

Association of p27 and cyclin D1 expression and benefit from adjuvant trastuzumab treatment in HER2-positive early breast cancer: a TransHERA study

Martin Filipits PhD¹, Urania Dafni ScD², Michael Gnant MD³, Varvara Polydoropoulou MSc², Margaret Hills⁴, Astrid Kiermaier PhD⁵, Evandro de Azambuja MD⁶, Denis Larsimont MD⁶, Federico Rojo MD⁷, Giuseppe Viale MD⁸, Masakazu Toi MD⁹, Nadia Harbeck MD¹⁰, Kathleen I Prichard MD¹¹, Richard D Gelber PhD¹², Phuong Dinh MD¹³, Dimitrios Zardavas MD¹⁴, Brian Leyland-Jones MBBS¹⁵, Martine J Piccart-Gebhart PhD^{6, 14}, Mitch Dowsett PhD⁴ on behalf of the TransHERA investigators

¹Departments of Medicine I and ³Surgery, Medical University of Vienna, Comprehensive Cancer Center, Austria; ²Frontier Science Foundation-Hellas, Greece; ⁴The Royal Marsden Hospital, United Kingdom; ⁵Oncology Biomarker Development, Genentech, Switzerland; ⁶Institut Jules Bordet, Université Libre de Bruxelles (U.L.B), Belgium; ⁷Pathology Department, University Hospital “Fundacion Jimenez Diaz”, Spain; ⁸University of Milan, European Institute of Oncology, Italy; ⁹Department of Surgery, Kyoto University, Japan. ¹⁰University Hospital Munich, Germany; ¹¹Sunnybrook Odette Cancer Centre, Toronto Sunnybrook Regional Cancer Centre, Canada; ¹²Department of Biostatistics and Computational Biology, Dana-Farber Cancer Institute, Harvard Medical School, Harvard TH Chan School of Public Health and Frontier Science and Technology Research Foundation, USA; ¹³Westmead Hospital, University of Sydney, Australia; ¹⁴Breast International Group (BIG), Belgium; ¹⁵Avera Cancer Institute, USA.

Correspondence to:

Martin Filipits, PhD
Institute of Cancer Research
Department of Medicine I
Comprehensive Cancer Center Vienna
Medical University of Vienna
Borschkegasse 8a, A-1090 Vienna, Austria
Phone: +43 1 40160 57528
E-mail: martin.filipits@meduniwien.ac.at

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Conflict of interests

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Translational relevance

Patients with HER2-positive early breast cancer benefit from treatment with anti-HER2 therapies but long-term follow up indicates that a significant proportion of women will experience recurrence of their disease. Identification of predictive biomarkers may help identifying those patients most likely to benefit from adjuvant trastuzumab. In the TransHERA study, we observed a significant interaction between p27 and treatment, independent of known prognostic clinicopathological variables. Patients with HER2-positive early breast cancer with low p27 expression in their tumors benefited from trastuzumab treatment whereas patients with high p27 expression did not. This finding may help to optimize the adjuvant treatment of patients with HER2-positive breast cancer in the future.

Abstract

Purpose: To assess the prognostic and predictive value of selected biomarkers involved in cell cycle regulation or proliferation in HER2-positive early breast cancer patients.

Methods: Protein expression of TOP2A, Ki67, cyclin D1 and p27 was immunohistochemically determined in tissue microarrays of surgical specimens from 862 patients randomized to trastuzumab (1 or 2 year; N=561) and observation (N=301) arms of the HERA trial. The primary analysis endpoint was disease-free survival (DFS). Biomarkers were examined as continuous or categorical variables (pre-defined cutoffs). Interaction terms between biomarkers and treatment were assessed in multivariate Cox models adjusted for variables of clinical interest.

Results: A significant interaction was detected between p27 and treatment (adjusted $p=0.0049$). Trastuzumab effect was significant in the p27 low subgroup ($\leq 70\%$ p27-positive tumor cells; N=318). $HR_{\text{Comb Trast vs. Obs}} = 0.44$, 95% CI: 0.29-0.65 ($p < 0.001$). No trastuzumab effect was observed in the p27 high subgroup N=435; $HR_{\text{Comb Trast vs. Obs}} = 0.97$, 95% CI: 0.66-1.44, $p=0.89$), indicating that these patients derived little or no benefit from trastuzumab treatment. A prognostic effect of p27 on DFS was observed, with p27 high patients experiencing half the hazard of a DFS event compared to low ones ($HR_{\text{p27 High vs. Low}} = 0.49$, 95% CI: 0.32-0.75). TOP2A, Ki67 and cyclin D1, as categorical variables were not predictive, whereas cyclin D1 as continuous variable was predictive of trastuzumab benefit.

Conclusions: In TransHERA, HER2-positive early breast cancer patients with low p27 expression in their tumors benefited from trastuzumab treatment, whereas patients with high p27 expression did not.

Introduction

Amplification or overexpression of the human epidermal growth factor receptor 2 (HER2) oncogene and oncoprotein, respectively, occurs in about 15-20% of primary breast cancers. Patients with HER2-positive breast cancer benefit from treatment with anti-HER2 therapies including the monoclonal antibody trastuzumab (1,2). In the adjuvant setting, pivotal randomized clinical trials including the HERceptin Adjuvant (HERA) trial (1) showed that 1 year is the optimal duration for trastuzumab therapy in these patients (3). Despite the improvements of outcome achieved by anti-HER2 therapies, clinical resistance to trastuzumab remains a significant problem; long-term follow up indicates that a significant proportion of patients with early HER2-positive breast cancer will experience recurrence of their disease (4). The overall objective of TransHERA, the translational research sub-study of HERA, was to identify markers that can predict sensitivity or resistance to adjuvant trastuzumab therapy.

Some clinical parameters, including histologic type and tumor stage are associated with the magnitude of benefit from adjuvant trastuzumab because of their relationship with prognosis but they do not relate to proportional benefit from trastuzumab (5). We have reported that HER2-positive early breast cancers that are estrogen receptor (ER)-positive by immunohistochemistry with low HER2:CEP17 FISH ratio, or with higher mRNA expression of *ESR1* levels derived significantly less benefit from adjuvant trastuzumab after chemotherapy in TransHERA (6).

Several mechanisms of resistance to HER2 blockade have been described to date including activation of the phosphatidylinositol-3-kinase (PI3K) pathway (7,8). Cell cycle regulators have also been suggested to predict resistance or response to trastuzumab treatment (9,10). Cyclins, their associated cyclin-dependent kinases (CDKs), and CDK inhibitory proteins play a central role in cell cycle progression and may also affect response to trastuzumab (10). In addition, topoisomerase 2-alpha (TOP2A) alterations may also have the potential to act as a predictive biomarker of trastuzumab benefit (11,12).

In this study, we evaluated the prognostic/predictive value of p27, cyclin D1, TOP2A, and Ki67 protein expression in patients with HER2-positive primary breast cancer who were enrolled in the HERA and participated in TransHERA.

Patients and Methods

Study design and participants

The TransHERA sub-study included patients who participated in the HERA study (Breast International Group [BIG] 01-01-ClinicalTrials.gov NCT00045032), an international, intergroup, open-label, phase III randomized study enrolling 5102 women with HER2-positive (overexpressed or amplified) primary breast cancer (1), and who consented to evaluation of their tumor tissue for research purposes. All patients had at least 4 courses of adjuvant standard chemotherapy, and were randomized to one year or two years of adjuvant trastuzumab administered intravenously every 3 weeks, or to observation (no trastuzumab arm). The observation patients were allowed to selectively crossover to trastuzumab (either 1 or 2 year), if they were alive and disease free as of May 16th 2005, based on the significant benefit in disease-free survival (DFS) for patients treated with 1-year trastuzumab versus observation shown at the first interim analysis. The study was conducted according to HERA master and Trans-HERA sub-study protocols; with adherence to country specific ethics, regulatory requirements and REMARK recommendations (13).

Specimen collection and immunostaining for biomarkers

Formalin-fixed, paraffin-embedded (FFPE) tumor tissue blocks were obtained at the time of surgery before adjuvant therapy. Paraffin blocks were stored at room temperature and were identifiable only by an identification number assigned to each patient at randomization.

Tissue microarrays were constructed with a manual tissue arrayer (MTA-1; Beecher Instruments, Sun Prairie, Wisconsin, USA) using 600 µm cores. Immunohistochemistry was performed and evaluated in the Austrian Breast cancer Study Group (ABCESG) central research laboratory at the Medical University of Vienna (p27, TOP2A, cyclin D1) and at the Royal Marsden Hospital, London (Ki67) by means of standard protocols.

Briefly, tissue microarray sections were deparaffinized and rehydrated. After heat-induced epitope retrieval in 10 mM citrate buffer (pH 6.0) for 10 minutes, the tissue sections were incubated at room temperature with the particular monoclonal antibodies specific for p27 (clone SX53G8; Dako, Glostrup, Denmark; dilution 1:100), TOP2A (Ki-S1; Thermo Scientific; dilution 1:200), cyclin D1 (SP4; NeoMarkers; dilution 1:100), and Ki67 (MIB1; Dako, Glostrup, Denmark; dilution 1:40). Antibody binding was detected by means of the UltraVision LP detection system (p27, TOP2A, cyclin D1; Lab Vision Corporation) or with the Dako REAL Detection System (Ki67, Dako) according to the manufacturer's recommendations. Color development was performed by 3,3'-diaminobenzidine and counterstaining by hematoxylin. Immunostaining was evaluated without knowledge of the patients' clinical data. All invasive tumor cells on each core were evaluated. Interpretation of the results was limited to the invasive part of the tumor and only nuclear staining was scored as positive. The results were documented as the percentage of stained nuclei regardless of staining intensity.

Statistical analyses

The primary analysis was an intention-to-treat analysis. To take into account selective crossover, additional analyses were performed. These included a censored, an IPCW (Inverse Probability Censored Weighting), and an early events analysis. In the censored analysis, no further follow-up is taken into account for observation patients that crossed over to a trastuzumab arm, at the time of crossing over. In the IPCW analysis, the additional follow-up for these patients is substituted by weighting appropriately the patients who did not cross over. In the early events analysis, only

events occurring up to 2.2 years of median follow-up were taken into account. These events, important in the context of translational research, occurred at a time when crossover was minimal.

The primary endpoint of the study was DFS, defined as time from randomization to first occurrence of local, regional, distant recurrence, contralateral breast cancer including ductal carcinoma in situ (DCIS), second non-breast malignant disease or death from any cause. Overall survival (OS) was a secondary endpoint, defined as the time from randomization to death from any cause. In the present analysis the 1-year and 2-year trastuzumab arms were combined into a single arm (3).

Survival outcomes were obtained from the HERA database with clinical cut-off date the 12th of April 2012 and a median follow-up time of 8 years (IQ range with interquartile range). The representativeness of the present cohort, with respect to the original HERA population was explored via the comparison of patients' baseline characteristics and outcomes.

Fisher's exact and Mantel-Haenszel or Kruskal-Wallis tests were used in order to explore potential differences between groups. The distributions of the markers were shown graphically via histograms, whereas their associations were explored by scatter plots, box-plots, and the Spearman correlation coefficient. In all exploratory analyses, results with a two-sided p-value less than 0.05 were considered significant.

Cox proportional hazards regression for DFS and OS, was the main analysis tool in order to assess prognostic or predictive effect of the markers. Marker and treatment effects were added into the models, along with their interaction terms. Hazard ratios (HRs), along with corresponding 95% confidence intervals (CIs), were used to show the relationship between markers and outcome. The observed differences in hazards between groups are presented through Kaplan-Meier curves. In multivariate models, adjustment for variables of clinical interest [age, pathological tumor size, progesterone receptor (PgR) local, tumor grade, menopausal status, nodal status, prior-(neo) adjuvant chemotherapy, ECOG performance status, race, region, ER

local and ESR1 (categorization based on tertiles) (6) main and predictive effects] was performed, and the best model was chosen by the backward selection method with removal criterion a p-value > 0.10. Departures from the proportional hazards assumption were assessed based on the Schoenfeld residuals (14). The False Discovery Rate (FDR) method was implemented to account for multiple testing (15).

Biomarkers were analyzed either as continuous or as categorical variables, based on the following prospectively specified categorization scheme: TOP2A percent: ≤ 5 versus > 5 , Ki67 according to tertiles, cyclin D1 percent: ≤ 10 versus > 10 , p27 percent: ≤ 70 versus > 70 (12,16-18). Whenever a continuous variable showed a significant interaction with treatment not translated when the marker was categorized in two groups, further exploration using tertiles for the corresponding marker was performed.

Results

Study Population

Out of the 5099 HERA patients (excluding three patients due to missing informed consent documentation), there were 1162 participating in TransHERA. From these, 862 patients (17% of the whole study population) had available information on the protein expression on at least one of the following biomarkers: TOP2A, Ki67, cyclin D1, or p27.

Patient and tumor baseline characteristics, as well as information on DFS and OS for the current analysis cohort and the remainder of the HERA population are shown in Tables S1-4. Tumor size, ER measured locally, race, region, and prior neoadjuvant chemotherapy were baseline characteristics differing substantially between the TransHERA group and the remainder of HERA cohort. For both DFS and OS, the observed magnitude of the trastuzumab benefit in the TransHERA group is similar to that detected in the HERA cohort. In the current analysis cohort, the treatment effect is significant for DFS, whereas for OS the indicated beneficial effect does not reach significance, probably due to the small event number (DFS: TransHERA group: HR

Comb Trast vs. Obs = 0.74, 95% CI: 0.57-0.95, Remainder of HERA cohort: HR_{Comb Trast vs. Obs} = 0.76, 95% CI 0.68-0.85; OS: TransHERA group HR_{Comb Trast vs. Obs} = 0.73, 95% CI: 0.51-1.03, Remainder of HERA cohort: HR_{Comb Trast vs. Obs} = 0.75, 95% CI: 0.65-0.86).

In the current analysis cohort, 301 patients were randomized to the observation arm and 561 to the combined trastuzumab arm (1- or 2-year trastuzumab). All characteristics of the patients were balanced between the two treatment groups (Table 1). Out of the 248 observation patients eligible to cross over, 188 (74.3%) switched to trastuzumab treatment.

Biomarker distributions and associations

Frequency distribution on biomarker categories overall and according to treatment arms are shown in Table 2, while distributions of TOP2A, Ki67, cyclin D1, and p27, used as continuous variables are presented in figures S1A-D. No difference in the expression of the biomarkers was seen by treatment allocation.

Significant positive associations were detected for TOP2A with Ki67 ($r_{\text{Spearman}} = 0.47$, $p < 0.001$), cyclin D1 ($r_{\text{Spearman}} = 0.22$, $p < 0.001$), p27 ($r_{\text{Spearman}} = 0.12$, $p = 0.0017$); and cyclin D1 with Ki67 ($r_{\text{Spearman}} = 0.15$, $p < 0.001$), and p27 ($r_{\text{Spearman}} = 0.56$, $p < 0.001$), (Figures S2A-C and S3A-B). When biomarkers were treated as categorical variables, all associations remained significant except the association between p27 and TOP2A. In addition, a significant association between p27 and Ki67 (Fisher's exact $p = 0.043$) was observed.

Biomarker association with baseline characteristics

Significant associations were observed between p27 and ER, PgR and tumor grade ($p < 0.001$). P27 high cases are more frequent in ER positive (76.3% vs. 39.3% for ER negative), PgR positive (76.7% vs. 47.3% for PgR negative) and tumor grade 2 groups (67.4% vs. 53.1% for grade 3). Higher cyclin D1 values are more frequent in ER and PgR positive subgroups (31.3% vs. 11.7% for ER negative and 30.9% vs. 14.3% for PgR negative, $p < 0.001$ for both). Ki67 is

significantly associated with PgR ($p=0.017$), tumor grade ($p<0.001$), race ($p=0.0039$) and region ($p<0.001$). No associations between Topo2A and any baseline characteristic was detected.

Biomarker associations with outcome

A total of 249 DFS events (28.9%) and 129 (15.0%) deaths were observed in our cohort. The 8-year DFS and OS estimates were 70.5% (95% CI: 67.3%-73.6%) and 84.5% (95% CI: 82.0%-87.0%), respectively. TOP2A, Ki67 and cyclin D1 when used as categorical variables were neither prognostic nor predictive of adjuvant trastuzumab benefit (adjusted interaction $p=0.80$, 0.81 and 0.17 respectively; Figures 1 A-B). However, a significant interaction was detected between p27 classified as categorical variable and treatment (interaction $p=0.036$; FDR $p=0.17$; Table S5). This interaction was also significant after adjusting for variables of clinical interest, i.e. ($p=0.0049$, FDR $p=0.045$; Figure 1D & 2, Table S6; significant variables: region, nodal status and pathological tumor size). Patients in the observation arm classified as p27 high (>70% p27-positive tumor cells) experience half the hazard of a DFS event compared to patients in the p27 low group ($HR_{p27 \text{ high vs. low}}=0.49$; 95% CI: 0.32-0.75, $p=0.012$; Table S6). No p27 effect is observed in the trastuzumab arm $HR_{p27 \text{ high vs. low}}=1.09$, 95% CI: 0.77-1.54, $p=0.64$; Table S6). For patients classified as p27 low, a significant treatment effect was observed ($HR_{\text{Comb Trast vs. Obs}}=0.44$, 95% CI: 0.29-0.65, $p<0.001$), while the treatment effect was not found significant for patients in the p27 high group ($HR_{\text{Comb Trast vs. Obs}}=0.97$, 95% CI: 0.66-1.44, $p=0.88$). The predictive ability of p27 was maintained when the remaining three markers were included in the model (adjusted interaction $p=0.0039$; FDR $p=0.095$), or when the predictive effect of ER Local or ERS1 gene expression was included in the model (Tables S7-8).

When biomarkers were treated as continuous variables, the only marker found to have a significant predictive effect on DFS was cyclin D1 (interaction $p=0.006$; Table S10). This effect remained statistically significant after adjusting for clinical parameters ($p=0.0046$, FDR $p=0.045$; Table S10). Higher levels of cyclin D1 were significantly associated with better DFS in the

observation arm (HR =0.96, 95% CI 0.94-0.99, p=0.0082) but not in the trastuzumab arm (HR =1.01, 95% CI: 0.99-1.02, p=0.31). Inference remains the same when accounting for the remaining three markers (adjusted p=0.0042, FDR p=0.05), or ER and ESR1 predictive effects (Tables S11-12).

To further investigate the predictive ability of cyclin D1, a categorization based on tertiles was also considered (low, intermediate and high; p=0.013, FDR p=0.078; Figure 1C). Adjusting for baseline characteristics, a significant treatment benefit was detected for cyclin D1 for the low and intermediate groups (low: HR_{Comb Trast vs. Obs} =0.43, 95% CI: 0.25- 0.74, p=0.0024 and intermediate HR_{Comb Trast vs Obs} 0.57, 95% CI: 0.36-0.90, p=0.015). For cyclin D1 high category, no treatment benefit is detected (HR_{Comb Trast vs. Obs} =1.08, 95% CI: 0.67-1.74, p=0.75). This corresponds to not observing any effect of cyclin D1 in the trastuzumab arm, while in the observation arm, the hazard of a DFS event was found significantly lower for the high group patients as compared to the low group patients (HR_{high vs. low} =0.51, 95% CI: 0.29-0.89, p=0.017). The above findings on cyclin D1 did not change after adjusting for the remaining three markers. In particular, the interaction term between treatment and cyclin D1 (high versus low) was found significant (p=0.010; FDR p=0.068).

No significant interaction was detected between treatment and any of the four biomarkers (both as continuous and categorical variable), with respect to OS. The results of the early events, censored and IPCW analyses did not lead to different conclusions from the ITT analysis.

Discussion

Results of the HERA trial demonstrated that one year of adjuvant trastuzumab treatment of early-stage HER2-positive breast cancer patients provides a significant DFS and OS benefit compared with observation and the degree of benefit was similar to that with 2 years' trastuzumab. This has resulted in one year of trastuzumab being the standard of care for patients with HER2-positive

early breast cancer (1,3). It also allowed us to analyze the outcome data from the 1 year and 2 year trastuzumab arms of HERA as a single treatment arm. Our study is a step forward in establishing the impact of molecular biomarkers on the outcome of a large group of patients in this study cohort. Of great importance we report for the first time within the context of an adjuvant randomized trastuzumab trial that the positive and negative cell cycle regulators p27 and cyclin D1 respectively have predictive value in HER2-positive early breast cancer: only patients with low p27 expression or lower levels of cyclin D1 expression benefited from trastuzumab treatment in the TransHERA cohort.

Strengths of this study were the randomized design of the clinical trial from which the patients were derived, the relatively large size of the TransHERA population, the biomarker analysis using validated assays in a central laboratory and the data analyses being conducted on a formal statistical analysis plan that included adjustment on standard prognostic variables. A weakness is that the TransHERA group was only a modest proportion of the HERA trial and although the outcome data were similar in those patients included or not included in TransHERA it cannot be concluded that our findings apply fully across the whole trial population. In addition, although the markers were selected as potential candidate markers on the basis of previous findings, the predictive role of p27 and cyclin D1 still requires validation before being accepted as clinically valid and worthy of investigation for clinical utility.

We applied a previously defined cut-off for p27 positivity (high >70% p27-positive tumor cells). This had previously been used to assess the clinical relevance of p27 in postmenopausal hormone receptor-positive breast cancer patients who received tamoxifen for 5 years in Austrian Breast and Colorectal Cancer Study Group Trial 06 (17). In that retrospective analysis, women with high p27 expression had a significantly longer DFS and OS as compared to women with low p27 expression; this finding is similar to what we report in this HER2-positive breast cancer cohort, where p27 showed strong favorable prognostic significance in the observation group.

It has previously been described that induction of p27, a CDK inhibitor functioning as distal downstream regulator of several oncogenic signaling pathways, is one of the key modes of action of HER2-targeting antibodies in breast cancer (19-22), with an increase in p27 thought to cause proliferating cells to exit the cell cycle and thereby undergo apoptosis (23). It may be that breast cancer patients with HER2-positive, p27 low expressing tumors benefit more from trastuzumab because the effect of p27 up-regulation may be greater in these patients compared to patients whose tumors have p27 high expression. Interestingly, down-regulation of p27 has been associated with trastuzumab resistance in breast cancer cells (9) and trastuzumab-resistant cells have been reported to have decreased levels of p27 and exogenous addition of trastuzumab to increase trastuzumab sensitivity (9). Thus it seems likely that decreased p27 levels in the tumors of trastuzumab-resistant patients are mediated by different mechanisms compared to low p27 expression in trastuzumab-naive patients. One potential mechanism in trastuzumab-resistant cells could be overexpression of insulin-like growth factor I receptor (IGF-IR) (24). It has been shown that the mechanism by which IGF-IR confers resistance to trastuzumab involves increased degradation of p27 by the ubiquitin/proteasome degradation machinery (24). Thus sensitivity and resistance to trastuzumab in HER2-overexpressing cells is critically dependent on p27 levels.

Only limited information on the clinical role of p27 as a predictive marker for trastuzumab therapy in breast cancer is available in the literature. In a Japanese trial, the association of p27 and other biomarkers with pathological complete response (pCR) and survival was studied in women with HER2-positive breast cancer who received neo-adjuvant trastuzumab-based chemotherapy (25). In this study, the pCR rate was significantly higher in patients whose tumors had low expression of p27 (defined as <75% p27-positive tumor cells) compared to patients with high p27 expression (71% versus 50%, respectively, $p=0.025$) (25). This is in line with our observation that patients with low p27 expression in their tumors benefit more from trastuzumab treatment than patients with high p27 expression in their tumors. High grading, low ER, low PgR

and high Ki67 also predicted pCR in the Japanese patient cohort. However, we could not confirm the predictive value of these biomarkers in TransHERA patients.

If the result that only HER2-positive early breast cancer patients with low p27 expression in their tumors benefit from trastuzumab treatment is validated, this finding may help to optimize the adjuvant treatment of patients with HER2-positive breast cancer in the future.

In summary, we have analyzed p27 protein expression in early stage HER2-positive breast cancers from women who had been enrolled into the HERA trial. We have demonstrated that, by using a predefined cut-off, low p27 expression may be associated with benefit from adjuvant trastuzumab treatment.

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Figure Legends

Figure 1 Survival curves for disease-free survival (DFS) according to TOP2A (A), Ki67 (B), cyclin D1 (C), and p27 expression (D) in the observation arm and the trastuzumab arm. The adjusted interaction P values and the 8-year DFS rates are reported.

Figure 2 Analyses of disease-free survival according to biomarker subgroups. The hazard ratios (with 95 percent confidence intervals) are for the patients assigned to trastuzumab arm, as compared with those assigned to observation arm. The adjusted interaction P values are reported. CI denotes confidence interval.

Table 1: Summary of baseline characteristics for current analysis cohort, overall and by treatment arm

(*)Mantel-Haenszel, (f)Fisher's exact, (∞)Excluding category "Missing", (£)Excluding category "Unknown", (©)Excluding category "Uncertain", (≠)Excluding category "Not Assessed"

Baseline characteristics	Observation (N=301)	Combined Trastuzumab (N=561)	Total (N=862)	p-value
Age in yrs				
Median	50	50	50	
<35	14 (4.65%)	42 (7.49%)	56 (6.50%)	0.61*
35-49	134 (44.52%)	238 (42.42%)	372 (43.16%)	
50-59	103 (34.22%)	185 (32.98%)	288 (33.41%)	
≥60	50 (16.61%)	96 (17.11%)	146 (16.94%)	
Pathological Tumor Size				
Median in mm	20.0	22.0	21.0	
0-2 cm	150 (49.83%)	261 (46.52%)	411 (47.68%)	0.49*
>2-5 cm	134 (44.52%)	261 (46.52%)	395 (45.82%)	0.29 ^{*,∞}
>5 cm	13 (4.32%)	31 (5.53%)	44 (5.10%)	
Missing	4 (1.33%)	8 (1.43%)	12 (1.39%)	
PgR Local				
Negative (-)	177 (58.80%)	307 (54.72%)	484 (56.15%)	0.47 ^{f,£}
Positive (+)	96 (31.89%)	202 (36.01%)	298 (34.57%)	
Unknown	28 (9.30%)	52 (9.27%)	80 (9.28%)	
ER Local				
Negative (-)	160 (53.16%)	277 (49.38%)	437 (50.70%)	0.32 ^f

Baseline characteristics	Observation (N=301)	Combined Trastuzumab (N=561)	Total (N=862)	p-value
Positive (+)	141 (46.84%)	284 (50.62%)	425 (49.30%)	
Tumor Grade				
G1	7 (2.33%)	8 (1.43%)	15 (1.74%)	0.42*
G2	99 (32.89%)	174 (31.02%)	273 (31.67%)	
G3	191 (63.46%)	375 (66.84%)	566 (65.66%)	
GX	4 (1.33%)	4 (0.71%)	8 (0.93%)	
Menopausal status				
Premenopausal	39 (12.96%)	61 (10.87%)	100 (11.60%)	0.61 ^f
Postmenopausal	132 (43.85%)	245 (43.67%)	377 (43.74%)	0.48 ^{f,Ⓞ}
Uncertain	130 (43.19%)	255 (45.45%)	385 (44.66%)	
Nodal status				
Not assessed (neoadjuvant chemotherapy)	11 (3.65%)	38 (6.77%)	49 (5.68%)	0.18*
Negative	102 (33.89%)	186 (33.16%)	288 (33.41%)	0.65 ^{*,‡}
1-3	88 (29.24%)	169 (30.12%)	257 (29.81%)	
≥4	100 (33.22%)	168 (29.95%)	268 (31.09%)	
Ethnicity				
Caucasian	281 (93.36%)	517 (92.16%)	798 (92.58%)	0.46 ^f
Oriental	19 (6.31%)	37 (6.60%)	56 (6.50%)	
Other	1 (0.33%)	7 (1.25%)	8 (0.93%)	
Region				
Western and Northern Europe, Canada, South Africa, Australia, New Zealand	248 (82.39%)	449 (80.04%)	697 (80.86%)	0.88 ^f

Baseline characteristics	Observation (N=301)	Combined Trastuzumab (N=561)	Total (N=862)	p-value
Asia Pacific, Japan	16 (5.32%)	33 (5.88%)	49 (5.68%)	
Eastern Europe	30 (9.97%)	65 (11.59%)	95 (11.02%)	
Central and South America	7 (2.33%)	14 (2.50%)	21 (2.44%)	
Prior (neo)adjuvant chemotherapy				
No anthracyclines	18 (5.98%)	30 (5.35%)	48 (5.57%)	0.90 ^f
Anthracyclines, no taxanes	216 (71.76%)	401 (71.48%)	617 (71.58%)	
Anthracyclines and taxanes	67 (22.26%)	130 (23.17%)	197 (22.85%)	
ECOG Performance Status				
0	270 (89.70%)	512 (91.27%)	782 (90.72%)	0.46 ^f
1	31 (10.30%)	49 (8.73%)	80 (9.28%)	

Table 2: Biomarker's level distributions, overall and by treatment arm*Fisher's exact test computed, ⊕ Excluding category "Missing"*

Biomarker level	Observation (N=301)	Combined trastuzumab (N=561)	Total (N=862)	p-value
p27				
Low (≤ 70)	113 (37.54%)	205 (36.54%)	318 (36.89%)	0.46
High (>70)	145 (48.17%)	290 (51.69%)	435 (50.46%)	0.54 \oplus
Missing	43 (14.29%)	66 (11.76%)	109 (12.65%)	
Cyclin D1				
Low (≤ 10)	207 (68.77%)	388 (69.16%)	595 (69.03%)	0.41
High (>10)	52 (17.28%)	110 (19.61%)	162 (18.79%)	0.58 \oplus
Missing	42 (13.95%)	63 (11.23%)	105 (12.18%)	
TOP2A				
Low (≤ 5)	119 (39.53%)	234 (41.71%)	353 (40.95%)	0.77
High (>5)	140 (46.51%)	256 (45.63%)	396 (45.94%)	0.65 \oplus
Missing	42 (13.95%)	71 (12.66%)	113 (13.11%)	
Ki67				
$0 \leq \text{Ki67} < 13.2$	92 (30.56%)	169 (30.12%)	261 (30.28%)	0.67
$13.2 \leq \text{Ki67} < 24.6$	88 (29.24%)	171 (30.48%)	259 (30.05%)	0.93 \oplus
$\text{Ki67} \geq 24.6$	89 (29.57%)	175 (31.19%)	264 (30.63%)	
Missing	32 (10.63%)	46 (8.20%)	78 (9.05%)	