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

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Running title: Biomarkers in HER2-positive early breast cancer

Key words: HER2-positive early breast cancer, trastuzumab, predictive biomarkers, CDK inhibitors, cell cycle

Funding: The HERA study was funded and sponsored by Hoffman-La Roche.

Conflict of interests

MF reports grants and personal fees from AstraZeneca, Boehringer Ingelheim, Eli Lilly, Merck Sharp & Dohme, Novartis, Ratiopharm, and Roche. MG reports grants and personal fees from Accelsior, AstraZeneca, Celgene, GlaxoSmithKline, Novartis, OBI Pharma, Pfizer, and Roche. AK is an employee and reports personal fees from F Hoffmann-La Roche. EdA reports grants and personal fees from Genentech/Roche. GV reports personal fees from Astellas Pharma, AstraZeneca, Bristol-Myers Squibb, Celgene, Dako, Genentech, MSD Oncology, and Roche. NH reports grants and personal fees from Agendia, Amgen, AstraZeneca, Celgene, Daiichi Sankyo, Eli Lilly, Genentech, Genomic Health, NanoString Technologies, Novartis, Pfizer, Roche, and Sandoz. KIP reports personal fees from Amgen, Eisai, Novartis, Pfizer, and Roche. RDG reports grants from AstraZeneca, Celgene, Ferring, Ipsen, Merck, Novartis, Pfizer and Roche. DZ reports grants from Genentech, Novartis, Pfizer, Roche, and Tesaro. MJP-G reports grants and personal fees from Genentech and Roche. MD reports grants and personal fees from AstraZeneca, Genetech, Genoptix, GTx, Jansen Oncology, Myriad Genetics, Novartis, Pfizer, Radius Health, and Roche. UD, VP, MH, DL, FR, MT, PD, and BL-J declare no potential conflicts of interests.

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 (≤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 (ABCSG) 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: $HR_{\text{Comb Trast vs. Obs}} = 0.74$, 95% CI: 0.57-0.95, Remainder of HERA cohort:$HR_{\text{Comb Trast vs. Obs}} = 0.76$, 95% CI

0.68-0.85; OS: TransHERA group $HR_{\text{Comb Trast vs. Obs}}$ =0.73, 95% CI: 0.51-1.03, Remainder of HERA cohort: $HR_{\text{Comb Trast vs. Obs}}$= 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 ($r_{Spearman}$ =0.47, p<0.001), cyclin D1 ($r_{Spearman}$ =0.22, p<0.001), p27 ($r_{Spearman}$=0.12, p=0.0017); and cyclin D1 with Ki67 ($r_{Spearman}$ =0.15, p<0.001), and p27 ($r_{Spearman}$ =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 ($HR_{p27\ high\ vs.\ low}$ =0.49; 95% CI: 0.32-0.75, p=0.012; Table S6). No p27 effect is observed in the trastuzumab arm $HR_{p27\ high\ vs.\ low}$ =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 ($HR_{Comb\ Trast\ vs.\ Obs}$ =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 ($HR_{Comb\ Trast\ vs.\ Obs}$ =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: $HR_{\text{Comb Trast vs. Obs}}$ =0.43, 95% CI: 0.25- 0.74, p=0.0024 and intermediate $HR_{\text{Comb Trast vs Obs}}$ 0.57, 95% CI: 0.36-0.90, p=0.015). For cyclin D1 high category, no treatment benefit is detected ($HR_{\text{Comb Trast vs. Obs}}$ =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 ($HR_{\text{high vs. low}}$ =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.

Acknowledgements

We thank the pathologists and investigators who helped in creating the TransHERA collection. TransHERA was supported by grants from the Royal Marsden NIHR Biomedical Research Centre and Roche.

References

1. Piccart-Gebhart MJ, Procter M, Leyland-Jones B, Goldhirsch A, Untch M, Smith I, *et al.* Trastuzumab after adjuvant chemotherapy in HER2-positive breast cancer. N Engl J Med **2005**;353(16):1659-72.

2. Romond EH, Perez EA, Bryant J, Suman VJ, Geyer CE, Jr., Davidson NE, *et al.* Trastuzumab plus adjuvant chemotherapy for operable HER2-positive breast cancer. N Engl J Med **2005**;353(16):1673-84.

3. Goldhirsch A, Gelber RD, Piccart-Gebhart MJ, de Azambuja E, Procter M, Suter TM, *et al.* 2 years versus 1 year of adjuvant trastuzumab for HER2-positive breast cancer (HERA): an open-label, randomised controlled trial. Lancet **2013**;382(9897):1021-8 doi S0140-6736(13)61094-6 [pii]
10.1016/S0140-6736(13)61094-6.

4. Cameron D, Piccart-Gebhart MJ, Gelber RD, Procter M, Goldhirsch A, de Azambuja E, *et al.* 11 years' follow-up of trastuzumab after adjuvant chemotherapy in HER2-positive early breast cancer: final analysis of the HERceptin Adjuvant (HERA) trial. Lancet **2017**;389(10075):1195-205 doi S0140-6736(16)32616-2 [pii]
10.1016/S0140-6736(16)32616-2.

5. Untch M, Gelber RD, Jackisch C, Procter M, Baselga J, Bell R, *et al.* Estimating the magnitude of trastuzumab effects within patient subgroups in the HERA trial. Ann Oncol **2008**;19(6):1090-6 doi mdn005 [pii]
10.1093/annonc/mdn005.

6. Loi S, Dafni U, Karlis D, Polydoropoulou V, Young BM, Willis S, *et al.* Effects of Estrogen Receptor and Human Epidermal Growth Factor Receptor-2 Levels on the Efficacy of Trastuzumab: A Secondary Analysis of the HERA Trial. JAMA Oncol **2016**;2(8):1040-7 doi 10.1001/jamaoncol.2016.0339.

7. Nagata Y, Lan KH, Zhou X, Tan M, Esteva FJ, Sahin AA, *et al.* PTEN activation contributes to tumor inhibition by trastuzumab, and loss of PTEN predicts trastuzumab resistance in patients. Cancer Cell **2004**;6(2):117-27.

8. Berns K, Horlings HM, Hennessy BT, Madiredjo M, Hijmans EM, Beelen K, *et al.* A functional genetic approach identifies the PI3K pathway as a major determinant of trastuzumab resistance in breast cancer. Cancer Cell **2007**;12(4):395-402.

9. Nahta R, Takahashi T, Ueno NT, Hung MC, Esteva FJ. P27(kip1) down-regulation is associated with trastuzumab resistance in breast cancer cells. Cancer Res **2004**;64(11):3981-6.

10. Scaltriti M, Eichhorn PJ, Cortes J, Prudkin L, Aura C, Jimenez J, *et al.* Cyclin E amplification/overexpression is a mechanism of trastuzumab resistance in HER2+ breast cancer patients. Proc Natl Acad Sci U S A **2011**;108(9):3761-6 doi 1014835108 [pii]
10.1073/pnas.1014835108.

11. Jones SE, Collea R, Paul D, Sedlacek S, Favret AM, Gore I, Jr., *et al.* Adjuvant docetaxel and cyclophosphamide plus trastuzumab in patients with HER2-amplified early stage breast cancer: a single-group, open-label, phase 2 study. Lancet Oncol **2013**;14(11):1121-8 doi S1470-2045(13)70384-X [pii]
10.1016/S1470-2045(13)70384-X.

12. Fountzilas G, Christodoulou C, Bobos M, Kotoula V, Eleftheraki AG, Xanthakis I, *et al.* Topoisomerase II alpha gene amplification is a favorable prognostic factor in patients with HER2-positive metastatic breast cancer treated with trastuzumab. J Transl Med **2012**;10:212 doi 1479-5876-10-212 [pii]
10.1186/1479-5876-10-212.

13. McShane LM, Altman DG, Sauerbrei W, Taube SE, Gion M, Clark GM, *et al.* REporting recommendations for tumor MARKer prognostic studies (REMARK). Breast Cancer Res Treat **2006**;100(2):229-35 doi 10.1007/s10549-006-9242-8.

14. Grambsch PM, Therneau TM. Proportional hazards tests and diagnostics based weighted residuals. Biometrika **1994**;81:515-26.

15. Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. J R Stat Soc Series B **1995**;57:289-300.

16. Rudas M, Lehnert M, Huynh A, Jakesz R, Singer C, Lax S, *et al.* Cyclin D1 expression in breast cancer patients receiving adjuvant tamoxifen-based therapy. Clin Cancer Res **2008**;14(6):1767-74 doi 14/6/1767 [pii]
10.1158/1078-0432.CCR-07-4122.

17. Filipits M, Rudas M, Heinzl H, Jakesz R, Kubista E, Lax S, *et al.* Low p27 expression predicts early relapse and death in postmenopausal hormone receptor-positive breast cancer patients receiving adjuvant tamoxifen therapy. Clin Cancer Res **2009**;15(18):5888-94 doi 1078-0432.CCR-09-0728 [pii]
10.1158/1078-0432.CCR-09-0728.

18. Jones RL, Salter J, A'Hern R, Nerurkar A, Parton M, Reis-Filho JS, *et al.* The prognostic significance of Ki67 before and after neoadjuvant chemotherapy in breast cancer. Breast Cancer Res Treat **2009**;116(1):53-68 doi 10.1007/s10549-008-0081-7.

19. Yakes FM, Chinratanalab W, Ritter CA, King W, Seelig S, Arteaga CL. Herceptin-induced inhibition of phosphatidylinositol-3 kinase and Akt Is required for antibody-mediated effects on p27, cyclin D1, and antitumor action. Cancer Res **2002**;62(14):4132-41.

20. Le XF, Claret FX, Lammayot A, Tian L, Deshpande D, LaPushin R, *et al.* The role of cyclin-dependent kinase inhibitor p27Kip1 in anti-HER2 antibody-induced G1 cell cycle arrest and tumor growth inhibition. J Biol Chem **2003**;278(26):23441-50.

21. Marches R, Uhr JW. Enhancement of the p27Kip1-mediated antiproliferative effect of trastuzumab (Herceptin) on HER2-overexpressing tumor cells. Int J Cancer **2004**;112(3):492-501 doi 10.1002/ijc.20378.

22. Le XF, Pruefer F, Bast RC, Jr. HER2-targeting antibodies modulate the cyclin-dependent kinase inhibitor p27Kip1 via multiple signaling pathways. Cell Cycle **2005**;4(1):87-95 doi 1360 [pii]
10.4161/cc.4.1.1360.

23. Lane HA, Motoyama AB, Beuvink I, Hynes NE. Modulation of p27/Cdk2 complex formation through 4D5-mediated inhibition of HER2 receptor signaling. Ann Oncol **2001**;12 Suppl 1:S21-2.

24. Lu Y, Zi X, Pollak M. Molecular mechanisms underlying IGF-I-induced attenuation of the growth-inhibitory activity of trastuzumab (Herceptin) on SKBR3 breast cancer cells. Int J Cancer **2004**;108(3):334-41.

25. Kurozumi S, Inoue K, Takei H, Matsumoto H, Kurosumi M, Horiguchi J, *et al.* ER, PgR, Ki67, p27(Kip1), and histological grade as predictors of pathological complete response in patients with HER2-positive breast cancer receiving neoadjuvant chemotherapy using taxanes followed by fluorouracil, epirubicin, and cyclophosphamide concomitant with trastuzumab. BMC Cancer **2015**;15:622 doi 10.1186/s12885-015-1641-y
10.1186/s12885-015-1641-y [pii].

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, (ƒ)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%)	0.47ƒ,£
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%)	0.32ƒ

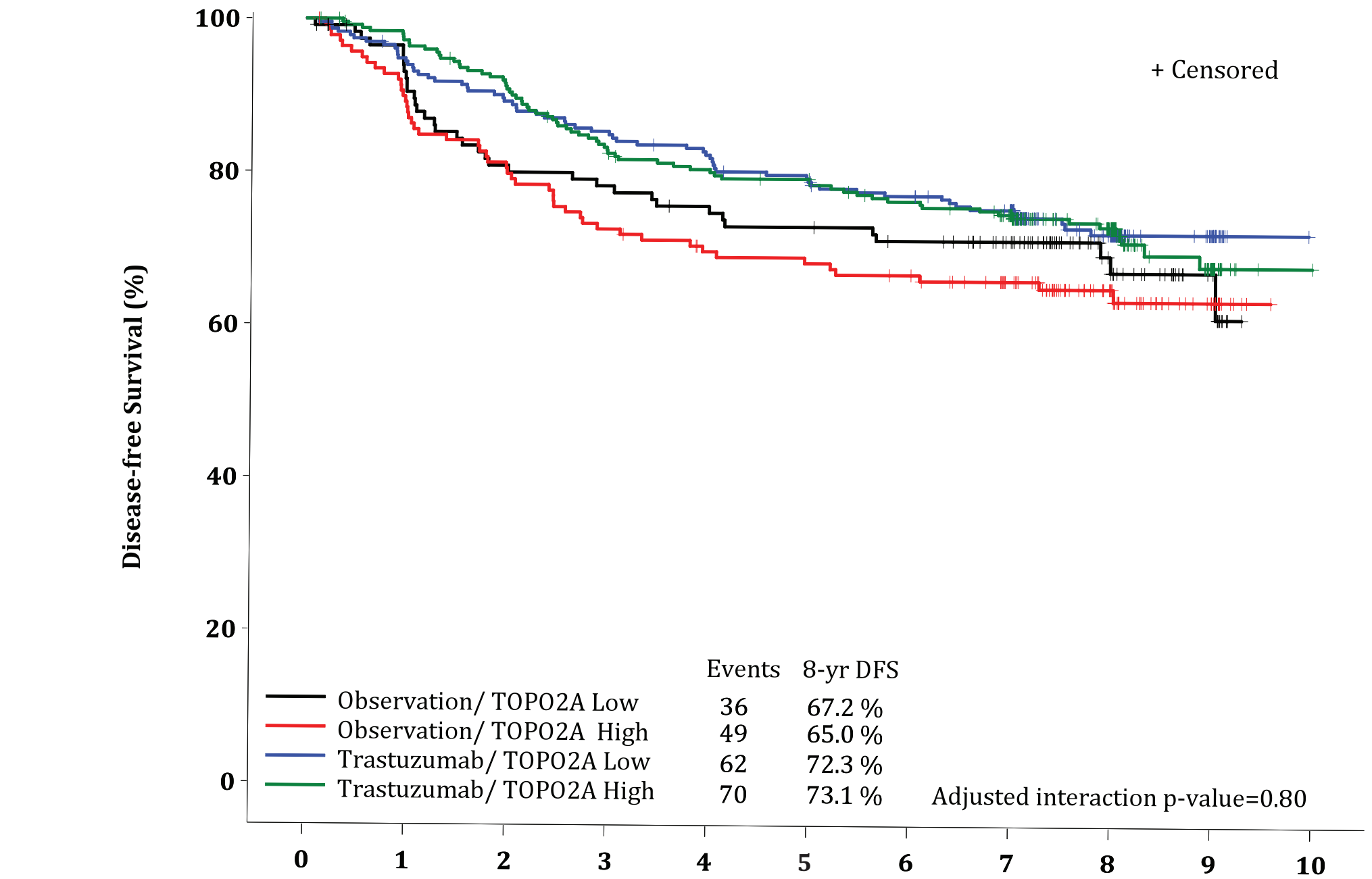
Baseline characteristics	Observation (N=301)	Combined Trastuzumab (N=561)	Total (N=862)	p-value
Positive (+)	141 (46.84%)	284 (50.62%)	425 (49.30%)	
Tumor Grade				
G1	7 (2.33%)	8 (1.43%)	15 (1.74%)	0.42*
G2	99 (32.89%)	174 (31.02%)	273 (31.67%)	
G3	191 (63.46%)	375 (66.84%)	566 (65.66%)	
GX	4 (1.33%)	4 (0.71%)	8 (0.93%)	
Menopausal status				
Premenopausal	39 (12.96%)	61 (10.87%)	100 (11.60%)	0.61ƒ
Postmenopausal	132 (43.85%)	245 (43.67%)	377 (43.74%)	0.48ƒ,©
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%)	0.46ƒ
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%)	0.88ƒ

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

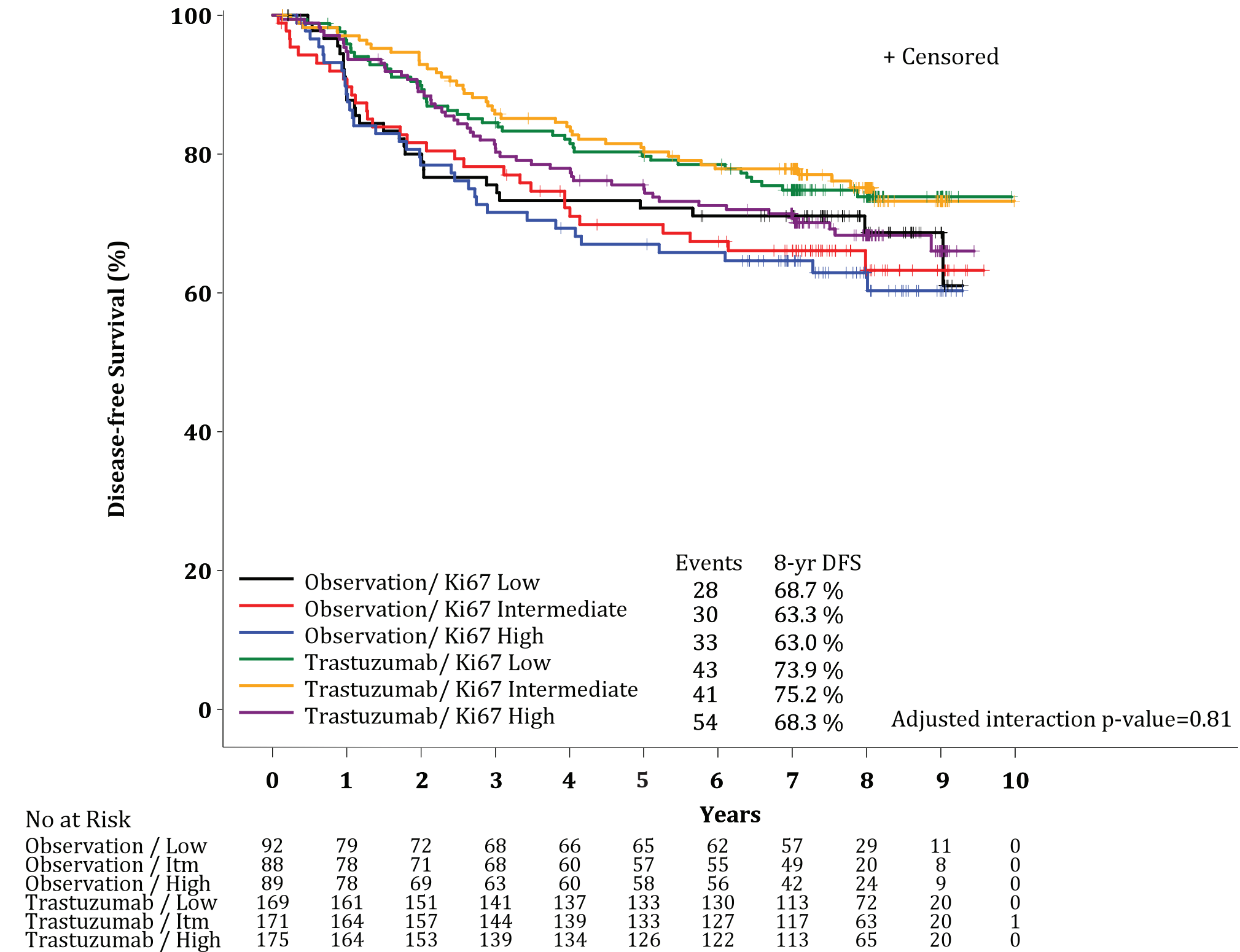
Fisher's exact test computed, ⊕ Excluding category "Missing"

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⊕
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⊕
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⊕
Missing	42 (13.95%)	71 (12.66%)	113 (13.11%)	
Ki67				
0≤Ki67<13.2	92 (30.56%)	169 (30.12%)	261 (30.28%)	0.67
13.2≤ Ki67<24.6	88 (29.24%)	171 (30.48%)	259 (30.05%)	0.93⊕
Ki67≥24.6	89 (29.57%)	175 (31.19%)	264 (30.63%)	
Missing	32 (10.63%)	46 (8.20%)	78 (9.05%)	



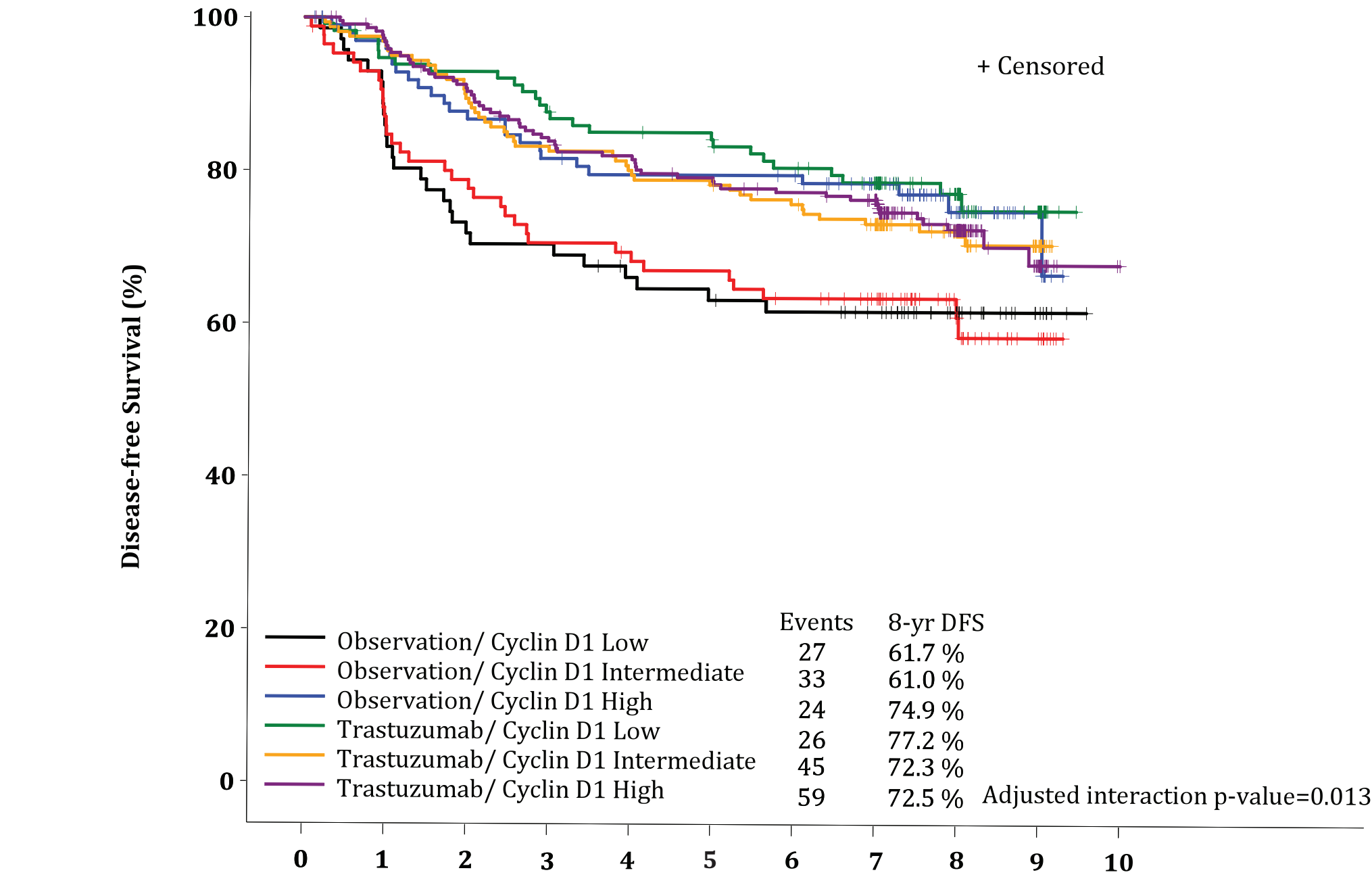
No at Risk

Observation/ Low	119	104	93	90	86	83	79	68	31	12	0
Observation/ High	140	122	111	101	94	92	89	76	40	13	0
Trastuzumab/ Low	234	219	208	199	191	182	176	161	82	25	0
Trastuzumab/ High	256	246	229	206	200	193	186	164	104	27	1



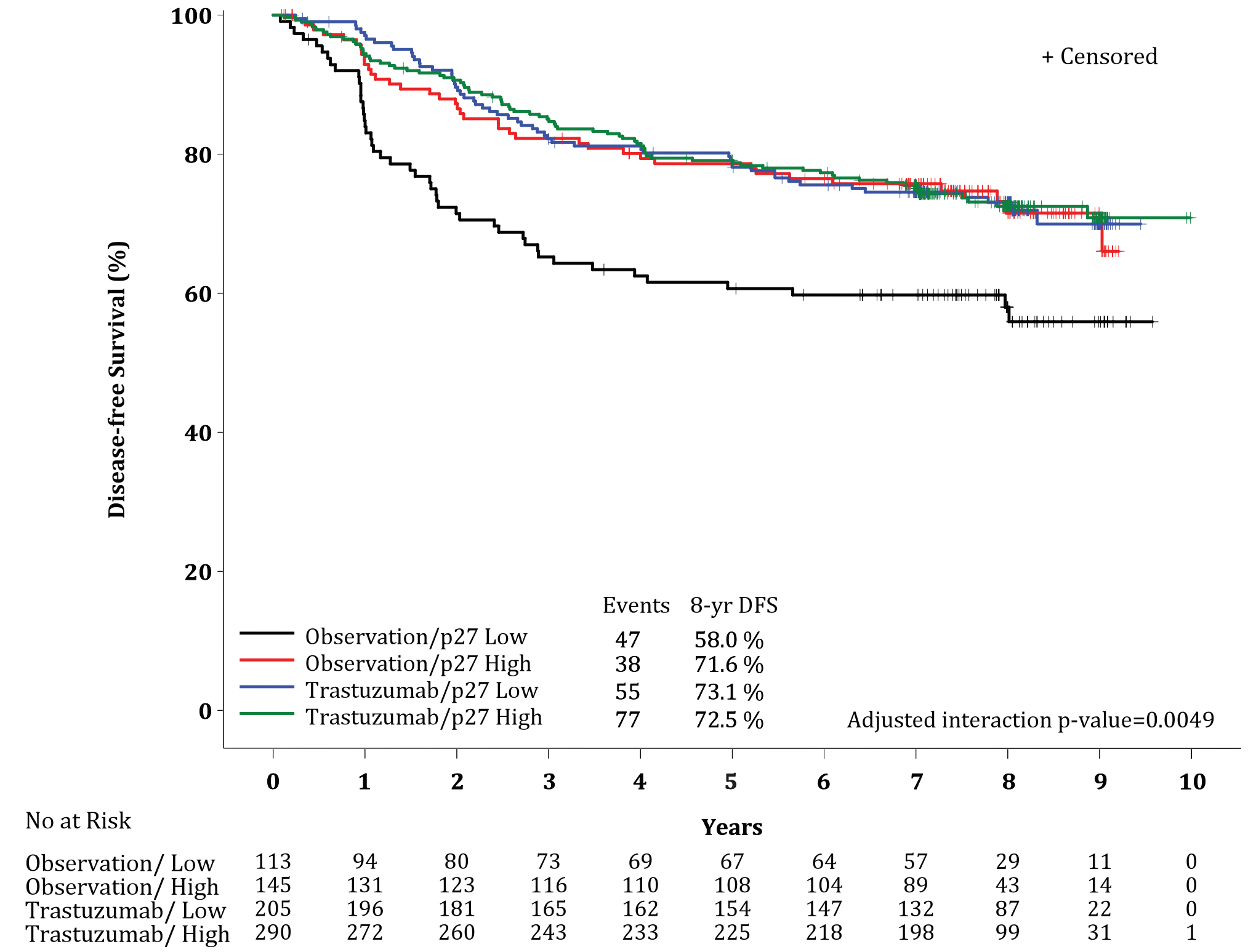
No at Risk

Observation / Low	92	79	72	68	66	65	62	57	29	11	0
Observation / Itm	88	78	71	68	60	57	55	49	20	8	0
Observation / High	89	78	69	63	60	58	56	42	24	9	0
Trastuzumab / Low	169	161	151	141	137	133	130	113	72	20	0
Trastuzumab / Itm	171	164	157	144	139	133	127	117	63	20	1
Trastuzumab / High	175	164	153	139	134	126	122	113	65	20	0

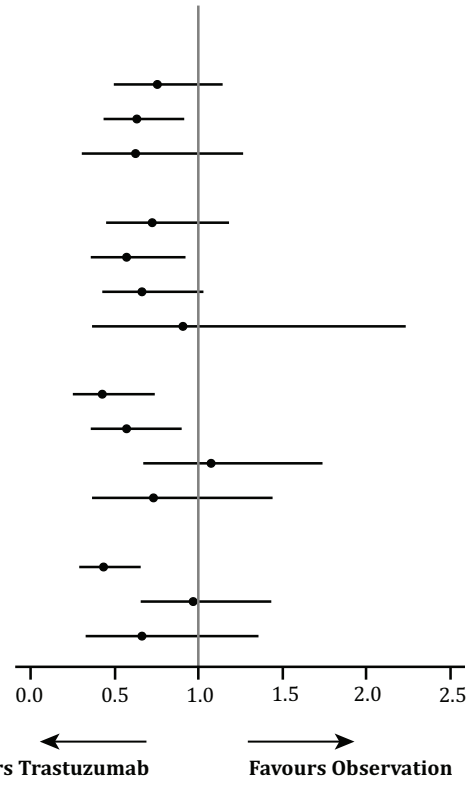


No at Risk

Observation/ Low	71	60	51	50	45	43	41	37	20	7	0
Observation/ Itm	86	72	67	60	58	56	52	46	24	8	0
Observation/ High	102	94	85	80	77	77	76	63	30	10	0
Trastuzumab/ Low	115	108	106	99	96	92	88	78	46	17	0
Trastuzumab/ Itm	161	155	144	133	129	126	121	111	68	19	1
Trastuzumab/ High	222	210	196	181	176	168	163	147	79	20	0


Disease-free Survival (%)
100
80
60
40
20
0
+ Censored
Events
8-yr DFS
Observation/p27 Low
47
58.0 %
Observation/p27 High
38
71.6 %
Trastuzumab/p27 Low
55
73.1 %
Trastuzumab/p27 High
77
72.5 %
Adjusted interaction p-value=0.0049
0
1
2
3
4
5
6
7
8
9
10
Years
No at Risk
Observation/ Low 113 94 80 73 69 67 64 57 29 11 0
Observation/ High 145 131 123 116 110 108 104 89 43 14 0
Trastuzumab/ Low 205 196 181 165 162 154 147 132 87 22 0
Trastuzumab/ High 290 272 260 243 233 225 218 198 99 31 1

Biomarker level	No. of pts by trt arm	No. of events by trt arm	HR 95% CI	Adjusted interaction p-value
TOPO2A				
≤ 5	234 vs 119	62 vs 36	0.75 (0.50, 1.14)	
> 5	256 vs 140	70 vs 49	0.63 (0.44, 0.91)	0.80
Missing	71 vs 42	18 vs 14	0.63 (0.31, 1.27)	
Ki67				
1st tertile	169 vs 92	43 vs 28	0.79 (0.45, 1.18)	
2nd tertile	171 vs 88	41 vs 30	0.57 (0.36, 0.92)	0.81
3rd tertile	175 vs 89	54 vs 33	0.66 (0.42, 1.03)	
Missing	46 vs 32	12 vs 8	0.90 (0.37, 2.23)	
Cyclin D1				
1st tertile	115 vs 71	26 vs 27	0.43 (0.25, 0.74)	
2nd tertile	161 vs 86	45 vs 33	0.57 (0.36, 0.90)	0.013
3rd tertile	222 vs 102	59 vs 24	1.08 (0.67, 1.74)	
Missing	63 vs 42	20 vs 15	0.73 (0.37, 1.44)	
p27				
≤ 70	205 vs 113	55 vs 47	0.44 (0.29, 0.65)	
> 70	290 vs 145	77 vs 38	0.97 (0.66, 1.44)	0.0049
Missing	66 vs 43	18 vs 14	0.66 (0.33, 1.35)	



Models adjusted for: Nodal status, Pathological tumor size & Region