Effects of Estrogen Receptor and Human Epidermal Growth Factor Receptor-2 Levels on the Efficacy of Trastuzumab
A Secondary Analysis of the HERA Trial

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IMPORTANCE  A number of studies suggest that response to antihuman epidermal growth factor receptor-2 (currently known as ERBB2, but referred to as HER2 in this study) agents differs by estrogen receptor (ER) level status. The clinical relevance of this is unknown.

OBJECTIVE  To determine the magnitude of trastuzumab benefit according to quantitative levels of ER and HER2 in the HERceptin Adjuvant (HERA) trial.

DESIGN, SETTING, AND PARTICIPANTS  The HERA trial was an international, multicenter, randomized trial that included 5099 patients with early-stage HER2-positive breast cancer, randomized between 2001 and 2005 to receive either no trastuzumab or trastuzumab, after adjuvant chemotherapy. This is a secondary analysis of the HERA study. Local ER immunohistochemical (IHC) analyses, HER2 fluorescence in situ hybridization (FISH) ratio, and copy number results were available for 3037 patients (59.6%) randomized to observation and trastuzumab (1 or 2 years) (cohort 1). Transcript levels of ESR1 and HER2 genes were available for 615 patients (12.1%) (cohort 2).

INTERVENTIONS  Patients were randomized to receive either no trastuzumab or 1 year vs 2 years of trastuzumab. Endocrine therapy was given to patients with hormone receptor–positive disease as per local guidelines.

MAIN OUTCOMES AND MEASURES  Disease-free survival (DFS) and overall survival (OS) were the primary and secondary end points in the intent-to-treat population (ITT). Analyses adjusting for crossover (censored and inverse probability weighted [IPW]) were also performed. Interactions among treatment, ER status, and HER2 amplification using predefined cutoffs were assessed in Cox proportional hazards regression models.

RESULTS  Median follow-up time was 8 years. Levels of FISH and HER2 copy numbers were significantly higher in ER-negative patients ($P < .001$). In cohort 1, for DFS and OS, a significant treatment effect was found for all ER, IHC, and FISH levels, except for the ER-positive/HER2 low FISH ratio ($\geq 2$ to $< 5$) group (DFS: 3-way ITT $P$ value for interaction = .07; censored = .02; IPW = .03; OS ITT $P$ value for interaction = .007; censored = .04; IPW = .03). In cohort 2, consistent with cohort 1, a significant predictive effect of the ESR1 gene for both end points was also observed (DFS $P$ value for interaction = .06; OS = .02), indicating that breast cancers with higher ESR1 levels also derive less benefit from trastuzumab.

CONCLUSIONS AND RELEVANCE  Patients with HER2-positive breast cancers that are ER-positive by IHC analyses with low FISH ratio ($\geq 2$ to $< 5$), or with higher ESR1 levels derive significantly less benefit from adjuvant trastuzumab after chemotherapy. These data may explain heterogeneity in response to anti-HER2 agents in HER2-positive, ER-positive breast cancers as some may be more luminal-like than HER2 driven.

TRIAL REGISTRATION  clinicaltrials.gov Identifier: NCT0045032
The use of trastuzumab, a monoclonal antibody targeted against the human epidermal growth factor receptor-2 (currently known as ERBB2, but referred to as HER2 in this study) receptor, in addition to cytotoxic chemotherapy, has radically changed the natural history of breast cancer that overexpresses the human epidermal growth factor-2 (HER2/neu or ERBB2) oncogene, resulting in considerable survival improvements. Despite concerted efforts to identify trastuzumab resistance mechanisms, the only biomarker currently validated to select patients for trastuzumab and other anti-HER2 therapy is still HER2 gene amplification and/or protein overexpression.

An inverse correlation between levels of the estrogen receptor (ER) and HER2 has been previously noted—patients with higher HER2 amplification have lower ER levels (both at the protein and messenger RNA [mRNA] level), which may explain their lower responses to hormonal agents. The biological reasons for this relationship are likely complex, but it suggests that interplay between ER and HER2 pathways could have an effect on the response to trastuzumab-based therapy. Despite this knowledge, the interaction between quantitative ER levels and trastuzumab benefit has not been extensively investigated in the large adjuvant trials. Recent data from neo-adjuvant clinical trials report lower rates of pathological complete response in patients with ER-positive compared with ER-negative/HER2-positive tumors. These data support the hypothesis that estrogen signaling may influence response to anti-HER2 based therapies.

In this study, we investigated the hypothesis that trastuzumab benefit would be lower in patients with HER2-positive tumors with a dominant estrogen signaling pathway. To this end, we evaluated trastuzumab benefit in subgroups defined by ER status and HER2 amplification (using copy number and fluorescence in situ hybridization [FISH] ratios) from patients enrolled in the HERceptin Adjuvant clinical trial (HERA), the largest randomized trial for adjuvant therapy with trastuzumab, to our knowledge. Because we also had gene expression levels of ESR1 and ERBB2 available from a subset of patients, we investigated if similar results could be observed using the corresponding transcript levels.

Methods

The HERA (Breast International Group [BIG] 01-01; NCT00045032) study was an international, intergroup, open-label, phase 3 randomized clinical trial enrolling 5102 women with HER2-positive (overexpressed defined as >10% + 3 staining by immunohistochemical analysis [IHC], or amplified defined as a FISH ratio of ≥2) primary breast cancer after a minimum of 4 courses of standard chemotherapy. These women were randomly assigned to 1 of 3 groups: observation (no trastuzumab), or 1 year or 2 years of adjuvant trastuzumab therapy administered intravenously every 3 weeks. The ethics committee and relevant health authorities at each participating site approved the study prior to start of recruitment. Patients gave written informed consent prior to enrollment in the study, and they were not compensated for their participation. There was also an optional consent to donate their breast tumor for future research purposes. The total number of tissue blocks collected from among the 5099 individual patients retrospectively (until December 2007) was 1229 (24%).

After an interim analysis showed a significant benefit in DFS for patients treated with 1 year of trastuzumab vs those in the observation group, a protocol amendment was implemented, allowing patients in the observation group to selectively crossover to trastuzumab (for 1 or 2 years), if they were alive and disease free as of May 16, 2005.7 The intention-to-treat population (ITT) consisted of 5099 patients, excluding 3 patients owing to missing informed consent documentation.6

The primary end point of the study is disease-free survival (DFS), defined as time from randomization to first occurrence of local, regional, distant recurrence, contralateral breast cancer including ductal carcinoma in situ, second non-breast malignant disease, or death from any cause. The secondary end point is overall survival (OS), defined as the time from randomization to death from any cause. Patients were censored on the last day of follow-up if the corresponding event had not occurred.

Recent follow-up data and efficacy data concerning 2-year vs 1-year adjuvant trastuzumab treatment have been reported and showed no superiority of 2 years vs 1 year. Therefore, in the present analysis, the 1- and 2-year trastuzumab arms are combined into a single arm.

Of the 5099 eligible patients randomized, 3037 (59.6%) had locally tested ER IHC analysis, with positive defined as greater than 10% at that time, and HER2 FISH ratio and copy number available (cohort 1; Figure 1). Central confirmation of the tumors’ HER2 status was required in all cases prior to randomization but this did not necessarily require FISH analysis. Additional FISH analyses were performed retrospectively centrally for patients not having a FISH result. A ratio of 2.0 or greater was regarded as positive according to US Food and Drug Administration recommendations.

We have previously defined groups using FISH ratio and HER2 copy number by using equal quartiles of the data.9 For the current analyses we used the same values (approximated to the closest median integer value) to define low and high groups, respectively: FISH ratio (low, ≥2 to <5 [hereafter...
Gene Expression Analysis of ESR1 and ERBB2

A total of 709 of the 1081 available samples had quality RNA available for the gene expression analyses. Included in these 709 were replicates as well as control samples, resulting in 615 patients with gene expression data available for these analyses (cohort 2) (Figure 1). Sample RNA was extracted using the High Pure RNA Paraffin Kit (Roche), which is optimized for isolation of total RNA specifically from formalin-fixed and paraffin-embedded (FFPE) tissue samples. Two to 4 tissue sections of 4 µm were used for extraction of RNA for gene expression analyses. The concentration of the RNA was determined using a spectrophotometer (NanoDrop ND-1000). We used a DASL array (Illumina) to extract ESR1 and ERBB2 transcript levels from molecular samples. For this study, molecular profiling was performed on 100 ng of RNA using Illumina whole-genome complimentary DNA (cDNA)-mediated annealing, selection, extension, and ligation (WG-DASL) assay, specifically designed to capture mRNA expression levels from archived FFPE tissue samples. The WG-DASL method uses biotinylated random nonamer and oligo (dT) primers to convert input RNA to cDNA. The biotinylated cDNA is then immobilized to a streptavidin-coated solid support and annealed to a pool of gene-specific oligonucleotides (DAP) for extension and ligation followed by polymerase chain reaction (PCR) amplification with a biotinylated and a fluorescently-labeled universal primer. Finally, the single-stranded PCR products are eluted and hybridized to the IlluminaHT-12 (version 3) BeadChip. Each oligonucleotide probe is represented, on average, by 30 beads per hybridized sample. Control (liver and brain) RNA samples were included for each processed batch of 48 samples to ensure that RNA processing was successful and for quality control and normalization of data between assay batches. Illumina probe-gene annotations were programmatically mapped to the current HUGO gene annotation. When replicate samples were available for a single patient, the first annotated replicate was used to represent the sample.

Statistical Analysis

The hypotheses of interest were tested using information from the HERA database with clinical cutoff date of April 12, 2012, and a median follow-up time of 8 years. The main analysis was an ITT analysis (ie, selective crossover was not taken into account and all patients were analyzed based on the initial treatment assignment). To circumvent the selective crossover effect, a censored and an inverse probability weighted (IPW) analyses were performed.

In the censored analysis, all observation patients who switched to trastuzumab were censored the first day they received active treatment.

The IPW approach is based on the idea of artificially censoring the follow-up of each patient at the time of crossover. Then, the real treatment effect is assessed by recreating the population that would have been observed without crossover through statistical modeling and weights assignment. In this way, the follow-up of patients whose experience receiving the control treatment could not be observed because they selectively switched to trastuzumab, was replaced by the follow-up of patients with similar characteristics (both baseline and postrandomization factors) who remained in the control arm at the time of treatment switching. Fundamental to the IPW approach is the assumption of no unmeasured confounders, which implies that all common predictors have been appropriately measured and accounted for in the analysis. Even though this assumption cannot be tested based on the observed data, the availability of a sufficient number of covariates and the use of both baseline and time-dependent covariates that are common predictors of the outcome of interest and artificial censoring limits the risk of important omissions.

The representativeness of the 2 cohorts with respect to the HERA population was explored through the comparison of patient baseline characteristics and outcome using Fisher exact, Mantel-Haenszel, and log-rank tests at the 5% level of significance. Normalization of the gene expression data was performed by the cubic-spline method. Cubic-spline has been shown to combine the positive effects of quantile normalization and avoiding the drawbacks of discontinuous mapping of intensity values and no rank preservation. If there were multiple probe sets per gene, the most variant probe for each gene per sample was used. We predefined categorized gene expression values of ERBB2 and ESR1 based on tertiles. ESR1 has previously been observed to show a nonlinear distribution as well as a nonlinear association with treatment effect.

Multivariate Cox proportional hazards regression models were used to model DFS and OS, and to obtain hazard ratios (HRs) along with corresponding 95% CIs. The terms of main interest were the 3-way interaction between treatment, ER local, and either FISH ratio or mean HER2 copy number, and the
Results

Study Population

Two different cohorts, subsets of the 5099 randomized, eligible, HERA patients at a median follow-up of 8 years (interquartile range [IQR], 7.1-8.3 years), were used in the analysis. The 1- and 2-year trastuzumab arms were combined. Cohort 1 comprised the 3037 patients with available local results on ER level and considered HER2-positive, which were either FISH-low or HER2-low (observation group, 1008 cases; trastuzumab group, 414 cases) and cohort 2 (201 cases; trastuzumab group, 414 cases) (eFigure 1 in the Supplement).

In cohort 1, the distribution of FISH and HER2 copy number, according to ER-positive or ER-negative status, is shown in Figure 1. The effect of the combined levels of HER2 amplification and ER expression (both as continuous and categorical) was significantly associated with both the outcome (DFS or OS) and the probability of crossover. Weighting was based on time-dependent Cox regression models with age at study entry and prior neoadjuvant chemotherapy included as time-fixed covariates. No other baseline characteristics were significantly associated with both the outcome (DFS or OS) and the probability of crossover. Weights were set equal to 1 for noneligible patients (no disease-free and with left ventricular ejection fraction <55%) and noneligible time intervals (ie, before May 16, 2005).

The ITT and censored analyses were carried out using SAS statistical software (version 9.3) while the IPW analysis was implemented in R statistical software using the IPW package (version 2.15.0). Differences were noted in tumor size, region, and patient race (see eTables 1 and 2 in the Supplement) indicating bigger participation in tissue provision in certain parts of the world, while ER-positive status was more prevalent among patients in cohort 1 (P < .001). Other baseline characteristics were similar among those with and without HER2 FISH central review, as well as those with and without gene expression data. A similar proportion of patients in the observation group selectively crossed over in the full HERA cohort (884 patients [52%]) and in cohort 1 (542 patients [54%]), whereas a higher proportion crossed over in cohort 2 (129 patients [64%]).

FISH Ratio, HER2 Copy Number Distributions, and Associations With ER IHC Analysis and Related Genes

In cohort 1, the distributions of FISH and HER2 copy number, according to ER-positive or ER-negative status, are shown in eFigure 1 in the Supplement. Significantly higher levels of HER2 amplification were detected in patients with ER-negative tumors (ER-negative vs ER-positive: FISH-high: 1030 of 1560 [66.0%] vs 791 of 1458 [54.3%]; P < .001).

In cohort 2, significant correlations were detected between ESR1 and ER IHC and ERBB2 and either FISH or HER2 copy number (P < .001). Distributions of ESR1 and ERBB2 genes did not differ significantly by treatment. As expected, a significant moderate inverse correlation was detected between ERBB2 and ESR1 expression (Spearman ρ = −0.261; P < .001).

Relationship Between ER IHC Analysis, HER2 Using IHC, DNA, and RNA Levels With Adjuvant Trastuzumab Benefit

The effect of the combined levels of HER2 amplification and ER status, or ERBB2 and ESR1 genes, on DFS and OS, is explored through Cox proportional hazards models by examining the significance of the corresponding 3-way interaction.

HER2 Amplification as Defined by FISH Ratio and ER by IHC Analysis

In cohort 1, based on the ITT analysis, differential treatment effect by subgroup on DFS was not statistically significant (P value for interaction = .07) (Figure 2). The trastuzumab benefit was apparent in all subgroups, although it was not significant for the patients who are ER-positive/FISH-low (HR, 0.92; adjusted [AHR], 0.89; 95% CI, 0.65-1.21) (eFigure 2 in the Supplement).

However, using a censored analysis to account for crossover, a statistically significant 3-way interaction was found (P value for interaction = .02, Figure 2). Similarly, inference remains the same with the IPW results (P value for interaction = .03). This remained significant in Cox models adjusted for pathological tumor size, age (or menopausal status), nodal status, and prior neoadjuvant chemotherapy.

Regarding OS, based on the ITT analysis, the effect of trastuzumab was found to be significantly different between the 4 subgroups created by the combination of FISH-low vs FISH-high and ER-negative vs ER-positive (P value for interaction = .006, Figure 3). In particular, significant benefit from trastuzumab was observed for the ER-positive/FISH-high patients (HR, 0.51; AHR, 0.48; 95% CI, 0.34-0.69; P < .001), with less pronounced benefit for the ER-negative categories, and no benefit for the ER-negative/FISH-low patients (HR, 1.34; AHR, 1.27; 95% CI, 0.81-2.01; P = .30) (eFigure 3 in the Supplement).
When adjusting for crossover either through the censored or the IPW analysis, a significant 3-way interaction for OS was also found (P value for interaction = .04 and .03, respectively), similar to the DFS result, with a significant treatment benefit for all subgroups except for the ER-positive/FISH-low patients (Figure 3).

HER2 Amplification as Defined by HER2 Copy Number and ER by IHC Analysis

Interestingly, for both DFS and OS, the interaction between treatment and the combination of ER IHC analysis with HER2 copy number was not significant (P value for interaction = .73; OS, P = .01) (eFigure 4 in the Supplement). These results are confirmed by additional analyses (censored and IPW).

HER2 Amplification as Defined by ERBB2 mRNA, and ER Defined by the ESR1 Gene

In cohort 2, Cox models were used to explore whether the effect of trastuzumab on either DFS or OS differs by the combination of ESR1 gene and ERBB2 transcript values. The corresponding 3-way interaction term is found to be statistically nonsignificant for both end points. However, a significant 2-way interaction was observed between treatment and ESR1 gene (DFS-adjusted P value for interaction = .01; OS-adjusted P value for interaction = .005; Figure 4) (eFigure 5 in the Supplement).

In addition, a statistically significant interaction between treatment and ERBB2 (continuous) was detected for OS for the simple model (P = .047). The significant findings for treatment by ESR1 gene (tertiles) interaction is confirmed by all additional analyses (IPW and censored), for both end points. Notably, 115 (57%) of the 202 patients classified as ER-positive/FISH-low were also categorized as ESR1-high (P < .001).

Discussion

Large randomized clinical trials are critical for establishing the predictive value of a biomarker. In this study, we investigated the role of ER IHC status (locally tested) levels combined with HER2 levels (centrally tested), in predicting the magnitude of benefit from adjuvant trastuzumab in the HERA trial, the largest of the adjuvant trastuzumab trials. We show that patients with ER-positive tumors (IHC verified) with low FISH levels (though still HER2-overexpressing) derive less benefit from adjuvant trastuzumab after chemotherapy, with all of these patients receiving endocrine therapy. ESR1 transcript levels could also define a group that did not have significant benefit from the addition of trastuzumab, with ERBB2 levels not having an effect on this interaction. Our results support the potential importance of estrogen signaling for patients with HER2-positive disease. While there was a lack of significance for HER2 copy number, this potentially underscores the importance of correction for polysomy in establishing HER2 amplification status, and supports previous observations that polysomy does not equate functional HER2 signaling.

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In all adjusted Cox models, significant predictors were pathological tumor size, age, (or menopausal status), nodal status, and prior neoadjuvant chemotherapy. ER indicates estrogen receptor; FISH, fluorescence in situ hybridization; IHC, immunohistochemical analysis.
Our findings support the increasing amount of genomic and clinical data reporting that HER2-overexpressing tumors have distinct molecular and clinical profiles according to ER status. Molecular subtype classification of breast cancer using gene expression classify most patients with ER positive/HER2-positive breast cancers as “luminal-B” type breast cancers rather than HER2-enriched, as they had high expression of luminal type genes such as GATA3, BCL2, ESR1, as well as ERBB2.18,19 Some preliminary data support this concept by suggesting that the “HER2-enriched” subtype, which is predominantly ER negative, can achieve higher responses to HER2-directed therapy. 20 This is further reinforced by the Cancer Genome...
genomic instability.\textsuperscript{22} Amplification combined with ER positivity could also result in less HER2-mediated oncogenic signaling. Lower levels of HER2 amplification combined with ER positivity could also result in less genomic instability.\textsuperscript{22}

The strengths of our study include its size, centrally tested HER2, and a prospectively predefined hypothesis stating the ER and HER2 cutoffs. We acknowledge that further validation is required in other adjuvant and metastatic trastuzumab trials because we cannot exclude the possibility that we may have seen this effect owing to unique aspects of the HERA study: (1) trastuzumab was given without concurrent chemotherapy; and (2) this effect could be limited to the adjuvant setting. If confirmed, this subgroup of patients could be earmarked for investigation with therapeutics that are effective in endocrine positive disease.\textsuperscript{23}

Conclusions

We report for the first time, to our knowledge, using data from the HERA trial, that a subgroup of patients with ER-positive disease (with lower FISH ratios or highest $ESR1$ expression) derive less magnitude of benefit from adjuvant trastuzumab after prior adjuvant chemotherapy. Our data emphasize that more research into the clinical relevance of how ER influences HER2 oncogenic signaling and outcomes to anti-HER2 therapy are warranted.
Adjuvant Trastuzumab Benefit in Patients Diagnosed With Triple-Positive Breast Cancer

Anne F. Schott, MD

The article “Effects of Estrogen Receptor and Human Epidermal Growth Factor Receptor-2 Levels on the Efficacy of Trastuzumab: A Secondary Analysis of HERA Trial,” by Loi et al1 in this issue of JAMA Oncology entices the reader with the conclusion that certain human epidermal growth factor receptor-2 positive (HER2+, now known as ERBB2) breast cancers that are also estrogen receptor positive (ER+) “derive significantly less benefit from adjuvant trastuzumab after chemotherapy.” The phrase, “significantly less,” has special connotation for scientists and clinicians trained to disregard findings that do not meet statistical significance. This concluding statement questions whether trastuzumab is of value in the patients we have come to call “triple positive” (ER+, progesterone receptor positive, and HER2+), especially if ER expression is high and if concurrently the fluorescent in situ hybridization (FISH) ratio determining HER2 positivity is low (<2 to <5). Based on this article, should we rethink our use of trastuzumab in these patients?

The article by Loi et al1 presents a secondary analysis of tissues and clinical data from the HERceptin Adjuvant (HERA) trial.2 The HERA trial was an international, multicenter, randomized, open-label, phase 3 trial comparing treatment with trastuzumab for either 1 year or 2 years versus observation alone (i.e., without trastuzumab) after standard neoadjuvant chemotherapy, adjuvant chemotherapy, or both in 5102 patients with HER2+ early-stage breast cancer. The primary conclusions of the HERA trial were (1) trastuzumab for 1 year provides a significant disease-free survival (DFS) and overall survival (OS) benefit compared with observation, and (2) 2 years of adjuvant trastuzumab is not more effective than 1 year. The importance of the HERA trial was that it helped establish 1 year of trastuzumab as the standard of care for women with HER2+ early-stage breast cancer, irrespective of ER status.

The researchers designed the current study to address the “prospective-retrospective”3 a priori hypothesis that benefit from anti-HER2 agents (in this case, trastuzumab) differs depending on the level of ER expression. They used specimens collected on a subset of HERA patients to estimate the magnitude of trastuzumab benefit based on tumor ER protein content determined by local immunohistochemical analysis (positive >10%, vs negative <10%). In addition, magnitude of trastuzumab benefit was also estimated by quantitative ESR1 (determination of estrogen receptor RNA) in an even smaller subset of patients. All patients enrolled in the HERA trial were HER2+ by standard definitions; however, Loi et al1 also explored trastuzumab benefit in patients with varying degrees of positivity as defined by FISH ratio, as well as by HER2 copy number.

The key findings supporting the article’s concluding statement are presented in Figure 2 showing DFS, and Figure 3...