [1]. Achievement of a pathologic complete response (pCR) is a surrogate marker for long-term outcome, particularly for the most aggressive breast cancer subtypes such as those positive for human epidermal growth factor 2 (HER2) and triple-negative breast cancers [2]. Moreover, preoperative trials offer the opportunity to identify potential predictive biomarkers and to directly measure the pharmacodynamic effects of treatments. The possibility of identifying potential markers of treatment sensitivity or resistance is crucial when developing targeted agents, particularly for HER2-positive disease, for which several anti-HER2 agents and several combinations are now available. HER2 overexpression or amplification is currently the only validated marker to select patients for anti-HER2 therapy. Markers of the HER family and the downstream signaling pathways such as the truncated form of HER2 receptor, p95-HER2, and the AKT and phosphoinositide 3-kinase (PI3K) pathway have been studied as markers of trastuzumab resistance. p95-HER2 is an NH(2)-terminal truncated form of HER2 receptor that lacks the trastuzumab binding site, which is coexpressed in approximately 30% of HER2-positive breast cancer patients [3]. Preclinical and clinical evidence in the metastatic setting suggest that p95-HER2 confers resistance to trastuzumab without affecting sensitivity to kinase inhibitors [4–6]. PI3Ks are a superfamily of lipid kinases involved in the phosphorylation of the inositol head of the membrane phospholipids, regulated by growth factor receptors [7]. Loss of the phosphatase and tensin homolog (PTEN) has been associated with activation of the PI3K pathway, resulting in resistance to trastuzumab [8–10]. Moreover, activation of the PI3K pathway by *PIK3CA* mutation has a prognostic impact in advanced HER2-positive disease [11, 12]. The results of the CHER-LOB (Chemotherapy, Herceptin and Lapatinib in Operable Breast Cancer) study showed that dual HER2 blockade with trastuzumab and lapatinib combined with chemotherapy resulted in a significantly increased pCR rate compared with single HER2 blockade with either lapatinib or trastuzumab plus chemotherapy [13]. In this paper, we report the results of the preplanned translational biomarker program of the CHER-LOB study.

Methods

Clinical Platform

CHER-LOB is a phase II randomized multicenter trial in which 121 patients with primary HER2-positive breast cancer were randomized to receive preoperative chemotherapy with weekly paclitaxel for 12 weeks followed by 4 weekly courses over 3 weeks of the FEC regimen (fluorouracil, epirubicin, and cyclophosphamide) plus either trastuzumab (arm A), lapatinib (arm B), or the combination of trastuzumab and lapatinib (arm C). The trial design; eligibility criteria; statistical analysis; and clinical results, including response, surgery outcomes, and treatment safety, have been described in detail elsewhere [13]. Briefly, the main inclusion criteria included a diagnosis of breast cancer stage II to IIIA, HER2 positivity according to the local laboratory (immunohistochemistry [IHC] 3+ or fluorescence in situ hybridization [FISH] amplification), and no prior therapy for breast cancer. The translational biomarker program included the central reassessment of HER2 status, protein biomarker evaluation (p95-HER2, PTEN, phosphorylated AKT [pAKT], Ki67, terminal deoxynucleotidyl transferase dUTP nick end labeling [TUNEL]), the assessment of gene expression profile and copy number (CN) variations, and the study of somatic mutations of *PIK3CA*. The ethics committees of all participating sites approved the study, and informed consent was obtained from all patients prior to study entry.

Biomarkers and Genomic Analyses

Formalin-fixed paraffin-embedded (FFPE) tumor blocks from diagnostic core biopsies and from surgical specimens after preoperative study therapy were centralized and reviewed for quality and tumor content. Fresh-frozen samples from the diagnostic core biopsy were centralized for genomic studies.

Immunohistochemistry and Assessment of the Apoptotic Index

Hormone receptor (HR) status was locally evaluated by IHC, and the cutoff for positivity was immunostaining in ≥10% of tumor cells.

HER2 was centrally re-evaluated on diagnostic core biopsies and evaluated on surgical specimen following preoperative therapy by a dedicated breast pathologist. The following biomarkers were centrally evaluated on diagnostic core biopsies and on surgical specimens: Ki67, PTEN, pAKT. The following antibodies were used: HER2 (monoclonal antibody HER2, clone 4B5; Ventana Medical Systems, Inc., Tucson, AZ, http://www.ventana.com), Ki67 (clone Ki67-MIB-1; Dako, Glostrup, Denmark, http://www.dako.com), pAKT (Ser 473; Cell Signaling Technology, Beverly, MA, http://www.cellsignal.com), PTEN (clone 28H6, Novocastra; Buffalo Grove, IL, http://www.leicabiosystems.com). Immunohistochemical staining was performed according to the avidin-biotin method, using tissue sections of 3 μm in thickness. The following parameters were recorded: presence or absence of immunoreactivity (diffuse or focal), cell types exhibiting a positive reaction (tumor, endothelial, stromal, and inflammatory cells), and percentage of immunostained tumor cells.

PTEN loss was defined as IHC staining in <10% of cancer cells.

HER2 FISH analysis (PathVysion HER-2 DNA Probe Kit; Vysis Inc., Downers Grove, IL, https://www.abbottmolecular.com/) was performed in any case of HER2 IHC 2+ and in cases of discordance between the local and central laboratories.

Expression of p95-HER2 was determined on the diagnostic core biopsy by an IHC assay that detects a C-terminal fragment (CTF) of HER2 using a monoclonal antibody that specifically recognizes the 611 CTFs (BioTheranostics, San Diego, CA, http://www.biotheranostics.com). Tumors were scored according to the intensity of membrane staining as follows: 0, no staining; 1+, faint or barely perceptible membrane staining; 2+, weak to moderate complete membrane staining; 3+, strong and complete membrane staining. Different cutoffs

for binary score of positive or negative were tested from 30% to 80% of tumor cells with strong immunoreactivity. For correlation with clinical outcomes, we considered the 80% cutoff. p95-HER2 scoring was independently completed by two pathologists; a third independent pathologist examined discordant cases.

Tissue sections were stained using the TUNEL method, according to the standard procedure included in the Apop Tag Plus In Situ Apoptosis Detection Kit (Chemicon International, Billerica, MA, http://www.millipore.com). The percentage of apoptotic events per cells population was recorded by counting at least 3,000 malignant cells randomly selected at ×400 magnification. Gene expression and CN data have been deposited in the Gene Expression Omnibus (GEO) database (accession number GSE66399).

PIK3CA Mutation Analysis

Three 5-μm FFPE sections of a primary lesion containing at least 50% tumor cells were deparaffinized and incubated in lysis buffer with proteinase K (50 mM Tris, 1 mM EDTA, 05% TWEEN 20) at 56°C overnight. Genomic DNA was extracted with QIAmpl DNA Mini Kit (Qiagen, Valencia, CA, https://www.qiagen.com). DNA concentration was determined using the NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Freemont, CA, https://www.thermofisher.com). Genetic analysis of *PIK3CA* gene was performed using a commercially available "*PIK3CA* status kit" (certified CE-IVD for diagnostic use; Diatech Pharmacogenetics, Jesi, Ancona, Italy, http://www.diatechpharmacogenetics.com/en/). The kit permits the identification of mutations in codons 542, 545, and 546 of exon 9 (E542K, E545K, E545A, E545G, Q546E, Q546K) and codons 1043, 1047, and 1049 of exon 20 (M1043I, H1047Y, H1047R, H1047L, G1049R, G1049S) of the *PIK3CA* gene. Real-time polymerase chain reaction (RotorGene 6000; Qiagen) was carried out using 30-ng DNA as template. Specific mutations were subsequently identified by pyrosequencing on a PyroMark Q96 ID (Qiagen).

Statistical Analysis

pCR was defined as the absence of invasive breast cancer in both the breast and the axilla.

The association between baseline biomarkers with tumor characteristics and pCR was made by Pearson's chi-square. Biomarker change from baseline to surgery was evaluated by the Wilcoxon signed-rank matched-pair test. Difference in biomarker inhibition among arms was evaluated by the Student *t* test.

Genomic Studies

Genomic DNA and total RNA were extracted from pretreatment frozen core biopsies of the primary tumor using the commercial kit DNeasy and RNeasy Mini Kit (Qiagen). Each biopsy was pulverized in liquid nitrogen and processed following the manufacturer's protocol. Total RNA was eluted in a final volume of 35-μL of RNase-free water. DNA was eluted in a final volume of 35-μL of Tris-EDTA buffer. Total RNA was quantified using the NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific). RNA quality was assessed through evaluation of the A260:A280 and A260:A230 ratios of each sample and by means of capillary electrophoresis using the Agilent 2100 Bioanalyzer with the RNA 6000 Nano Assay Kit (Agilent Technologies, Santa Clara, CA, http://www.agilent.com), providing the resultant RNA integrity number (RIN) parameter. RIN is a quality score of nucleic acid integrity, independent from the amount of RNA loaded on the support and from sample origin, and is assigned by an algorithm that analyzes the entire electrophoretic trace of the RNA sample, including the 28S:18S ribosomal RNA ratio and the presence or absence of degradation products. RIN values span 1–10, in which 10 indicates that the nucleic acid is completely intact. The concentration of genomic DNA was measured using a spectrophotometer (NanoDrop ND-1000; Thermo Fisher Scientific). DNA quality was assessed through the evaluation of the A260:A280 and A260:230 ratios and by agarose gel electrophoresis.

For gene expression analyses, samples that met the quality requirements were further processed according to the Affymetrix GeneChip 3′ IVT Express Kit (Affymetrix, Santa Clara, CA, http://www.affymetrix.com) user's manual, starting from 150 ng of total RNA for each sample. The arrays were hybridized, washed, stained, using the Affymetrix Model 450 Fluidics Station using the manufacturer's recommended protocols. After hybridization, wash, and staining, probe array images were acquired using the laser scanner GCS 3000 7G (Affymetrix). The Affymetrix Gene Chip software Command Console was used to create the raw CEL files. Probe-level data were normalized and converted to expression values using the robust multiarray average procedure. Quality-control assessment was performed in the R statistical environment using *affy*, *affyQCReport*, and *affyPLM* Bioconductor packages (R Foundation, Vienna, Austria, https://www.r-project.org). Sample data were then filtered to remove probe sets with a coefficient of variation <0.8 or $>1{,}000$.

To identify a gene set for specifically predicting the response to lapatinib, the expression profiles of patients randomized to chemotherapy and lapatinib (arm B) were randomly split into two sets, called the *training* and *test* sets, including a balanced number of pCR in each group. Gene expression data from the training set were used to select differentially expressed genes (DEGs). DEGs were obtained by merging the gene lists derived from two different analyses. The first analysis was based on the analysis of variance procedure implemented in GeneSpring (Agilent Technologies) and allowed selection of those probe sets that showed a p value $<.01$ and a contrast fold change >2. The second analysis was based on the Compare Sample procedure implemented in dChip software (Harvard University, Cambridge, MA, http://www.hsph.harvard.edu/cli/complab/dchip/). The resulting list of DEGs was used to perform a class prediction analysis using the *support vector machines* algorithm implemented in GeneSpring. The agglomerative hierarchical clustering was performed using Euclidean distance and average as a similarity metric and a linkage method, respectively. The Golub method, as implemented in the GeneSpring package, was used to select the top 50 predictor genes from the DEG list.

For CN analyses, 250 ng of genomic DNA was digested with Sty I and Nsp I (New England BioLabs, Ipswich, MA, https://www.neb.com) for each individual assay. After the

reaction with restriction enzymes was completed, the manufacturer's instructions for the Affymetrix Genome-wide Human SNP (single nucleotide polymorphism) array 6.0 were followed. The concentration of polymerase chain reaction products after purification with magnetic beads (Agencourt Magnetic Beads; Beckman Coulter, Indianapolis IN, https://www.beckmancoulter.com) was measured using a spectrophotometer (NanoDrop ND-1000; Thermo Fisher Scientific). Purified polymerase chain reaction products were diluted 10-fold with TE buffer (pH 8.0; Wako Pure Chemical Industries, Ltd, Osaka, Japan, http://www.wako-chem.co.jp/english/) to have a suitable concentration for the spectrophotometer measurement. Log^2 ratios were determined using the algorithm embedded in the software Affymetrix Genotyping Console 2.0 (Affymetrix). CN data were analyzed using the *lokern smoothing copy number* (LSCN) procedure of the PREDA Bioconductor package to estimate CN gain or loss at gene loci in individual samples [14, 15]. In LSCN, CN data are transformed into a score that quantifies, for each SNP in any sample, the amplitude of the CN variation from the diploid status, estimated from the median CN calculated over all SNP probes. CN scores are then estimated at gene positions using a kernel regression estimator with an automatically adapted local plug-in bandwidth. Finally, a permutation scheme is used to identify genes with statistically significant CN imbalances under the assumption that each chromosomal position has a unique CN characteristic and that the corresponding score is not comparable with any score in other positions of the genome. The CN status of a gene can be thus defined as loss or gain when the statistical significance is below a given q value threshold ($1 \times 10 - 2$) and the CN score is smaller than the low-score threshold (10th quantile of scores) or larger than the high-score threshold (90th quantile of scores), respectively. To elevate the analysis from the single- to the multiple-sample level and to detect the presence of a common signature across an entire data set, CN imbalances from all single-sample analyses are aggregated to generate summary scores for amplifications and deletions using a binomial distribution test and the q value to correct for multiple hypothesis testing [14, 15].

Results

Of 113 samples available for centralized HER2 evaluation, 110 were confirmed as HER2 overexpressing (IHC 3+, $n = 82$), or FISH amplified ($n = 28$), for an overall concordance with local laboratories of 97%.

Pretreatment tumor characteristics are summarized in Table 1. The majority of tumors were classified as having ductal histology and grade 3. Sixty percent of the cases had positive expression of hormone receptor (estrogen or progesterone receptor), and 58.7% of the patients were estrogen receptor positive. A significant correlation between the expression of estrogen receptor and Ki67 ($\rho = -0.256, p = .006$), p95-HER2 ($\rho = -0.22, p = .018$), and pAKT ($\rho = -0.31$, $p = .0007$) was observed. A significant correlation between p95-HER2 and Ki67 ($\rho = 0.19, p = .046$) and between pAKT and Ki67 ($\rho = 0.24$, $p = .015$) was observed.

Table 1. Tumor characteristics at baseline

Characteristics	*n* (%)
Patients	121 (100)
Histology	
Ductal	111 (91.7)
Lobular/other	10 (8.3)
Histologic grade	
G1/2	23 (19)
G3	77 (63.6)
NA	21 (17.4)
Hormone receptor expression[a]	
ER and/or PgR positive	73 (60.3)
ER and PgR negative	48 (39.7)
ER positive	71 (58.7)
PgR negative	48 (39.7)
Central laboratory assessed	
p95-HER2 expression[b]	
Positive	36 (29.7)
Negative	70 (57.8)
NA	15 (12.4)
Ki67 expression, %, median (min; max)	25 (4; 90)
TUNEL, %, median (min; max)	0.6 (0.05; 2.6)
PTEN expression, %, median (min; max)	80 (0; 100)
pAKT expression, %, median (min; max)	4 (0; 100)

[a]Cutoff for ER and PgR positivity: ≥10% of staining tumor cells.
[b]Cutoff for p95-HER2 positivity: ≥80% of staining tumor cells.
Abbreviations: ER, estrogen receptor; max, maximum; min, minimum; NA, not available; pAKT, phosphorylated Akt; PgR, progesterone receptor; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling.

PI3K pathway analyses are summarized in Table 2. Overall, 20% of the tumors harbored a *PIK3CA* mutation. Specifically, 16 patients had mutation in exon 20, and 6 patients had mutation in exon 9. A trend for higher prevalence of PTEN loss was observed in the group of patients with HR-negative tumors compared with HR-positive cases (20.5% vs 8.5%, $p = .08$).

Prediction of Pathologic Response

As previously reported, pCR was documented in 33% of patients. The distribution of pCR was as follows: 25% (90% CI: 13.1%–36.9%) in patients randomized to chemotherapy plus trastuzumab (arm A), 26.3% (90% CI: 14.5%–38.1%) in patients randomized to chemotherapy plus lapatinib (arm B), and 46.7% (90% CI: 34.4%–58.9%) in patients randomized to chemotherapy plus trastuzumab and lapatinib (arm C) [13].

Figure 1 describes the rate of pCR overall and per treatment arm, according to the following parameters: expression of hormone receptor, p95-HER2 expression, *PIK3CA* mutations, and PI3K pathway deregulation (defined as *PIK3CA* mutation and/or PTEN loss). The pCR rate tended to be higher in case of absence of hormone receptor. The expression of p95-HER2 was not able to predict a different probability of achieving pCR by exposure to trastuzumab or lapatinib or both.

In the whole population, pCR rates were similar in *PIK3CA* wild-type and *PIK3CA*-mutated patients (33.7% vs. 22.7%;

Table 2. PI3K pathway alteration, overall and by expression of hormone receptor

Alteration	Overall, *n* (%)	HR negative, *n* (%)	HR positive, *n* (%)
Patients	108 (100)	32 (100)	76 (100)
PIK3CA status			
WT	86 (79.6)	25 (78.1)	61 (80.3)
Mutated[a]	22 (20.4)	7 (21.9)	15 (19.7)
PTEN status	98 (100)	39 (100)	59 (100)
PTEN loss	13 (13.2)	8 (20.5)	5 (8.5)
PI3K/PTEN dysfunction	95 (100)	37 (100)	58 (100)
PIK3CA mutation and/or PTEN loss	30 (31.6)	12 (32.4)	18 (31)

[a]Exon 9 mutations, $n = 6$ (locus E545K, $n = 5$; E545G, $n = 1$); exon 20 mutations, $n = 16$ (locus H1047R, $n = 15$, H1047L, $n = 1$).
Abbreviations: HR, hormone receptor; PI3K, phosphoinositide 3-kinase; PTEN, phosphatase and tensin homolog; WT, wild type.

$p = .323$); however, for patients receiving trastuzumab plus lapatinib ($n = 41$), the pCR rate was higher in *PIK3CA* wild-type tumors compared with *PIK3CA*-mutant tumors, although it did not reach statistical significance (48.4% vs. 12.5%; $p = .06$).

Treatment-Induced Modulation of Tumor Biomarkers

Treatment effects on HER2 expression, Ki67, apoptosis, and the expression of pAKT and PTEN were measured in residual disease after preoperative chemotherapy. Overall, 10 of the 69 evaluable patients (14.5%) showed a loss in HER2 expression (3 in arm A, 3 in arm B, and 4 in arm C). Table 3 summarizes treatment effect on the expression of tumor biomarkers overall and by treatment arm. Overall, Ki67, pAKT, and apoptosis were significantly decreased from baseline. No significant differences in the mean suppression of Ki67, pAKT, and apoptosis between the trastuzumab and lapatinib arms were observed. Mean Ki67 suppression was higher after dual HER2 blockade compared with combined single-agent arms (-19.9 vs. -8.5, $p = .0036$). Mean pAKT inhibition was significantly higher in cases of hormone receptor negativity (-40.3 vs. -8.0, $p = .0005$).

Genomic Analyses

Gene expression profiles were obtained from 88 samples deriving from 88 different patients. Patients randomized to receive chemotherapy plus lapatinib were split randomly into training and test sets, balanced for number of pCRs. A 50-gene predictor list was identified in the training set (supplemental online Tables 1, 2). This 50-gene predictor list was then used to predict pCR in the test set data, obtaining 93% correct predictions (sensitivity 0.75; specificity 1) (supplemental online Table 3). The same class-prediction algorithm and the same predictor gene list were applied to predict a pCR class for patients enrolled in arm A (chemotherapy plus trastuzumab) and arm C (chemotherapy plus trastuzumab and lapatinib) and obtained very low performance (arm A: sensitivity 0.16, specificity 0.85; arm C: sensitivity 0.38, specificity 0.80) (supplemental online Tables 4, 5), suggesting that our signature was specific for predicting response in the chemotherapy plus lapatinib arm (arm B).

CN data were obtained from 68 patients (18 in arm A, 23 in arm B, and 27 in arm C). A signature of CN alteration was present for patients not achieving pCR (Fig. 2A, 2C, 2E), whereas no significant copy number alterations were specifically obtained for patients achieving pCR independently from treatment arm (Fig. 2B, 2D, 2F). The copy number alterations present in samples from all trial arms are listed in supplemental online Table 6.

Discussion

The phase II randomized CHER-LOB study was designed to evaluate the role of neoadjuvant dual HER2 inhibition combined with chemotherapy in HER2-positive breast cancer. An extensive biomarker evaluation was planned prospectively to identify potential molecular predictors of different treatment sensitivity. Currently, HER2 overexpression or amplification represents the only validated predictor of response to anti-HER2 therapy. Although HER2 testing has become a routine part of breast cancer diagnosis, the reproducibility of HER2 assessment remains an issue. In our study, HER2 status was centrally confirmed in 97% of the cases. Our study was conducted in a relatively small number of high-qualified centers with dedicated breast pathologists, explaining the high concordance, which compares favorably with the literature [16, 17].

Tumor characteristics at baseline were consistent with a population of HER2-positive cases, with the majority of the cases being highly proliferative and poorly differentiated with ductal histology. The inverse relationship between the expression of hormone receptor and Ki67, p95-HER2, and pAKT confirms the different biology of hormone receptor-positive versus -negative tumors, even in the presence of HER2 overexpression and amplification. The prevalence of *PIK3CA* mutations in our study (20%) is consistent with reports from neoadjuvant studies in HER2-positive breast cancer populations (range: 19.2%–24.3%) [18, 19].

The identification of markers associated with different probabilities of achieving pCR was an aim of the CHER-LOB study. From this perspective, the three-arm design allowed for the exploratory comparison of different biomarkers and response to chemotherapy plus trastuzumab, lapatinib, or both.

One hypothesized biomarker of trastuzumab resistance is the deregulation of the PI3K pathway. In the present analysis, *PIK3CA* mutations were suggested to predict resistance to neoadjuvant chemotherapy plus the combination of trastuzumab and lapatinib. Although this result did not reach statistical significance, probably because of the limited sample size, it is in line with findings from the NeoALTTO trial and the combined analysis of the GeparQuattro, GeparQuinto, and GeparSixto studies [18, 19]. In both analyses, pCR rates after chemotherapy plus anti-HER2 treatment (either single or dual blockade) were numerically lower for PIK3CA-mutated patients compared with wild-type cases. The trend was particularly evident and significant in cases of dual HER2 blockade. The only difference with our data consists in the results we observed in arm A (chemotherapy

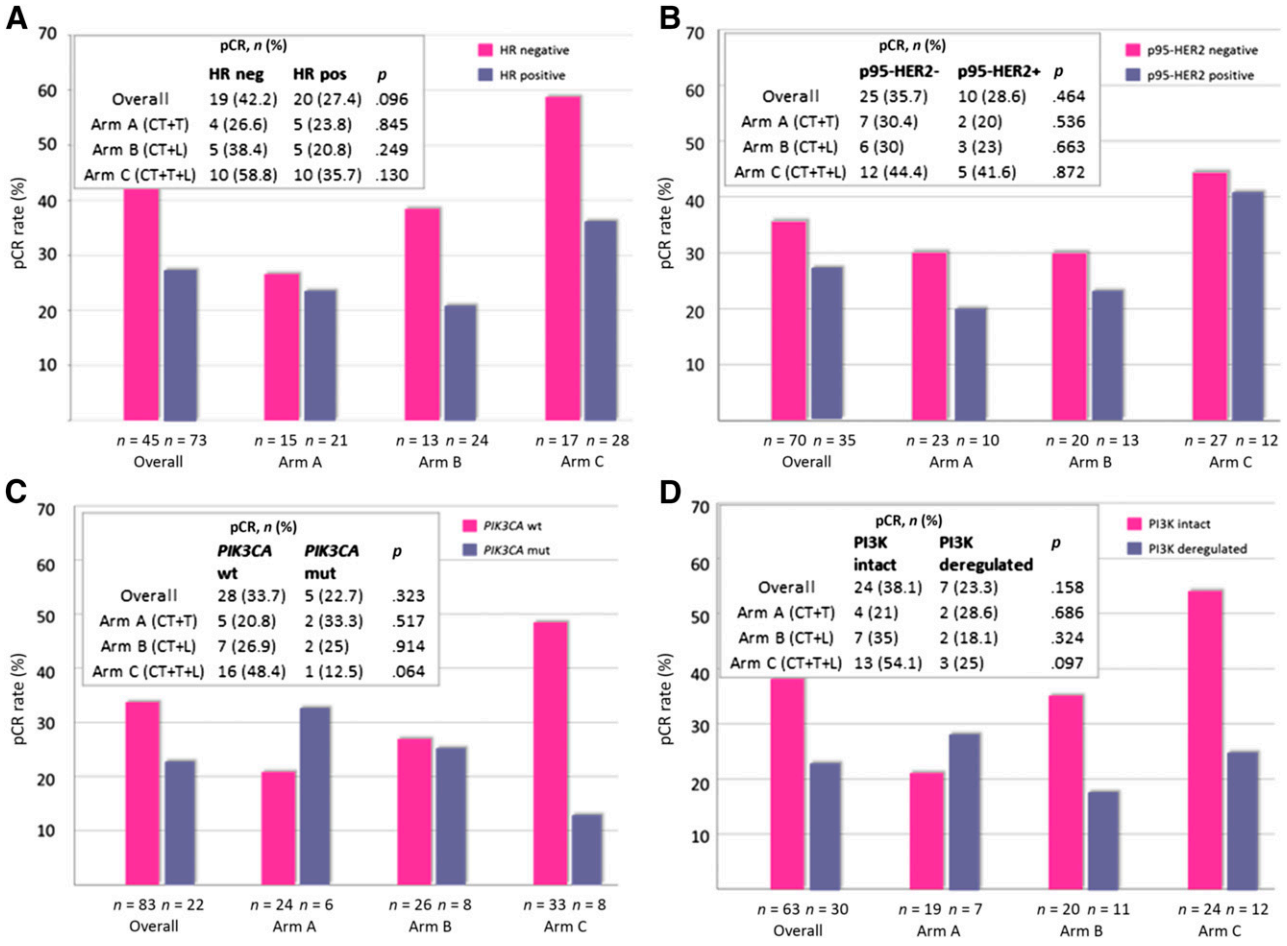

Figure 1. Pathologic complete remission rates according to biomarkers in the CHER-LOB study. Pathologic complete remission rates (ypT0/is ypN0) according to hormone receptor **(A)**, p95-HER2 **(B)**, *PIK3CA* status **(C)**, and PI3K pathway dysfunction **(D)**.

Abbreviations: −, negative; +, positive; CT, chemotherapy therapy; HR, hormone receptor; L, lapatinib; mut, mutated; neg, negative; pCR, pathologic complete response; PI3K, phosphoinositide 3-kinase; pos, positive; T, trastuzumab; wt, wild type.

and trastuzumab), with an apparently lower pCR rate for *PIK3CA* wild-type patients. It has to be taken into account that there were fewer correctly genotyped patients in arm A ($n = 30$) than in arms B ($n = 34$) and C ($n = 41$). Together with the low number of events in arm A, this suggests caution in interpreting the results. Moreover, it has been confirmed recently by combined analysis of nearly 1,000 patients from randomized trials (including the CHER-LOB study) that the *PIK3CA* gene status significantly predicts pCR after chemotherapy plus dual HER2 blockade and not after chemotherapy plus a single anti-HER2 agent [20, 21]. Some data, derived mainly from retrospective studies conducted in the advanced setting and small neoadjuvant series, suggest that by combining *PIK3CA* genotype and PTEN status, more information on the deregulation of the PI3K pathway can be obtained that could potentially predict for resistance to anti-HER2 agents better than either marker alone [8, 9, 22]. This result was not confirmed in our study. No significant difference in the probability of obtaining pCR was observed according to PI3K pathway deregulation, as defined by *PIK3CA* mutations and/or PTEN loss, overall and by treatment arm. Recently, the German Breast Group presented a similar analysis in the GeparQuattro study. In contrast with our data, those investigators found that PTEN assessment added information to *PIK3CA* genotype for the prediction of resistance to neoadjuvant chemotherapy plus trastuzumab [23]. The main issue with PTEN evaluation is the lack of a validated method of assessment and cutoff, which impairs a correct interpretation and limits the consistency of the results across studies.

In our study, expression of p95-HER2 was not associated with pCR overall and in each arm and did not predict for different sensitivity to trastuzumab, lapatinib, or the combination. We acknowledge the small size and the lack of a standardized p95-HER2 assay as potential limitations of the present analysis. Nevertheless, even when applying different cutoffs for defining p95-HER2 status, no differences were recorded (data not shown). Expression of p95-HER2 was previously identified as a marker of resistance to trastuzumab not affecting the activity of lapatinib [3]. In unplanned retrospective analyses, p95-HER2 correlated with worse outcome with trastuzumab in advanced disease [6]; however, data from neoadjuvant trials of chemotherapy plus anti-HER2 treatment provided results that contrast with the first evaluation of p95-HER2 as a marker of resistance to trastuzumab. Indeed, a positive expression of

Table 3. Treatment-induced modulation of tumor biomarkers

Biomarker	*n*	Post-treatment values				Mean change from baseline	*p* value[a]
		Mean	Median	Minimum	Maximum		
All patients							
Ki67, %	61	12	12	1	50	−11.8	<.0001
pAKT, %	55	8.8	0	0	90	−18.4	.0003
TUNEL test, %	29	0.21	0.1	0.05	1.1	−0.49	.0002
PTEN %	45	69.5	80	0	100	1.90	.8255
Arm A (CT + trastuzumab)							
Ki67, %	22	21.1	19	1	50	−6.04	.050
pAKT, %	18	1.1	0	0	10	−22.22	.0052
TUNEL test, %	7	0.4	0.1	0.05	1.1	−0.08	.3508
PTEN, %	14	76.4	100	10	100	22.1	.0388
Arm B (CT + lapatinib)							
Ki67, %	21	17.6	15	3	40	−11.2	.0037
pAKT, %	20	14.75	0	0	90	−16.58	.0620
TUNEL test, %	12	0.2	0.1	0.05	0.55	−0.05	.0107
PTEN, %	16	57.2	80	0	100	−4.0	.6683
Arm C (CT+ trastuzumab + lapatinib)							
Ki67, %	18	9.5	10	1	30	−19.9	.0003
pAKT, %	17	10	0	0	90	−16.25	.0818
TUNEL test, %	11	0.11	0.05	0.05	0.4	−0.76	.0076
PTEN, %	15	76.3	80	0	100	−8.33	.2763

[a]Wilcoxon rank-sign test.
Abbreviations: CT, chemotherapy; pAKT, phosphorylated Akt; PTEN, phosphatase and tensin homolog; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling.

p95-HER2 in patients enrolled in the GeparQuattro trial predicted for a higher pCR rate after chemotherapy plus trastuzumab [24]. Similarly, in the NeoALTTO trial, p95-HER2 expression was found to positively correlate with pCR in multivariate analysis. The association was seen in the trastuzumab-containing arms and not in the lapatinib arm [25]. Taken together, these data do not endorse p95-HER2 as a marker of resistance to trastuzumab, at least in the neoadjuvant setting.

In addition to the investigation for predictive biomarkers, the advantage of the neoadjuvant approach allows for a direct measurement of treatment effect on biomarker expression in residual disease. In our study, a loss of HER2 positivity was reported in 14% of the cases. No significant differences in HER2 loss according to exposure to trastuzumab or lapatinib or both were observed. The loss of HER2 expression after neoadjuvant anti-HER2 therapy has been described previously and seems to be associated with poorer prognosis [26, 27].

The inhibition of Ki67 after neoadjuvant therapy has a prognostic role. Several observations have shown that patients with a high Ki67 level after neoadjuvant chemotherapy have worse prognosis [28, 29]. In our study, we observed a significant reduction in Ki67 and apoptosis from baseline values in all three arms. The mean Ki67 suppression was similar between the trastuzumab and lapatinib arms but was significantly higher in the dual-blockade arm. It would be of interest to observe whether this greater proliferation inhibition will be associated with better survival in patients not achieving pCR.

In the last few years, high-throughput technologies have been applied extensively in early breast cancer to identify subgroups of patients with different prognoses or treatment sensitivities. The search for predictive signatures has been investigated particularly in the setting of neoadjuvant trials, in which pCR is considered a marker for treatment activity.

Another aim of our study was to identify a signature potentially able to discriminate sensitivity to lapatinib. A 50-gene signature was identified as a predictor of pCR in the chemotherapy plus lapatinib arm. As expected, the same signature was unable to predict pCR in the chemotherapy plus trastuzumab arm. The same low performance was observed with chemotherapy plus trastuzumab and lapatinib. Two hypotheses may exist: (a) that the genes involved in the response to trastuzumab and not to lapatinib are the main determinants of the activity of the dual combination; (b) that the genes that correlate with response to dual combination may not necessarily overlap with those involved in response to either single agent alone, suggesting that the dual combination results in synergistic rather than merely additive biological activity. Consequently, the identified 50-gene signature seems specific for predicting response to single-agent lapatinib plus chemotherapy only. A 30-gene paclitaxel, fluorouracil, doxorubicin, and cyclophosphamide chemotherapy response predictor (DLDA-30) in a multicenter randomized trial in breast cancer [30] was recently reported to demonstrate very good performance in the identification of patients with greater-than-average sensitivity to T/FAC

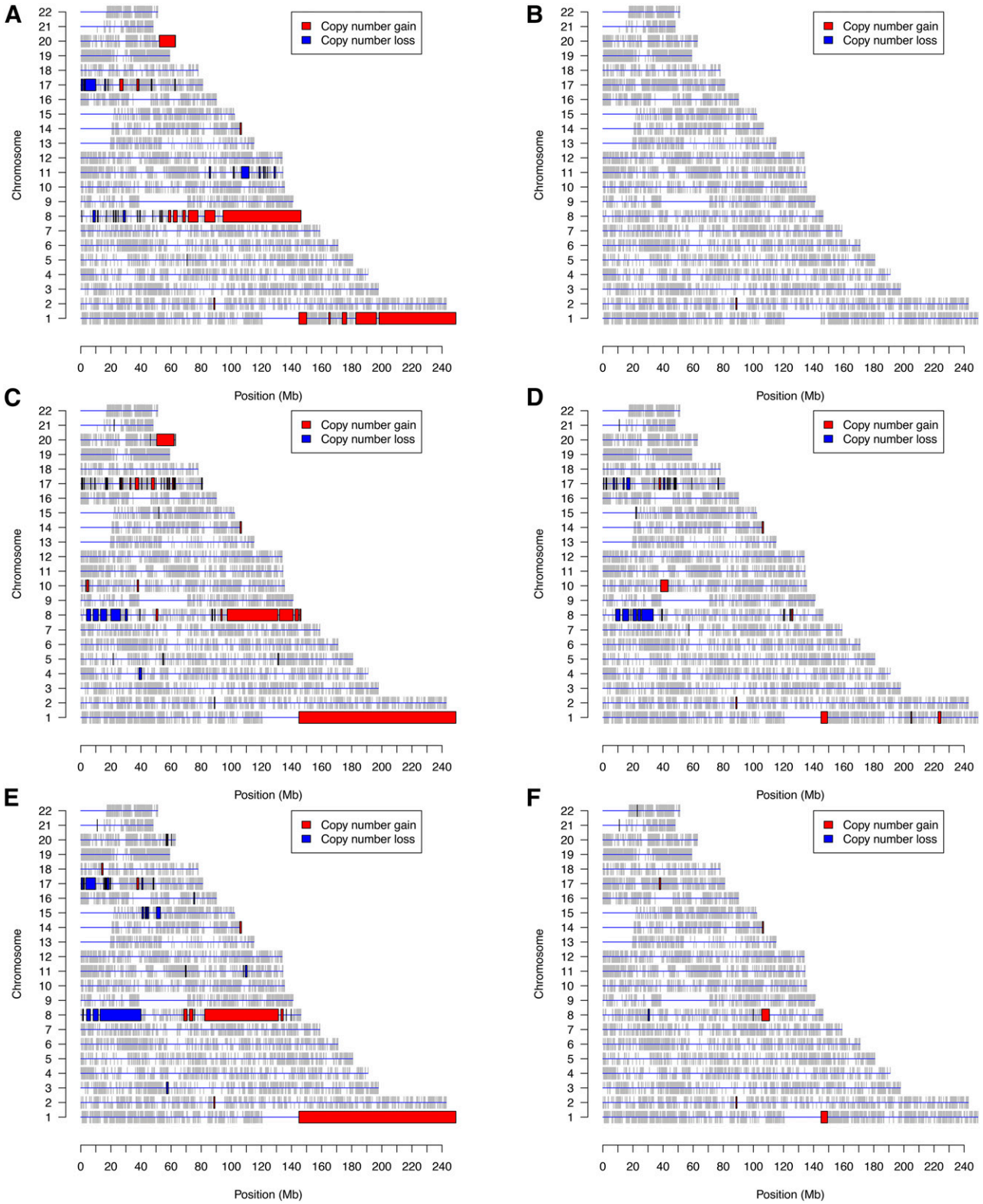

Figure 2. Copy number (CN) alteration and pathologic complete response (pCR). Results of the CN study obtained using the lokern smoothing copy number procedure. Each chromosome (*y*-axis) is represented along with its length (*x*-axis). CN loss is represented in the specific position as a blue bar, whereas CN gain is represented as a red bar. Panels refer to results for 68 breast cancer samples derived from 68 different patients who did not achieve a pCR (left column) **(A, C, E)** or who achieved a pCR (right column) **(B, D, F)** and were enrolled in arm B (23 samples; first row) **(A, B)**, in arm A (18 samples; second row) **(C, D)**, and in arm C (27 samples; third row) **(E, F)**.

chemotherapy. We applied the support vector machines algorithm for pCR prediction in all arms using this DLDA-30 predictor to further demonstrate that the response of patients to chemotherapy did not represent a bias for the correct evaluation of lapatinib-induced pCR. The results showed lower performance of DLDA-30 in predicting either lapatinib- or trastuzumab-induced pCR (data not shown).

The CN analysis identified a CN signature in patients not achieving pCR independently from the treatment arm, suggesting that such genomic alterations may contribute to a composite resistance to treatment and thus may be predictive of worse outcome. These new intriguing data deserve to be confirmed in larger analyses.

In conclusion, the presence of *PIK3CA* mutations may identify patients who are less likely to benefit from dual anti-HER2 inhibition. Taking into account the availability of agents specifically targeting the PI3K pathway, *PIK3CA* status seems to be the only marker in this analysis to be studied further in HER2-positive disease.

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Disclosures

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References

1. Guarneri V, Broglio K, Kau SW et al. Prognostic value of pathologic complete response after primary chemotherapy in relation to hormone receptor status and other factors. J Clin Oncol 2006;24:1037–1044.

2. Cortazar P, Zhang L, Untch M et al. Pathological complete response and long-term clinical benefit in breast cancer: The CTNeoBC pooled analysis. Lancet 2014;384:164–172.

3. Scaltriti M, Rojo F, Ocaña A et al. Expression of p95HER2, a truncated form of the HER2 receptor, and response to anti-HER2 therapies in breast cancer. J Natl Cancer Inst 2007;99:628–638.

4. Arribas J, Baselga J, Pedersen K et al. p95HER2 and breast cancer. Cancer Res 2011;71:1515–1519.

5. Sperinde J, Jin X, Banerjee J et al. Quantitation of p95HER2 in paraffin sections by using a p95-specific antibody and correlation with outcome in a cohort of trastuzumab-treated breast cancer patients. Clin Cancer Res 2010;16:4226–4235.

6. Duchnowska R, Sperinde J, Chenna A et al. Quantitative measurements of tumoral p95HER2 protein expression in metastatic breast cancer patients treated with trastuzumab: Independent validation of the p95HER2 clinical cutoff. Clin Cancer Res 2014;20:2805–2813.

7. Amzel LM, Huang CH, Mandelker D et al. Structural comparisons of class I phosphoinositide 3-kinases. Nat Rev Cancer 2008;8:665–669.

8. Esteva FJ, Guo H, Zhang S et al. PTEN, PIK3CA, p-AKT, and p-p70S6K status: Association with trastuzumab response and survival in patients with HER2-positive metastatic breast cancer. Am J Pathol 2010;177:1647–1656.

9. Dave B, Migliaccio I, Gutierrez MC et al. Loss of phosphatase and tensin homolog or phosphoinositol-3 kinase activation and response to trastuzumab or lapatinib in human epidermal growth factor receptor 2-overexpressing locally advanced breast cancers. J Clin Oncol 2011;29:166–173.

10. Razis E, Bobos M, Kotoula V et al. Evaluation of the association of PIK3CA mutations and PTEN loss with efficacy of trastuzumab therapy in metastatic breast cancer. Breast Cancer Res Treat 2011;128:447–456.

11. Baselga J, Cortés J, Im SA et al. Biomarker analyses in CLEOPATRA: A phase III, placebo-controlled study of pertuzumab in human epidermal growth factor receptor 2-positive, first-line metastatic breast cancer. J Clin Oncol 2014;32:3753–3761.

12. Xu B, Guan Z, Shen Z et al. Association of phosphatase and tensin homolog low and phosphatidylinositol 3-kinase catalytic subunit alpha gene mutations on outcome in human epidermal growth factor receptor 2-positive metastatic breast cancer patients treated with first-line lapatinib plus paclitaxel or paclitaxel alone. Breast Cancer Res 2014;16:405.

13. Guarneri V, Frassoldati A, Bottini A et al. Preoperative chemotherapy plus trastuzumab, lapatinib, or both in human epidermal growth factor receptor 2-positive operable breast cancer: Results of the randomized phase II CHER-LOB study. J Clin Oncol 2012;30:1989–1995.

14. Bicciato S, Spinelli R, Zampieri M et al. A computational procedure to identify significant overlap of differentially expressed and genomic imbalanced regions in cancer datasets. Nucleic Acids Res 2009;37:5057–5070.

15. Ferrari F, Solari A, Battaglia C et al. PREDA: An R-package to identify regional variations in genomic data. Bioinformatics 2011;27:2446–2447.

16. McCullough AE, Dell'orto P, Reinholz MM et al. Central pathology laboratory review of HER2 and ER in early breast cancer: An ALTTO trial [BIG 2-06/NCCTG N063D (Alliance)] ring study. Breast Cancer Res Treat 2014;143:485–492.

17. Perez EA, Press MF, Dueck AC et al. Immunohistochemistry and fluorescence in situ hybridization assessment of HER2 in clinical trials of adjuvant therapy for breast cancer (NCCTG N9831, BCIRG 006, and BCIRG 005). Breast Cancer Res Treat 2013;138:99–108.

18. Majewski IJ, Nuciforo P, Mittempergher L et al. *PIK3CA* mutations are associated with decreased benefit to neoadjuvant human epidermal growth factor receptor 2-targeted therapies in breast cancer. J Clin Oncol 2015;33:1334–1339.

19. Loibl S, von Minckwitz G, Schneeweiss A et al. PIK3CA mutations are associated with lower rates of pathologic complete response to anti-human epidermal growth factor receptor 2 (her2) therapy in primary HER2-overexpressing breast cancer. J Clin Oncol 2014;32:3212–3220.

20. Guarneri V, Dieci MV, Carbognin L et al. Activity of neoadjuvant lapatinib (L) plus trastuzumab (T) for early breast cancer (EBC) according to PIK3CA mutations: Pathological complete response (pCR) rate in the CherLOB study and pooled analysis of randomized trials [abstract 254O]. Presented at: European Society of Medical Oncology meeting; September 26–30, 2014, Madrid, Spain.

21. Loibl S, Majewski I, Guarneri V. PIK3CA mutation correlates with pathological complete

response in primary HER2-positive breast cancer – combined analysis of 967 patients from three prospective clinical trials. J Clin Oncol 2015;33 (suppl):511a.

22. Berns K, Horlings HM, Hennessy BT et al. A functional genetic approach identifies the PI3K pathway as a major determinant of trastuzumab resistance in breast cancer. Cancer Cell 2007;12: 395–402.

23. Loibl S, Darb-Esfahani S, Klimowicz A et al. *PIK3CA* but not p4EBP1 are associated with low rates of pathological complete response (pCR) to trastuzumab based chemotherapy in primary HER2-overexpressing breast cancer [abstract PD5-7]. Presented at: San Antonio Breast Cancer Symposium; December 9–13, 2014; San Antonio, TX.

24. Loibl S, Bruey J, von Minckwitz G et al. Validation of p95 as a predictive marker for trastuzumab-based therapy in primary HER2-positive breast cancer: A translational investigation from the neoadjuvant GeparQuattro study. J Clin Oncol 2011;29(suppl):530a.

25. Scaltriti M, Nuciforo P, Bradbury I et al. High HER2 expression correlates with response to the combination of lapatinib and trastuzumab. Clin Cancer Res 2015;21:569–576.

26. Mittendorf EA, Wu Y, Scaltriti M et al. Loss of HER2 amplification following trastuzumab-based neoadjuvant systemic therapy and survival outcomes. Clin Cancer Res 2009;15:7381–7388.

27. Guarneri V, Dieci MV, Barbieri E et al. Loss of HER2 positivity and prognosis after neoadjuvant therapy in HER2-positive breast cancer patients. Ann Oncol 2013;24:2990–2994.

28. Jones RL, Salter J, A'Hern R et al. The prognostic significance of Ki67 before and after neoadjuvant chemotherapy in breast cancer. Breast Cancer Res Treat 2009;116:53–68.

29. Guarneri V, Piacentini F, Ficarra G et al. A prognostic model based on nodal status and Ki-67 predicts the risk of recurrence and death in breast cancer patients with residual disease after pre-operative chemotherapy. Ann Oncol 2009;20: 1193–1198.

30. Tabchy A, Valero V, Vidaurre T et al. Evaluation of a 30-gene paclitaxel, fluorouracil, doxorubicin, and cyclophosphamide chemotherapy response predictor in a multicenter randomized trial in breast cancer. Clin Cancer Res 2010;16:5351–5361.

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For Further Reading:

Mellissa Hicks, Erin R. Macrae, Mahmoud Abdel-Rasoul et al. Neoadjuvant Dual HER2-Targeted Therapy With Lapatinib and Trastuzumab Improves Pathologic Complete Response in Patients With Early Stage HER2-Positive Breast Cancer: A Meta-Analysis of Randomized Prospective Clinical Trials. ***The Oncologist*** 2015;20:337–343.

Implications for Practice:

This meta-analysis reports that the addition of lapatinib to trastuzumab and neoadjuvant chemotherapy improves pathologic complete response (pCR) rates in patients with HER2-positive breast cancer, regardless of the pCR definition or hormone receptor status.