Impact of RNA Signatures on pCR and Survival after 12-Week Neoadjuvant Pertuzumab plus Trastuzumab with or without Paclitaxel in the WSG-ADAPT HER2$^+$/HR$^-$ Trial

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ABSTRACT

Purpose: To identify associations of biological signatures and stromal tumor-infiltrating lymphocytes (sTIL) with pathological complete response (pCR; ypT0 ypN0) and survival in the Phase II WSG-ADAPT HER2$^+$/HR$^-$ trial (NCT01817452).

Experimental Design: Patients with cT1-cT4c, cN0–3 HER2$^+$/HR$^-$ early breast cancer (EBC) were randomized to pertuzumab+trastuzumab (P+T, $n = 92$) or P+T+paclitaxel ($n = 42$). Gene expression signatures were analyzed in baseline biopsies using NanoString Breast Cancer 360 panel ($n = 117$); baseline and on-treatment (week 3) sTIL levels were available in 119 and 76 patients, respectively. Impacts of standardized gene expression signatures on pCR and invasive disease-free survival (iDFS) were estimated by logistic and Cox regression.

Results: In all patients, *ERBB2* [OR, 1.70; 95% confidence interval (CI), 1.08–2.67] and estrogen receptor (ER) signaling (OR, 1.72; 95% CI, 1.13–2.61) were favorable, whereas *PTEN* (OR, 0.57; 95% CI, 0.38–0.87) was unfavorable for pCR. After 60 months median follow-up, 13 invasive events occurred (P+T: $n = 11$, P+T+paclitaxel: $n = 2$), none following pCR. Gene signatures related to immune response (IR) and ER signaling were favorable for iDFS, all with similar HR about 0.43–0.55. These patterns were even more prominent in the neoadjuvant chemotherapy-free group, where additionally BRCAness signature was unfavorable (HR, 2.00; 95% CI, 1.04–3.84). IR signatures were strongly intercorrelated. sTILs (baseline/week 3/change) were not associated with pCR or iDFS, though baseline sTILs correlated positively with IR signatures.

Conclusions: Distinct gene signatures were associated with pCR versus iDFS in HER2$^+$/HR$^-$ EBC. The potential role of IR in preventing recurrence suggests that patients with upregulated IR signatures could be candidates for de-escalation concepts in HER2$^+$ EBC.

Introduction

Dual HER2 blockade combined with polychemotherapy is standard of care in high-risk HER2$^+$ early breast cancer (EBC). Nevertheless, these regimens are associated with toxicities caused mainly by the chemotherapy component. As HER2$^+$ EBC is a highly heterogenous disease, also regarding treatment efficacy and prognosis, there is a need to develop new treatment algorithms that could further improve outcomes while minimizing toxicities. Several trials demonstrated that chemotherapy-free neoadjuvant therapy (NAT) can induce clinically meaningful pathological complete response (pCR) rates; however, the most optimal approach for chemotherapy de-escalation is still unknown. NeoSphere was one of the first trials, indicating that there is a population particularly sensitive to chemotherapy-free anti-HER2

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Clin Cancer Res 2023;29:805–14

doi: 10.1158/1078-0432.CCR-22-1587

Translational Relevance

Limited data are available on predictive biomarkers for dual HER2 blockade in HER2$^+$/HR$^-$ early breast cancer (EBC). The present analysis aimed to identify associations of gene signatures in tumor tissue and of stromal tumor-infiltrating lymphocytes (sTIL) with pathologic complete response (pCR) and invasive disease-free survival (iDFS) in the Phase II WSG-ADAPT HER2$^+$/HR$^-$ trial, investigating neoadjuvant pertuzumab+trastuzumab with or without paclitaxel. We demonstrate that distinct signatures are associated with pCR and with iDFS, with immune response (IR) gene signatures correlating with survival but not tumor sensitivity to therapy. IR signatures strongly correlated with each other and were associated with baseline sTILs. The interaction between IR signatures and iDFS was more pronounced in the chemotherapy-free arm. This indicates that patients with upregulated immune-related signatures could be candidates for further de-escalation concepts in HER2$^+$/HR$^-$ EBC.

NAT (1). In the KRISTINE trial, pCR rates were 44.4% after trastuzumab emtansine + pertuzumab and 55.7% after docetaxel, carboplatin, and trastuzumab + pertuzumab, both given for 18 weeks (2). This trial also showed a similar invasive disease-free survival (iDFS) in both arms, thus indicating that omission of systemic chemotherapy does not compromise long-term outcomes (3). In addition, chemotherapy-free adjuvant regimes in patients with pCR are currently investigated in the ongoing CompassHER2-pCR trial (NCT04266249).

To identify patients who could benefit from de-escalation/escalation treatment strategies, robust prognostic and predictive biomarkers are urgently needed. A recent meta-analysis demonstrated that HER2-enriched status according to PAM50 is associated with the likelihood of a pCR after anti-HER2 NAT ± chemotherapy (4). Other studies found that higher levels of stromal tumor-infiltrating lymphocytes (sTIL) are associated with increased pCR rates and survival (5, 6). Moreover, several genomic alterations and gene expression signatures were identified as potential predictors of tumor response to NAT (7–10).

WSG-ADAPT HER2$^+$/HR$^-$ trial is a Phase II de-escalation trial. Following a 12-week neoadjuvant regimen with dual HER2 blockade (pertuzumab and trastuzumab) ± weekly paclitaxel, further chemotherapy could be omitted in patients with no invasive tumor cells in breast and lymph nodes (ypT0/is ypN0). Primary analysis of our WSG-ADAPT HER2$^+$/HR$^-$ trial revealed that early response (by low cellularity or Ki-67 decrease) after only three weeks of NAT is associated with ypT0/is ypN0, particularly if associated with a high HER2 protein expression (IHC 3+; refs. 11, 12). Moreover, patients with ypT0/is ypN0 had excellent 5-year outcome, independent of trial arm or further chemotherapy administration (13). In the present translational analysis of the WSG-ADAPT HER2$^+$/HR$^-$ trial, we investigated gene expression signatures and sTIL levels to identify new prognostic biomarkers associated with pCR (defined as absence of both any residual invasive cancer and ductal carcinoma *in situ* in breast and lymph nodes, ypT0 ypN0) and improved survival.

Materials and Methods

Trial design

The WSG-ADAPT HER2$^+$/HR$^-$ trial (NCT01817452) is part of prospective, controlled, randomized, non-blinded, multicenter, investigator-initiated umbrella clinical trial. A total of 134 patients were randomized (5:2) to 12 weeks of pertuzumab + trastuzumab (Arm A, $n = 92$) or pertuzumab + trastuzumab + paclitaxel (Arm B, $n = 42$). Patients received 840-mg loading dose pertuzumab, then 420-mg every 3 weeks (q3w); 8 mg/kg loading dose trastuzumab, then 6 mg/kg q3w and 80 mg/m^2 weekly ± paclitaxel. Surgery was performed within three weeks after the end of NAT. In patients with residual-invasive tumor cells, further standard chemotherapy was mandatory; in case investigators opted for further NAT, prior histologic confirmation of residual tumor by core needle biopsy was required. Recommended post-trial therapy (neoadjuvant and/or adjuvant) followed national guidelines. The primary trial endpoint was comparison of ypT0/is ypN0 rates among the arms. Secondary endpoints included 5-year iDFS (defined as the time interval from randomization until local, regional or distant relapse, second malignancy, or death, whichever occurred first), overall survival (OS), and safety. A further key secondary endpoint of the trial was translational research, specifically assessment of factors affecting clinical endpoints. To this end, core biopsies were performed at baseline (time of diagnosis) and on treatment (3-week biopsy) and analyzed as described below.

The trial was approved by the Ethics Committee of the medicinal department of University Cologne (approval number: 11–283) and by the independent ethics committee or institutional review board at each participating site. The trial was conducted in accordance with the principles of the Declaration of Helsinki and the International Conference on Harmonization of Good Clinical Practice guidelines.

Patients

Enrolled patients were females, ages ≥18 years with histologically confirmed unilateral, primary invasive, cT1-cT4c, cN0–3 breast carcinoma and centrally confirmed HR-negative [estrogen receptor (ER)- and progesterone receptor–negative <1% of tumor nuclei] and HER2-positive status (IHC 3+ positive or ISH positive). Eastern Cooperative Oncology Group Performance Status ≤1 or Karnofsky Performance Status ≥80% and normal organ function were required for inclusion. Detailed inclusion and exclusion criteria have been previously published (11, 14). All patients provided written informed consent before enrollment in the trial.

Gene expression and bioinformatics analysis

Formalin-fixed, paraffin-embedded (FFPE) tissue slides from core biopsies obtained at baseline were prepared at Institute of Pathology, University of Hannover (Germany), and used for isolation of total RNA using the FFPET RNA Isolation Kit (Roche Diagnostics GmbH, Mannheim, Germany). The relative gene expression was analyzed in 25–500 ng of RNA using the NanoString Breast Cancer 360 assay (BC360) on a NanoString nCounter prepstation and digital analyzer (NanoString Technologies Inc.). The expression of 758 genes across 41 gene signatures related to breast cancer–specific pathways and processes (Supplementary Table S1) was evaluated using nSolver 4.0 software (15). Intrinsic molecular subtypes were determined with the PAM50 predictor as previously described (16). Gene expression data were quality controlled on the basis of housekeeper geomean expression of greater than 220 counts. Standard nCounter quality controls were performed to assess control linearity, limit of detection, and number of fields of view. The normalization was performed in two steps after all zero counts on the raw scale were converted to ones. In the first step, genes were normalized using a ratio of the expression value to the geometric mean of the housekeeper genes. In the second step, single genes were normalized with panel standard using the reference sample comprised of *in vitro* transcripts for each gene.

Afterwards, the data were Log(2) transformed. A constant of eight was added to tumor inflammation signature (TIS) so that it was on the same scale as investigational use only (IUO) TIS, making scores comparable across research use only and IUO assays. Gene signatures were predefined on the basis of NanoString proprietary signature weights.

Assessment of sTIL levels

sTIL levels were measured according to current guidelines of the International TILs Working Group 2014 (17). Briefly, sTILs were counted on H&E-stained tissue sections obtained at baseline and after 3 weeks of NAT, as reported previously (18). Measurements were performed in triplicate and the median of the three measurements was used. Afterwards, an independent evaluator cross-checked plausibility considering the previous scoring, using additional information such as CD45 and CK5/14 staining to evaluate tumor composition. In line with current guidelines (17), "Hot spots" (small areas with increased TILs) were included in the average TIL count in the tumor area. In cases where multiple tumor samples were available, the tumor site with the highest sTIL count was selected for further analysis.

Statistical analysis

The present translational analysis focused on pCR and iDFS. For pCR, the stricter definition (ypT0 ypN0) was used due to higher statistical power. Impacts of standardized gene expression signatures, as assessed by the BC360 panel, and sTILs (both considered as continuous variables) on pCR were estimated by logistic regression and expressed as odds ratios (OR); impacts on survival endpoints (iDFS or OS) were estimated by Cox regression and expressed as hazard ratios (HR); 95% confidence intervals (95% CI) were computed for these impacts. Impacts are reported in the trial as a whole and in Arm A, because low event rates in Arm B precluded further detailed analyses.

Multivariate analyses of factors for prediction of pCR and of iDFS (dependent variables) were performed for all patients and for arm A only. Independent variables entering the analysis included the RNA signatures identified to be associated with pCR and iDFS by univariate analysis, and additionally age (≥50 vs. <50 years) and lymph node status (N+ vs. N0). Because of the explorative character of this investigation, no adjustments for multiple testing were performed; P values <0.05 were considered significant. Variables corresponding to RNA signatures were removed sequentially from the multivariable model using the backward selection method with a threshold for removal of $P < 0.1$. Age and lymph node status were forced to stay in the model.

Spearman's rank correlation coefficients were calculated to evaluate the intercorrelations among gene expression signatures and their relationship with sTILs.

Data availability statement

Deidentified participant data and study protocol will be made available upon a reasonable request to the corresponding author. Proposals for any purpose will be considered.

Results

Patient characteristics

Of 134 patients randomized into the trial, sufficient amount and quality of RNA from baseline core biopsies allowed BC360 analysis in 117 patients who were included in the present analysis ($n = 80$ in Arm A, $n = 37$ in Arm B; **Table 1** and Supplementary Fig. S1). In Arms A and B, 63.8% and 64.9% of patients were ages ≥50 years, 51.3% and 59.5% were postmenopausal, 53.8% and 54.1% had cT2 tumors, and 46.3% and 35.1% were N+, respectively. A total of 92.3% of tumors had grade 3. HER2 status was 3+ in most tumors (84.7% in local and 96.6% in central laboratory testing). A total of 82.9% of tumors were HER2-enriched according to PAM50. Information on pCR was available for 115 patients (78 in Arm A, 37 in Arm B); 2 patients in Arm A with missing information on pCR withdrew their consent before trial treatment. A total of 45 patients had pCR (ypT0 ypN0, $n = 17$, 21.3%, in Arm A; $n = 28$, 75.7%, in Arm B). A total of 59 patients had ypT0/is ypN0 ($n = 26$, 32.5%, in Arm A; $n = 33$, 89.2%, in Arm B). Compared with the 17 patients without BC360 data, patients included in the present analysis had a higher tumor grade ($P = 0.014$) and a higher central HER2 status ($P = 0.032$; Supplementary Table S2).

Table 1. Patient characteristics for subjects with BC360 analysis in the WSG-ADAPT HER2$^+$/HR$^-$ trial.

	Arm A ($n = 80$)	Arm B ($n = 37$)	Total ($n = 117$)
Age group (y)			
<50	29 (36.3)	13 (35.1)	42 (35.9)
≥50	51 (63.8)	24 (64.9)	75 (64.1)
Menopausal status			
Postmenopausal	41 (51.3)	22 (59.5)	63 (53.9)
Premenopausal	35 (43.8)	14 (37.8)	49 (41.9)
Unknown	4 (5)	1 (2.7)	5 (4.3)
cT, *n* (%)			
1	31 (38.8)	15 (40.5)	46 (39.3)
2	43 (53.8)	20 (54.1)	63 (53.9)
3	5 (6.3)	2 (5.4)	7 (6)
4	1 (1.3)	0 (0)	1 (0.9)
cN, *n* (%)			
0	43 (53.8)	24 (64.9)	67 (57.3)
1	32 (40)	12 (32.4)	44 (37.6)
2	5 (6.3)	1 (2.7)	6 (5.1)
Grade, *n* (%)			
1	0 (0)	0 (0)	0 (0)
2	6 (7.5)	3 (8.1)	9 (7.7)
3	74 (92.5)	34 (91.9)	108 (92.3)
HER2 status, local, *n* (%)			
1+	1 (1.3)	0 (0)	1 (0.9)
2+	8 (10)	3 (8.1)	11 (9.4)
3+	71 (88.8)	34 (91.9)	105 (84.7)
HER2 status, central, *n* (%)			
1+	1 (1.3)	0 (0)	1 (0.9)
2+	2 (2.5)	1 (2.7)	3 (2.6)
3+	77 (96.3)	36 (97.3)	113 (96.6)
PAM50 subtype, *n* (%)			
Basal	7 (8.8)	5 (13.5)	12 (10.3)
HER2-enriched	66 (82.5)	31 (83.8)	97 (82.9)
Luminal A	7 (8.8)	1 (2.7)	8 (6.8)
pCR, *n* (%)			
ypT0 ypN0	17 (21.3)	28 (75.7)	45 (38.5)
ypT0/is ypN0	26 (32.5)	33 (89.2)	59 (50.4)

Association between RNA expression signatures and pCR

In combined analysis of Arms A and B, ER signaling (OR, 1.72; 95% CI 1.13–2.61) and *ERBB2* (OR, 1.70; 95% CI, 1.08–2.67) were positively associated with pCR whereas *PTEN* was negatively associated (OR, 0.57; 95% CI, 0.38–0.87; **Fig. 1A**). ER signaling (OR, 2.57; 95% CI, 1.23–5.36) and *ERBB2* (OR, 2.24; 95% CI, 1.03–4.91) were positively associated with pCR also when only patients in the Arm A

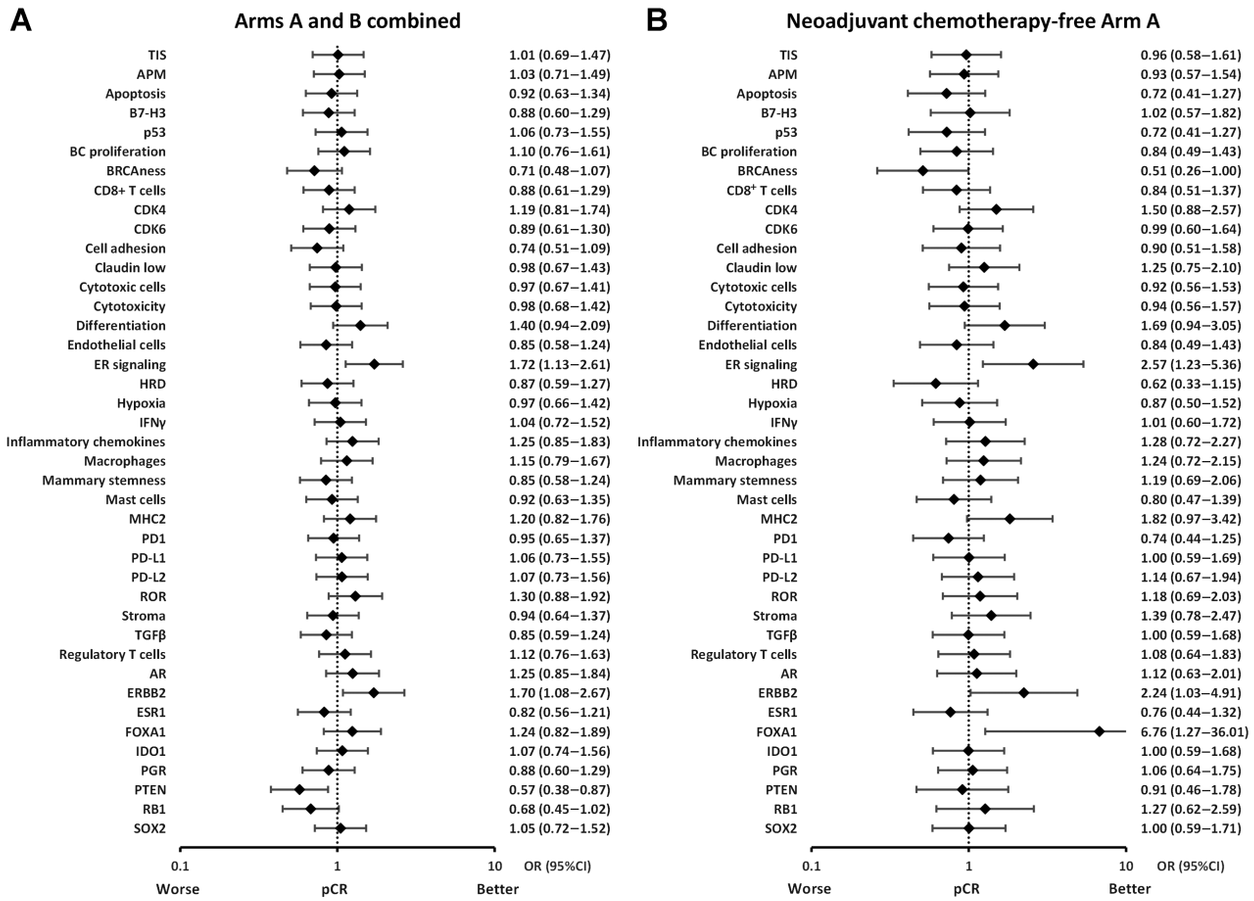

Figure 1.

Associations between RNA signatures and pCR. Data are shown for combined analysis of Arms A and B (**A**) and for the neoadjuvant chemotherapy-free Arm A only (**B**). ORs are expressed in terms of standardized variables.

were analyzed (**Fig. 1B**). In addition, *FOXA1* was positively (OR, 6.76; 95% CI, 1.27–36.01) and BRCAness was negatively associated with pCR (OR, 0.51; 95% CI, 0.26–0.99). Only ER signaling (among all patients) and *ERBB2* (among all patients and in Arm A) were associated with ypT0/is ypN0 (Supplementary Fig. S2).

Association between RNA expression signatures and survival

During a median follow-up of 60 months, 10 iDFS events occurred in the 117 patients with NanoString BC360 data, including eight events in Arm A and two events in Arm B. None of the iDFS events occurred after pCR. Twenty-six (57.8%) of the 45 patients with pCR received no further chemotherapy (5 in Arm A, 21 in Arm B). Two iDFS events (one local relapse in Arm A, and one distant relapse in Arm B) were observed in patients with ypT0/is ypN0.

Eight RNA signatures in combined Arms A and B (**Fig. 2A**) and 16 RNA signatures in Arm A only were associated with iDFS (**Fig. 2B**). In combined analysis, ER signaling (HR, 0.49; 95% CI, 0.25–0.95), and several signatures related to immune response (IR), including TIS, *IDO1*, macrophages, cytotoxic cells, cytotoxicity, PD1 (*PDCD1*), and MHC2, were positively associated with iDFS (HR, 0.43–0.55). In a separate analysis of the Arm A, these IR signatures, and additionally CD8 T cells, IFNγ, inflammatory chemokines, PD-L1 (*CD274*), PD-L2 (*PDCD1LG2*), and regulatory T cells, were associated with improved iDFS (HR, 0.37–0.57). Furthermore, ER signaling (HR, 0.47; 95% CI, 0.23–0.96) and APM (HR, 0.57; 95% CI, 0.35–0.93) were favorable for iDFS whereas BRCAness was unfavorable (HR, 2.00; 95% CI, 1.04–3.84).

Overall, we found 9 RNA signatures to be associated with pCR and/or iDFS in Arms A and B (**Fig. 3A**), and 17 signatures in the analysis of Arm A (**Fig. 3B**). ER signaling was positively associated with both pCR and iDFS in the combined analysis of Arms A and B and in Arm A. BRCAness was unfavorable for both pCR and iDFS in Arm A. Only ER signaling (in combined Arms A and B analysis) was favorably associated with both ypT0/is ypN0 and iDFS.

In addition, we analyzed the relationship between RNA signatures and OS. During the median follow-up of 60 months, there were 5 deaths (4 in Arm A, 1 in Arm B). There were no deaths after pCR; one patient in Arm A died after ypT0/is ypN0 and a distant relapse. In the combined analysis of Arms A and B, ER signaling (HR, 0.42; 95% CI, 0.19–0.89) and MHC2 (HR, 0.35; 95% CI, 0.15–0.79) were associated with improved OS, whereas p53 (HR, 2.38; 95% CI, 1.03–5.48), BC proliferation (HR, 2.59; 95% CI, 1.14–5.85), and BRCAness (HR, 2.08; 95% CI, 1.04–4.16) were unfavorable (Supplementary Fig. S3). In Arm A, TIS (HR, 0.47; 95% CI, 0.23–0.97), IFNγ (HR, 0.46; 95% CI, 0.22–0.95), MHC2 (HR, 0.38; 95% CI, 0.17–0.86), and PD-L2 (HR, 0.45; 95% CI, 0.22–0.96) were associated positively, whereas p53 (HR, 3.90; 95%

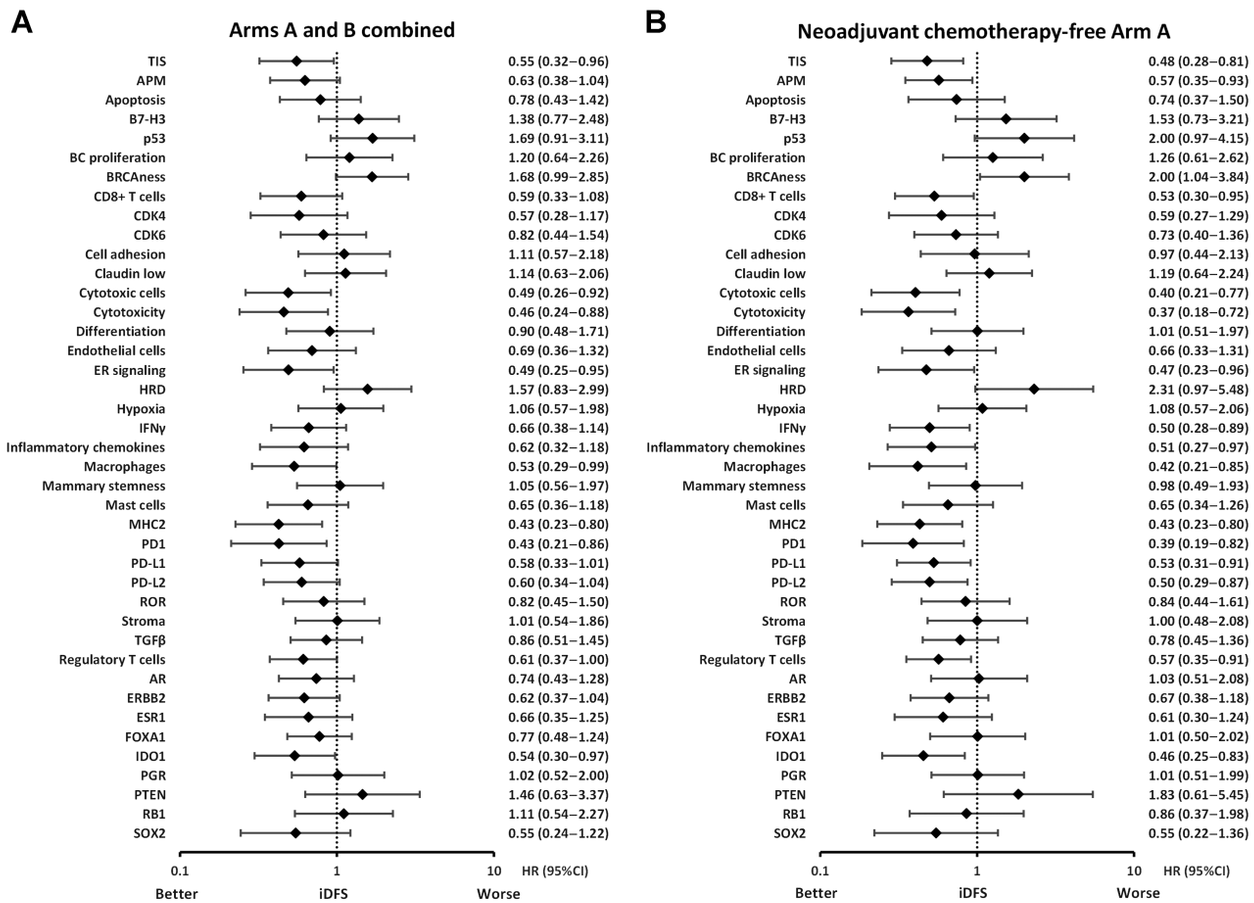

Figure 2.

Associations between RNA signatures and iDFS. Data are shown for combined analysis of Arms A and B (**A**) and for the neoadjuvant chemotherapy-free Arm A only (**B**). HRs are expressed in terms of standardized variables.

CI, 1.10–13.86), BC proliferation (HR, 4.89; 95% CI, 1.35–17.76), and BRCAness (HR, 2.46; 95% CI, 1.05–5.75) were associated negatively with OS.

Given the fact that ER signaling signature was predictive for pCR and survival in this HR$^-$ cohort, we analyzed single genes comprising this signature. We found that *CDCA8* was associated with pCR in combined analysis of Arms A and B and in Arm A (Supplementary Table S3). *ADCY9*, *DNAJC12*, *WDR77*, and *BORCS7* were associated with pCR in combined Arms A and B. Furthermore, *ADCY9*, *IFT140*, and *TBC1D9* genes were associated with iDFS in Arm A (Supplementary Table S4).

Correlations within RNA expression signatures

In the correlation analyses of RNA signatures that associated with pCR and/or iDFS, IR signatures intercorrelated positively and formed a separate cluster (**Fig. 4**). There was only a weak negative correlation between some of the IR-elated signatures and *ERBB2*, BRCAness, and *FOXA1*. Moreover, ER signaling and *PTEN* did not correlate with other signatures.

Association of sTILs with pCR, survival, and RNA expression signatures

Analysis of sTIL levels was possible in 119 patients at baseline and in 76 patients at week 3. sTIL levels at baseline or week 3 and the change from baseline to week 3 were not associated with pCR, iDFS, or OS, neither in regression analysis of combined Arms A and B nor in Arm A (Supplementary Table S5). Baseline sTIL levels positively correlated with gene expression signatures related to IR; however, there was no association between baseline sTILs and *ERBB2*, ER signaling, *PTEN*, BRCAness, or *FOXA1* (**Fig. 4**). Week 3 sTIL levels and change in sTILs number from baseline did not correlate with any of the gene expression signatures associated with pCR or iDFS.

Multivariate analysis for prediction of pCR and iDFS

In multivariate analysis, *ERBB2*, ER signaling, *PTEN*, and lymph node status (N+ vs. N0) were significant independent predictors of pCR in a combined analysis of Arms A and B (**Table 2**). *ERBB2* was also predictive for pCR in Arm A. These factors, and additionally age (≥50 vs. <50 years, in Arm A), were also predictive for ypT0/is ypN0 (Supplementary Table S6). MHC2 was the only significant predictor of iDFS in Arms A and B combined and in Arm A (**Table 2**).

Discussion

The introduction of anti-HER2 agents and their combination with chemotherapy has improved long-term outcomes in HER2$^+$ BC (19). However, some patients still experience disease recurrence, even

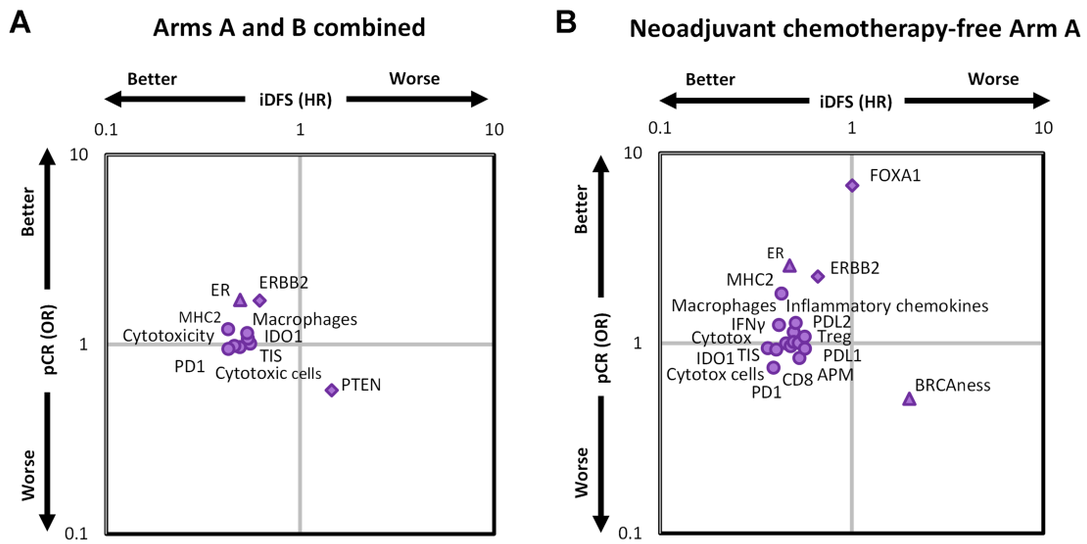

Figure 3.

RNA signatures associated with pCR and/or iDFS. Data are shown for combined analysis of Arms A and B (**A**) and for the neoadjuvant chemotherapy-free Arm A only (**B**). OR and HR are expressed in terms of standardized variables. Symbols denote RNA signatures associated only with pCR (◆), only with iDFS (●), or with both pCR and iDFS (▲).

among those with pCR after NAT. We now have compelling evidence that chemotherapy-free anti-HER2 regimes achieve pCR in approximately one third of patients with $HR^-/HER2^+$ EBC (1, 20–22). Thus, there is a need to identify prognostic/predictive markers associated with therapy response to avoid under- or over-treatment.

The WSG-ADAPT $HER2^+/HR^-$ trial originally aimed to identify an early-response biomarker defining a subgroup of $HER2^+/HR^-$ EBC patients who could be safely spared chemotherapy, for example, a subgroup of Arm A with pCR to anti-HER2 treatment alone similar to that obtained after a chemotherapy-containing regimen in Arm B. Efficacy regarding primary and secondary clinical endpoints, including univariate impacts of clinical risk factors and of early response (defined by Ki-67 dynamics or cellularity) were previously reported (11, 13).

The current translational analysis of the WSG-ADAPT $HER2^+/HR^-$ trial has evaluated the prognostic impact of baseline gene expression signatures and changes in sTIL levels regarding pCR and survival. The results include assessment of gene signatures associated with favorable outcomes specific to chemotherapy-free NAT in Arm A; the low number of non-pCR cases and iDFS events did not allow testing for these associations specifically in the neoadjuvant chemotherapy-containing Arm B.

Both ER signaling and BRCAness (in chemotherapy-free NAT) were associated with both pCR and iDFS: ER signaling favorably, BRCAness unfavorably. Disruption of BRCA-mediated DNA repair processes results in a higher susceptibility to DNA damage. This selected group of patients could thus be candidates for future de-escalated NAT concepts within randomized studies with dual-HER2 blockade ± chemotherapy. The ER signature in the BC360 panel captures regulation of transcription factors downstream of ERs and crosstalk between estrogen and other pathways. Previously, ER signaling was reported to associate negatively with pCR after chemotherapy and trastuzumab and/or lapatinib in the NSABP B-41 trial in $HER2^+$ and HR^+ or HR^- EBC (23). In NSABP B-41, tumors with high *ESR1* gene expression had a lower probability for a pCR; we did not see such an association in our collective. It is important to note, however, that NSABP B-41 enrolled patients with $HER2^+$ EBC irrespectively of HR status and, therefore, tumors were driven by functional ER in at least some patients. In contrast, the WSG-ADAPT $HER2^+/HR^-$ trial was conducted specifically in the HR-negative population, which indicates that activation of ER signaling could result from crosstalk with other pathways in patients responding to dual anti-HER2 therapy. For example, analysis of single genes in ER signaling signature revealed that *ADCY9* associated with pCR. *ADCY9* gene encodes adenylyl cyclase type 9 that mediates signaling pathways of several chemokines. Further genes associated with pCR or iDFS encode proteins modulating lysosome positioning, protein transport, and chromatin structure and transcription—processes known to be regulated by several signaling pathways. Of note, the gene encoding ER 1 (*ESR1*) was not associated with pCR or iDFS in our analysis.

We also identified several gene signatures associated either with pCR or with iDFS, suggesting that distinct gene expression patterns largely determine tumor sensitivity to therapy and long-term outcomes. For instance, expression of *ERBB2* mRNA was predictive for pCR in the whole cohort as well as in the neoadjuvant chemotherapy-free arm A. Previously, *ERBB2* was associated with pCR in the NSABP B-41 (23), NeoALTTO (8, 24), and CALGB 40601 trials (9). Recently, pooled analysis of SOLTI-PAMELA, TBCRC023, TBCRC006, and PER-ELISA trials showed that 44.5% of tumors with both HER2-enriched subtype and a high *ERBB2* expression (defined as mRNA levels in the upper two tertiles) achieved pCR after trastuzumab + lapatinib (25). High expression of *ERBB2* in these tumors indicates that cancer pathogenesis is driven by HER2 signaling, and therefore particularly sensitive to anti-HER2 agents. Regarding *FOXA1*, there is much less evidence for an association of this gene signature with response to NAT in $HER2^+$ EBC. In contrast with our findings, *FOXA1* mRNA levels were not predictive for pCR in the NSABP B-41 trial (23). Interestingly, *FOXA1* mediates ER-regulated gene expression and is one of the genes defining the luminal subtype (26). Given that both *FOXA1* and ER signaling were associated with pCR in our trial, there

	ERBB2	ER signaling	PTEN	BRCAness	FOXA1	APM	IDO1	PD-L1	TIS	IFNγ	MHC2	Cytotoxicity	Inflammatory chemokines	PD-L2	PD1	Cytotoxic cells	CD8 T cells	Macrophages	Regulatory T cells	Baseline sTILs	Cycle 2 sTILs	Change sTILs
ERBB2		−0.10	0.13	−0.15	0.46	−0.26	−0.29	−0.30	−0.32	−0.19	−0.16	−0.26	−0.11	−0.29	−0.30	−0.29	−0.31	−0.25	−0.11	−0.24	−0.09	−0.05
ER signaling	−0.10		0.04	−0.12	0.07	0.14	0.20	0.10	0.17	0.14	0.09	0.19	−0.08	0.10	0.14	0.20	0.16	0.13	0.19	0.09	0.08	−0.04
PTEN	0.13	0.04		−0.10	−0.07	−0.08	−0.04	−0.21	−0.06	0.05	−0.15	−0.09	−0.11	0.02	−0.23	−0.07	−0.06	−0.13	0.01	−0.01	−0.12	−0.13
BRCAness	−0.15	−0.12	−0.10		−0.20	−0.21	−0.19	−0.16	−0.28	−0.34	−0.35	−0.24	−0.14	−0.38	−0.09	−0.21	−0.11	−0.36	−0.24	−0.23	−0.24	−0.03
FOXA1	0.46	0.07	−0.07	−0.20		−0.37	−0.31	−0.27	−0.29	−0.32	0.01	−0.21	−0.22	−0.34	−0.18	−0.25	−0.25	−0.25	−0.19	−0.31	−0.01	0.18
APM	−0.26	0.14	−0.08	−0.21	−0.37		0.79	0.72	0.85	0.78	0.47	0.76	0.45	0.67	0.63	0.77	0.70	0.67	0.69	0.56	0.22	−0.13
IDO1	−0.29	0.20	−0.04	−0.19	−0.31	0.79		0.78	0.89	0.80	0.43	0.84	0.51	0.70	0.69	0.85	0.75	0.66	0.66	0.62	0.26	−0.08
PD-L1	−0.30	0.10	−0.21	−0.16	−0.27	0.72	0.78		0.84	0.68	0.45	0.81	0.59	0.75	0.83	0.82	0.78	0.73	0.70	0.50	0.19	−0.10
TIS	−0.32	0.17	−0.06	−0.28	−0.29	0.85	0.89	0.84		0.86	0.61	0.93	0.51	0.80	0.77	0.94	0.86	0.80	0.75	0.63	0.20	−0.17
IFNγ	−0.19	0.14	0.05	−0.34	−0.32	0.78	0.80	0.68	0.86		0.42	0.77	0.59	0.80	0.51	0.76	0.62	0.67	0.60	0.56	0.21	−0.13
MHC2	−0.16	0.09	−0.15	−0.35	0.01	0.47	0.43	0.45	0.61	0.42		0.49	0.31	0.42	0.43	0.49	0.44	0.54	0.45	0.29	0.15	0.04
Cytotoxicity	−0.26	0.19	−0.09	−0.24	−0.21	0.76	0.84	0.81	0.93	0.77	0.49		0.43	0.74	0.83	0.99	0.90	0.74	0.73	0.60	0.16	−0.18
Inflammatory chemokines	−0.11	−0.08	−0.11	−0.14	−0.22	0.45	0.51	0.59	0.51	0.59	0.31	0.43		0.59	0.36	0.41	0.33	0.63	0.36	0.20	0.24	0.22
PD-L2	−0.29	0.10	0.02	−0.38	−0.34	0.67	0.70	0.75	0.80	0.80	0.42	0.74	0.59		0.58	0.74	0.64	0.82	0.67	0.48	0.26	−0.04
PD1	−0.30	0.14	−0.23	−0.09	−0.18	0.63	0.69	0.83	0.77	0.51	0.43	0.83	0.36	0.58		0.84	0.81	0.66	0.63	0.52	0.09	−0.19
Cytotoxic cells	−0.29	0.20	−0.07	−0.21	−0.25	0.77	0.85	0.82	0.94	0.76	0.49	0.99	0.41	0.74	0.84		0.93	0.74	0.75	0.61	0.16	−0.19
CD8 T cells	−0.31	0.16	−0.06	−0.11	−0.25	0.70	0.75	0.78	0.86	0.62	0.44	0.90	0.33	0.64	0.81	0.93		0.65	0.70	0.53	0.11	−0.22
Macrophages	−0.25	0.13	−0.13	−0.36	−0.25	0.67	0.66	0.73	0.80	0.67	0.54	0.74	0.63	0.82	0.66	0.74	0.65		0.69	0.45	0.28	−0.02
Regulatory T cells	−0.11	0.19	0.01	−0.24	−0.19	0.69	0.66	0.70	0.75	0.60	0.45	0.73	0.36	0.67	0.63	0.75	0.70	0.69		0.54	0.17	−0.21
Baseline sTILs	−0.24	0.09	−0.01	−0.23	−0.31	0.56	0.62	0.50	0.63	0.56	0.29	0.60	0.20	0.48	0.52	0.61	0.53	0.45	0.54		0.15	−0.50
Cycle 2 sTILs	−0.09	0.08	−0.12	−0.24	−0.01	0.22	0.26	0.19	0.20	0.21	0.15	0.16	0.24	0.26	0.09	0.16	0.11	0.28	0.17	0.15		0.69
Change sTILs	−0.05	−0.04	−0.13	−0.03	0.18	−0.13	−0.08	−0.10	−0.17	−0.13	0.04	−0.18	0.22	−0.04	−0.19	−0.19	−0.22	−0.02	−0.21	−0.50	0.69	

Figure 4.
Spearman Correlations within RNA expression signatures. Shown are RNA signatures that associated with pCR or iDFS.

might be a synergistic effect between these two signatures that mediate response to NAT even in clinically HR$^-$ tumors.

The majority of all prognostic gene signatures identified in our trial were associated with iDFS; they were predominantly related to IR, namely inhibitory immune mechanisms (*IDO1* and PD-L1), antitumor immune activity (TIS, IFNγ, MHC2, and cytotoxicity), inhibitory immune signaling (inflammatory chemokines, PD-L2, and PD1), and immune cell abundance (cytotoxic cells, CD8 T cells, macrophages, and regulatory T cells). Moreover, these gene signatures were strongly intercorrelated, suggesting an intricate and complex relationship between different immune mechanisms in some HER2$^+$ tumors. Given that these signatures were associated with iDFS but not with pCR, our data indicate that activation of IR mechanisms has only a limited effect on tumor response to dual HER2 blockade but has a profound impact on recurrence. Other studies provided conflicting results on interaction between immune-related gene signatures and pCR or survival. On the one hand, several single-gene and metagene signatures were positively associated with pCR in the NSABP B-41, CherLOB, and CALGB 40601 trials (9, 10, 23, 27). On the other hand, no interaction between immune-related signatures and pCR was found in the NeoALTTO trial, similar to our results (24). Furthermore, expression of IR genes correlated with both pCR and recurrence-free

Table 2. Multivariate logistic regression models for predicting pCR and iDFS.

Endpoint/variable	Arms A and B combined		Neoadjuvant chemotherapy-free Arm A	
pCR	**OR (95% CI)**	***P***	**OR (95% CI)**	***P***
ERBB2	2.30 (1.34–3,94)	0.002	2.45 (1.07–5.58)	0.033
ER signaling	1.77 (1.09–2.86)	0.020	—	—
PTEN	0.47 (0.28–0.79)	0.004	—	—
Age (≥50 vs. <50 years)	0.77 (0.32–1.87)	0.566	0.61 (0.18–2.06)	0.430
Clinical lymph node status (N+ vs. N0)	0.41 (0.17–1.00)	0.049	0.22 (0.06–0.79)	0.020
iDFS	**HR (95% CI)**	***P***	**HR (95% CI)**	***P***
ER signaling	0.61 (0.35–1.08)	0.093	—	—
MHC2	0.33 (0.15–0.75)	0.008	0.32 (0.16–0.66)	0.002
Age (≥50 vs. <50 years)	3.37 (0.60–18.79)	0.166	3.78 (0.63–22.67)	0.145
Clinical lymph node status (N+ vs. N0)	0.64 (0.16–2.62)	0.532	0.48 (0.10–2.22)	0.346

Note: Number of patients analyzed in Arms A and B combined: $n = 115$ for pCR and $n = 117$ for iDFS; in neoadjuvant chemotherapy-free Arm A: $n = 78$ for pCR and $n = 80$ for iDFS.

survival (RFS) in the CALGB 40601 trial (28) and with pCR but not with EFS in the NeoALTTO trial (8). Of note, a higher expression of genes mediating IR resulted in a longer RFS after adjuvant trastuzumab in the NCCTG-N9831 trial (29). These conflicting findings could be attributed to various NAT regimens involving different chemotherapy drugs and combinations of single- or double anti-HER2 targeting approaches used in these studies. Furthermore, several of above-mentioned studies included patients with HR^+ and HR^- $HER2^+$ EBC, whereas our trial specifically enrolled HR^- disease.

Interestingly, the number of IR signatures that associated with improved iDFS was higher in the chemotherapy-free NAT Arm A than in both arms combined. This indicates that HER2 blockade may be the primary driver of engaging the ongoing IR processes and in turn reducing the risk of cancer recurrence. Although the underlying mechanisms remain to be identified, IR to antibody-dependent cellular cytotoxicity induced by anti-HER2 agents could be involved in this phenomenon (30). Furthermore, 26 out of 45 patients (57.8%) with pCR in our trial received no further chemotherapy (5 in chemotherapy-free NAT Arm A and 21 in Arm B). In the NCCTG-N9831 trial, activation of IR genes after adjuvant chemotherapy + trastuzumab was associated with delayed recurrence whereas no such benefit was observed in patients treated with chemotherapy only (29). Therefore, a more tolerable therapy involving only anti-HER2 agents could potentially be used in the neoadjuvant setting, with chemotherapy reserved as an adjuvant regimen in high-risk patients who did not have a pCR. The potential role of IR in preventing recurrences could pave the way for further de-escalation concepts in $HER2^+$ EBC. For instance, the ongoing Keyriched-1 trial (NCT03988036) investigates neoadjuvant immunotherapy with pembrolizumab in combination with dual anti-HER2 blockade in HER2-enriched EBC. Nevertheless, the benefits of immunotherapy need to be balanced with the risks of immune-related toxicities.

In addition to the distinct expression patterns of immune-related genes, available evidence highlights the association of sTILs with therapy response and survival in $HER2^+$ EBC. Higher levels of sTILs at baseline were predictive for pCR and survival after anti-HER2 treatment and chemotherapy in the neoadjuvant NeoALLTO and CherLOB trials and in the adjuvant APHINITY trial (31). In the PAMELA trial, an increase in sTILs (alone or in combination with a decrease in tumor cellularity) during the chemotherapy-free anti-HER2 NAT was associated with pCR (6). However, we could not confirm these findings as neither baseline, nor on-treatment sTILs nor changes in sTIL levels had an impact on pCR or survival in our trial. Nevertheless, we found a strong correlation between sTIL levels and immune-related signatures. Therefore, analysis of gene expression could augment the morphological information of sTILs and provide further details on processes shaping the tumor microenvironment (32).

Our trial has some limitations. First, the small number of patients in Arm B together with the high pCR rate in this arm precluded definitive conclusions regarding the impact of chemotherapy on the observed interactions of gene expression and sTIL levels with pCR and survival. Second, additional sequential analysis of gene expression in on-treatment biopsies could identify further signatures associated with tumor response and survival. Third, fortunately for the patients participating in our trial, there were only few iDFS and OS events during the 60-month follow-up. Nevertheless, we could identify several gene signatures associated with survival thus highlighting their robust prognostic potential. Finally, given the exploratory nature of these analyses, no alpha adjustments were made.

In conclusion, immune activation appears to select a group of $HER2^+$ patients for whom therapy de-escalation could potentially be feasible. These results warrant further randomized clinical trials in $HER2^+$ EBC investigating chemotherapy-free neoadjuvant concepts in patients selected according to baseline gene expression signatures.

Authors' Disclosures

O. Gluz reports personal fees from Roche, AstraZeneca, Daiichi Sankyo, Amgen, MSD, Novartis, Pfizer, Lilly, Gilead, Exact Science, Pierre Fabre, Seagen, and Agendia outside the submitted work. M. Christgen reports other support from WSG during the conduct of the study. S. Kuemmel reports personal fees from Roche, Lilly, Genomic Health, Novartis, Amgen, Celgene, Daiichi Sankyo, AstraZeneca, Somatex, MSD, Pfizer, Exact Science, Seagen, pfm medical, Gilead, and Sonoscape, as well as non-financial support from Roche, Daiichi Sankyo, Lilly, Gilead, and Sonoscape outside the submitted work; S. Kuemmel is also WSG Study Group co-director. R. Wuerstlein reports personal fees and non-financial support from Agendia and Amgen, as well as personal fees from Aristo, AstraZeneca, Celgene, Clovis Oncology, Daiichi Sankyo, Eisai, Exact Sciences, Gilead, Hexal, Sandoz, Lilly, Medstorm Medical, MSD, Mundipharma, Mylan, Nanostring, Novartis, Odonate, Paxman, Palleos, Pfizer, Pierre Fabre, Puma Biotechnology, Riemser, Roche, Sanofi Genzyme, Seagen, Tesaro Bio, Teva, Veracyte, Viatris, Pomme Med, Clinsol, Aurikamed, FOMF, Medconcept, and MCI outside the submitted work. K. Krauss reports personal fees from Roche, Pierre Fabre, MSD, Celgene, and Pfizer, as well as non-financial

support from Lilly and Novartis outside the submitted work. H. Forstbauer reports personal fees from Roche and BMS outside the submitted work. T. Reimer reports grants from German Cancer AID and Else Kroener-Fresenius Foundation, as well as personal fees from Roche, Novartis, AstraZeneca, and Daiichi Sankyo outside the submitted work. H.H. Fischer reports personal fees from Roche, Novartis, AstraZeneca, Daiichi Sankyo, Lilly, and Pfizer outside the submitted work. H. Ni reports personal fees from China Scholarship Council during the conduct of the study, as well as personal fees from China Scholarship Council outside the submitted work. C. Kolberg-Liedtke reports personal fees from Amgen, POMME, Seagen, Sanofi-Genzyme, NCO Hannover, AstraZeneca, Onkowissen, Novartis, MSD, Eli Lilly, Agendia, Exact Science, Carl Zeiss Meditec, Daiichi Sankyo, and Aurikamed; grants and personal fees from Roche and Gilead; and other support from Phaon Scientific GmbH during the conduct of the study. H.H. Kreipe reports personal fees from Roche Pharma, Exact Sciences, Lilly, AstraZeneca, Ventana, and Pfizer during the conduct of the study. U. Nitz reports grants from Roche and Amgen; personal fees from Celgene, Exact Science, and Teva; and non-financial support from AOK during the conduct of the study. U. Nitz also reports to be a shareholder of WSG. N. Harbeck reports personal fees from Amgen, AstraZeneca, Daiichi Sankyo, Gilead, Lilly, MSD, Novartis, Pierre Fabre, Pfizer, Roche, Sandoz, and Seagen outside the submitted work; in addition, N. Harbeck is a co-director of West German Study Group (WSG). No disclosures were reported by the other authors.

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Acknowledgments

The WSG-ASAPT-HER2$^+$/HR$^-$ trial was financially supported by Roche and Bayer. The funder of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report. Medical writing and editorial support were provided by Lukasz Wujak, Lukasz Wujak MedComms, Warsaw, Poland, and funded by West German Study Group, Moenchengladbach, Germany.

The publication costs of this article were defrayed in part by the payment of publication fees. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 USC Section 1734.

Note

Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

Received May 18, 2022; revised August 26, 2022; accepted November 21, 2022; published first November 28, 2022.

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