An RB-1 loss of function gene signature as a tool to predict response to neoadjuvant chemotherapy plus anti-HER2 agents: a substudy of the NeoALTTO trial (BIG 1-06)

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Abstract

Background: Chemotherapy added to anti-HER2 agents (H) is the treatment of choice in patients with HER2+ early breast cancer. However, HER2+ tumours are clinically and biologically heterogeneous, and treatment response varies significantly by hormone receptor (HR) status and molecular subtype. Predictive biomarkers are needed in this context. This study assessed whether an RB-1 loss of function gene signature (RBsig) is predictive of response to neoadjuvant chemotherapy in combination with trastuzumab, lapatinib or both, within the NeoALTTO trial.

Methods: We collected RNA-sequencing data from pretreatment biopsies derived from the NeoALTTO trial. RBsig expression was computed retrospectively and correlated with pathological complete response (pCR) using receiver-operating characteristic (ROC) curves. The RBsig was dichotomised as High/Low in correspondence to the 25th percentile. Reported *p* values resulted from Fisher's exact test.

Results: Of 455 NeoALTTO patients, 244 were eligible for this substudy (HR+ $n=129$; HR– $n=115$). Overall, pCR rate was significantly higher in patients with RBsig High tumours than those with RBsig Low (35% *versus* 18% respectively; $p=0.01$). The area under the ROC curve (AUC) was 0.60 (95% CI 0.52–0.67). A remarkably low pCR rate of 11% was seen in HR+/RBsig Low patients *versus* 28% in HR+/RBsig High.

Conclusions: These results indicate RBsig may add valuable information to HER2 and HR expression, which may in turn inform treatment choices. HR+/HER2+/RBsig Low breast cancers exhibited the poorest pathological response following chemotherapy plus H. Accordingly, in such patients, endocrine therapy in combination with H and, possibly, a CDK4/6 inhibitor, may potentially prove to be a more effective treatment.

Keywords: gene expression profiling, HER2+ breast cancer, neoadjuvant chemotherapy, predictive biomarker, RB pathway

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Introduction

Of all invasive breast cancers, approximately 20% present with, and are characterized by, human epidermal growth factor receptor 2 (HER2) overexpression and/or HER2 gene amplification.[1] Anti-HER2 agents (H) given in combination with chemotherapy (CT) represent the current standard of care for patients with early HER2+ breast cancer.[2] The phase III neoadjuvant NeoALTTO trial showed that pathological complete response

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(pCR) rates were significantly improved in patients who received paclitaxel plus dual HER2 blockade by way of the anti-HER2 monoclonal antibody, trastuzumab, plus the small molecule tyrosine kinase inhibitor, lapatinib, compared with paclitaxel plus either anti-HER2 agent alone.[3,4] This outcome was observed across all subgroups; however, a meaningful difference in terms of response was observed between hormone receptor positive (HR+) and HR negative (HR–) tumours, in favour of the latter.[3] Similar results have been reported by several other neoadjuvant trials, confirming that a substantial proportion of patients with HR+/HER2+ disease do not respond to H combined with CT.[5–8] Preclinical data suggest that this difference in response between HR+ and HR– tumours could be partly attributed to bidirectional crosstalk between the ER and HER2 pathways and as such, targeting both pathways simultaneously may be a superior therapeutic strategy than therapy directed at a single pathway.[9] Clinical trials have examined the addition of endocrine therapy (ET) to H in both early and advanced HR+/HER2+ breast cancer, consistently showing a significant benefit from the combination.[10–17] However, whether the combination of ET and H would prove superior to the clinically established combination of H and CT remains an open question, as there have been no direct comparisons made between these two approaches. In addition to HR expression, different molecular features also contribute to the heterogeneous response to treatment observed in HER2+ tumours. Gene expression profiling by PAM50 can divide HER2+ breast cancers into five intrinsic molecular subtypes (luminal-A, luminal-B, HER2-enriched, basal-like and normal-like) with a different subtype distribution between HR+ and HR– tumours. Several neoadjuvant trials have shown HER2-enriched (HER2-e) disease is associated with the highest pCR rate after CT in combination with H, out of the four molecular subtypes.[8,12,18]

Within the NeoALTTO trial, multiple efforts have focused on the secondary endpoint of predictive biomarker discovery.[19–25] However, no biomarker has been shown to be clinically effective in identifying subgroups who benefit the most (or least) from CT and H. RB pathway alterations occur frequently in HER2+ tumours.[26] Preclinical and clinical data suggest that loss of function of the tumour suppressor *RB1* and RB pathway alterations are linked to higher sensitivity to CT in breast cancer.[27–29] Our group has developed a gene signature of RB1 loss of function (RBsig), which includes 87 E2F1/E2F2-associated genes.[30] A previous analysis conducted within a meta-dataset of 10 neoadjuvant trials showed RBsig expression is predictive of response to CT plus H in HR+/HER2+ breast cancer patients.[31] In that study, gene expression data from pretreatment biopsies of 514 HER2+ patients undergoing neoadjuvant CT were retrospectively analysed. In patients with RBsig Low expression, the pCR rate after CT plus H was significantly lower than in patients with RBsig High expression. Of note, this correlation between RBsig and pCR outcome was observed only in HR+/HER2+ tumours, and not in those with HR–/HER2+ expression. Additionally, preclinical data obtained on breast cancer cell lines demonstrated RBsig Low expression correlates with response to the CDK4/6 inhibitor palbociclib.[30] Collectively, these data suggest that RBsig may potentially identify a subset of HR+/HER2+ patients (RBsig Low) who derive little benefit from CT plus H, and who might theoretically benefit from alternative treatments such as CDK 4/6 inhibitors in combination with ET and H.

The results observed within the aforementioned meta-dataset[31] were limited by way of the heterogeneity of the samples analysed, and the different treatments received by the patients. The present study aimed to validate those previous results within the NeoALTTO trial, a phase III randomised study, in which frozen tissue samples were prospectively collected, and all patients received the same CT regimen. RBsig expression was determined by RNA-sequencing (RNA-seq) data derived from pretreatment biopsies, and was correlated with pCR rates following CT in combination with H. As a secondary objective, the correlation of RBsig with event-free survival (EFS) was also evaluated.

Materials and methods

The NeoALTTO study [ClinicalTrials.gov identifier: NCT00553358] was a randomised, open-label, multicentre phase III trial in which patients with HER2+ early BC were allocated to receive neoadjuvant lapatinib, trastuzumab or both for an initial 6 weeks, followed by the addition of weekly paclitaxel for 12 weeks. After definitive surgery, patients received adjuvant chemotherapy followed by the same anti-HER2 therapy as previously assigned for a total of 1 year. The primary endpoint was pCR.[3,4] The NeoALTTO trial was

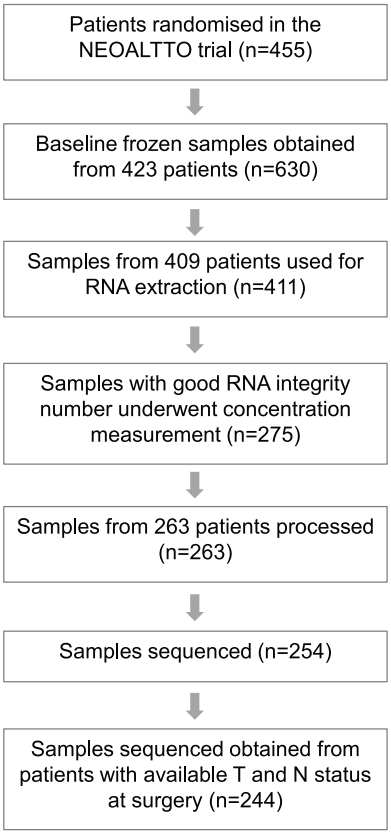

Figure 1. Consort diagram.

approved by the ethics committee and relevant health authorities of all the participating sites. Written informed consent, covering future biomarker research, was obtained from all patients at study entry.

We collected clinical data and RNA-sequencing (RNA-seq) data from pretreatment biopsies derived from 254 participants to NeoALTTO, which was previously processed by Istitut Jules Bordet (Brussels, Belgium). PAM50 classification was also provided, with the subtypes determined on a merged dataset composed of NeoALTTO and The Cancer Genome Atlas, as previously described.[23] The present substudy defined pCR as the absence of invasive tumour cells in both the breast and axillary lymph nodes at the time of surgery (ypT0/is ypN0).

RBsig expression was computed retrospectively, and correlated with pCR or EFS. The RBsig score was computed by calculating the average (i.e. mean) Z-score transformed expression levels across each score of the gene list, as previously described.[30] Data were available for 85 out of 87 genes of RBsig. RBsig was tested for its predictive and prognostic value as a continuous variable and as a classifier. For this purpose, RBsig was dichotomised as High or Low in correspondence to the 50th and the 25th percentile. RBsig was also investigated with a data-driven approach, computing all possible points of separation, and selecting the optimal cutoff.[32]

The receiver-operating characteristic (ROC) curve and the area under the curve (AUC) were used to assess the prediction performance of the RBsig score. The two-sided Wilcoxon Mann–Whitney test (WMW) was used to check for significant differences between two distributions that were represented both as box plots and density plots. Fisher's exact test was performed to check the independence of nominal variables. The distribution of EFS was estimated using the Kaplan–Meier method and compared with the log-rank test. The relationship between pCR, EFS and RBsig score was assessed using logistic regressions and Cox proportional hazard models. The univariate effect of major clinicopathological parameters and of treatment arms was evaluated and a multivariate model was fitted with those covariates that reached a statistically significant effect.

Results

Patient characteristics and pCR rates

The NeoALTTO trial enrolled 455 patients in total; RNA-seq data were obtained for 254 patients. Of these, 10 with non-evaluated nodal status at time of surgery were excluded, thus bringing the total population of this substudy to 244 (Figure 1). Overall, 85 patients had been assigned to the lapatinib arm, 77 to the trastuzumab arm, and 82 to the combination arm. Baseline characteristics of this substudy cohort did not significantly differ from that of the overall NeoALTTO population. Of the substudy cohort, 129 were HR+, and 115 were HR–. Overall, 75 patients obtained a pCR (pCR rate = 31%); of these, 30 were HR+ (pCR rate = 23%) and 45 were HR– (pCR rate = 39%). According to treatment arm, the pCR rates were 19% in the lapatinib arm, 25% in the trastuzumab arm and 49% in the lapatinib–trastuzumab arm (Table 1).

Correlation between pCR and RBsig

RBsig expression and its correlation with pCR was evaluated in the overall substudy population,

Table 1. Patient characteristics.

	HR positive *n* (%) (*n* = 129)	HR negative *n* (%) (*n* = 115)	*p* value
Age (years)			0.09
Median	47	51	
Range	23–73	23–79	
T			0.52
T2	73 (57)	70 (61)	
⩾T3	56 (43)	45 (39)	
N			0.48
N0/1	111 (86)	95 (83)	
⩾N2, Nx or missing	18 (14)	20 (17)	
Grade			0.63
G1/G2	62 (48)	37 (32)	
G3	52 (40)	64 (56)	
Gx	15 (12)	14 (12)	
Treatment arm			0.61
Lap	48 (37)	37 (32)	
Trast	41 (32)	36 (31)	
Trast + Lap	40 (31)	42 (37)	
Treatment response			0.008
RD	99 (77)	70 (61)	
pCR	30 (23)	45 (39)	

G, grade; Lap, lapatinib; N, lymph node status; pCR, pathological complete response; RD, residual disease; T, tumour status; Trast, trastuzumab.

as well as in the HR+ and HR– subgroups. As a standardized RBsig cutoff score to define "High" and "Low" expression levels is not yet established, samples were classified as RBsig High or Low initially based on the mean RBsig cutoff (50th percentile), then on a lower cutoff (25th percentile), and finally, according to the optimal cutoff (22nd percentile). The 25th percentile threshold performed better than the 50th percentile threshold, while producing similar results to the optimal cutoff (data not shown). For this reason, the 25th percentile threshold was selected as the final cutoff and is reported henceforth.

Overall population: of 244 patients included in the analysis, the classifier identified 182 patients with RBsig High tumours, and 62 patients with RBsig Low. pCR rates were significantly higher in patients with RBsig High tumours compared to those with RBsig Low (35% *versus* 18%, respectively; Fisher's exact test $p = 0.011$) (Figure 2(a)). A significant difference in RBsig distribution was observed between patients with pCR and those with residual disease (RD) (WMW $p = 0.01$) (Figure 2(b)). The distribution of RBsig across samples is illustrated in supplementary Figure 1(a). By ROC analysis, the AUC for RBsig was

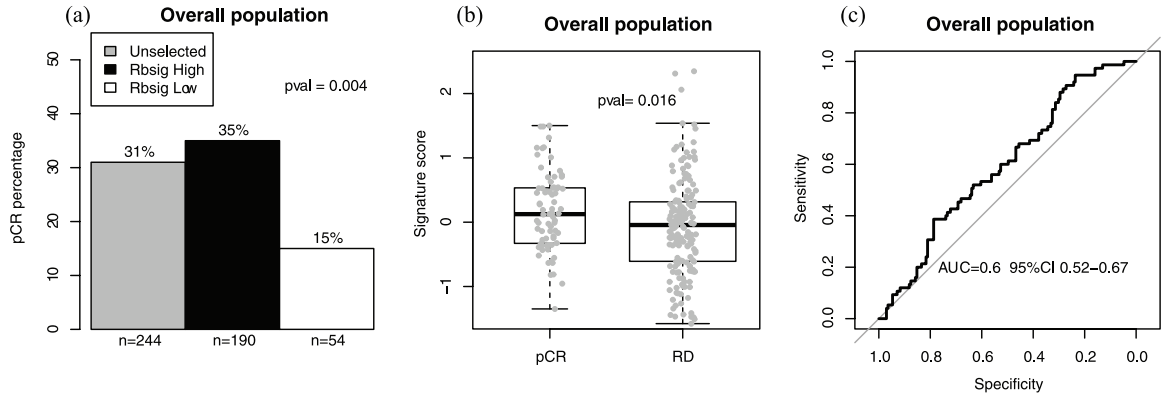

Figure 2. RBsig is associated with response to neoadjuvant chemotherapy plus anti-HER2 therapy in HER2+ breast cancer patients (overall population).
(a) Bar graphs showing the frequency of pathological complete response (pCR) in patients unselected for RBsig expression, RBsig High and RBsig Low; (b) box plots representing RBsig expression value as a function of pCR *versus* residual disease (RD); width of boxes is proportional to the number of samples; the whiskers mark 1.5 * IQR (interquartile range); (c) receiver-operating characteristic (ROC) analysis of RBsig.

0.60 [95% confidence interval (CI) 0.52–0.67], indicating modest sensitivity and specificity for predicting response (Figure 2(c)).

The association between RBsig and conventional clinicopathological parameters, treatment arm and pCR was explored by univariate and multivariate regression analyses. In the univariate model, RBsig was significantly associated with pCR, when considered as a continuous [RBsig cont $p = 0.021$, odds ratio (OR) 1.58] as well as a categorical (RBsig High/Low 25th percentile cut-off $p = 0.012$, OR 2.51) variable (Figure 3(a)). A significant correlation with pCR was also found for ER status ($p = 0.004$, OR 2.31), PR status ($p = 0.003$, OR 2.59) and the dual HER2-blockade treatment arm (lapatinib + trastuzumab *versus* trastuzumab $p = 0.002$, OR 2.91; lapatinib + trastuzumab *versus* lapatinib $p < 0.001$, OR 4.11). At multivariate analysis adjusted for HR status and treatment arm, RBsig was not found to be independently associated with pCR (RBsig High/Low 25th percentile cutoff $p = 0.089$, OR 1.94 95% C.I. 0.93–4.3).

HR+ subgroup: 129 tumours positively expressed HR; of these, 94 were classified as RBsig High, and 35 as RBsig Low. A remarkably low pCR rate of 11% was seen in the HR+/RBsig Low subgroup, *versus* 28% in HR+/RBsig High (Fisher's exact test $p = 0.06$) (Figure 4(a)). RBsig expression levels were higher in patients achieving a pCR *versus* those who did not (WMW $p = 0.09$) (Figure 4(b), Supplementary Figure 1(c)).The ROC curve AUC for RBsig was 0.60 (95% CI 0.49–0.72) (Figure 3(c)). At univariate analysis, RBsig was weakly associated with pCR in this subgroup of patients (RBsig cont $p = 0.049$) (Figure 3(c)).

HR– subgroup: of 115 patients with HR– disease, 88 were classified as RBsig High, and 27 as RBsig Low. The pCR rate was 43% in HR–/RBsig High patients *versus* 26% of the HR–/RBsig Low patients (Fisher's exact test $p = 0.1$) (Figure 4(d)). A nonsignificant difference in RBsig distribution was observed between patients with pCR and those with RD (WMW $p = 0.23$) (Figure 4(e), Supplementary Figure 1(b)). The ROC curve AUC for RBsig was 0.57 (95% CI 0.46–0.67) (Figure 4(f)). At univariate analysis, no association was found between RBsig and pCR (RBsig cont $p = 0.296$) (Figure 3(b)).

pCR rates according to the PAM50 classification and RBsig

The PAM50 classifier was used to define the molecular subtypes of the substudy population. Of 244 evaluable patients, 20 were classified as normal-like (normal), 24 as basal-like (basal), 39 as luminal-B (lum-B), 56 as luminal-A (lum-A), and 105 as HER2-e. Lum-A tumours dominated within the HR+ subgroup ($n = 42$), while HER2-e was the most represented subtype in the HR– subgroup ($n = 70$) (Supplementary Table 1). pCR was evaluated according to PAM50 subtype. Overwhelmingly, 47 of the 76 pCRs observed in the unclassified (overall) population occurred in patients with HER2-e tumours; the remainder

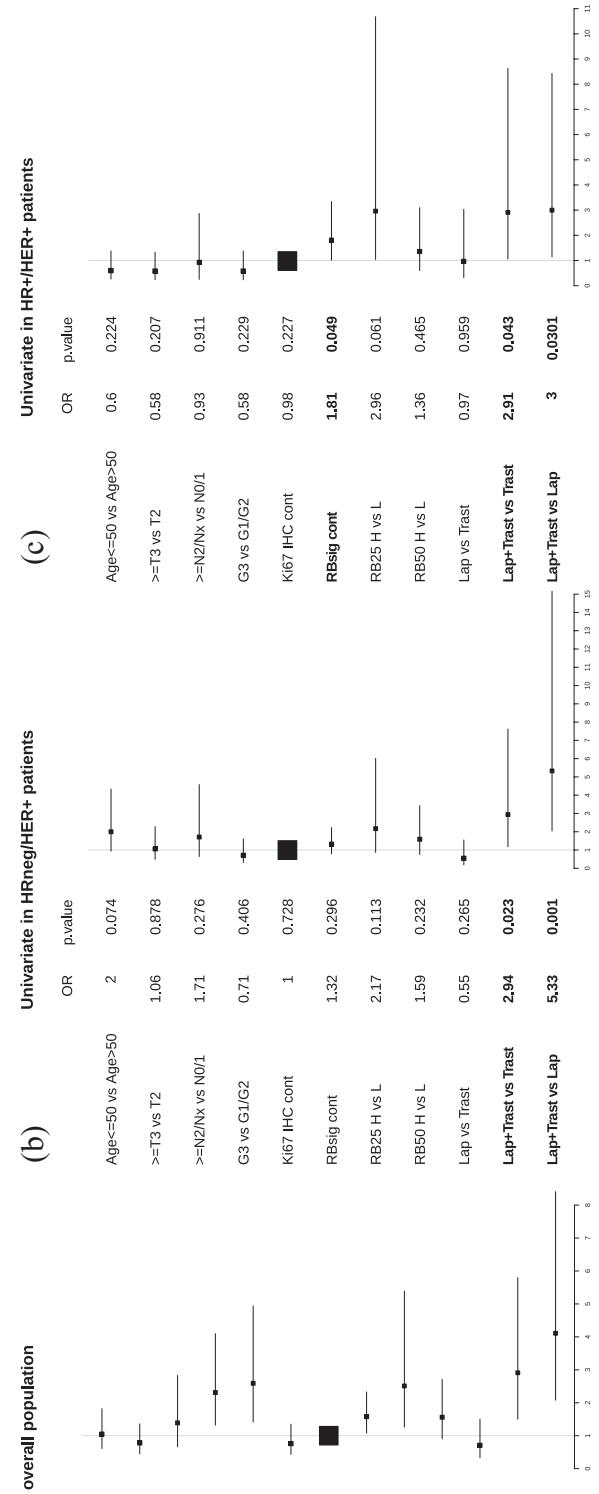

Figure 3. Effect of clinicopathological variables, treatment arm and RBsig on pathological complete response (pCR) in an univariate model in the overall population (a), HR− patients (b) and HR+ patients (c).
T, primary tumour size; N, lymph node status; ER, oestrogen receptor; PR, progesterone receptor; G, tumour grade; Ki67 IHC cont, Ki-67 expression detected by immunohistochemistry and considered as a continuous variable; RBsig cont, RBsig considered as a continuous variable; RB25 H *versus* L, RBsig considered as a categorical variable, with "High (H)" and "Low (L)" expression levels identified according to the 25th percentile cutoff; RB50 H *versus* L, RBsig considered as a categorical variable, with "High (H)" and "Low (L)" expression levels identified according to the 50th percentile cutoff; Lap, lapatinib; Trast, trastuzumab.

were distributed among the other four subtypes (lum-A, $n = 9$; basal, $n = 8$; lum-B, $n = 6$; normal, $n = 5$). (Supplementary Table 1).

Next, the distribution of RBsig expression within each PAM50 subtype was evaluated. As expected, RBsig levels varied significantly between molecular subtypes. Lum-A and normal tumours were mostly RBsig Low (70% and 65%, respectively), while RBsig High tumours dominated within the other subtypes (lum-B = 100%; basal = 96%; HER2-e = 91%) (Figure 5, Supplementary Table 1).

The correlation between RBsig expression and treatment response was evaluated within each subtype; the results were not significant, likely due to the small number of patients for each of the five subtypes and the high prevalence of RBsig High tumours in lum-B, basal and HER2-e. It was, however, noted that HR+/RBsig Low patients obtained lower pCR rates than HR+/RBsig High, regardless of the molecular subtype (Supplementary Table 1).

Correlation between EFS and RBsig

In the overall population ($n = 244$), EFS did not significantly differ between those tumours classified as RBsig Low and High; however, a trend towards significance was observed (hazard ratio = 0.65, 95% CI 0.39–1.08, $p = 0.09$). The 3-year EFS was 69% in RBsig Low ($n = 43$), and 74% in RBsig High patients ($n = 135$) (Figure 6(a)).

The correlation between EFS and RBsig was evaluated according to HR status. There was no significant difference between RBsig Low and RBsig High when EFS was analysed in the HR+ cohort (hazard ratio = 0.82, 95% CI 0.37–1.82, $p = 0.63$) (Figure 6(b)). The 3-year EFS in this cohort was 77% ($n = 27$) in RBsig Low and 75% ($n = 70$) in RBsig High. Differently, in the HR− cohort, RBsig Low patients showed a significantly worse EFS than RBsig High (hazard ratio = 0.50, 95% CI 0.25–0.98, $p = 0.04$). The 3-year EFS was 59% in RBsig Low ($n = 16$), and 74% in RBsig High ($n = 65$) (Figure 6(c)).

Univariate and multivariate regression analyses were performed to explore the association between conventional clinicopathological parameters and EFS. At univariate analysis, RBsig was significantly correlated with EFS in the HR− subgroup

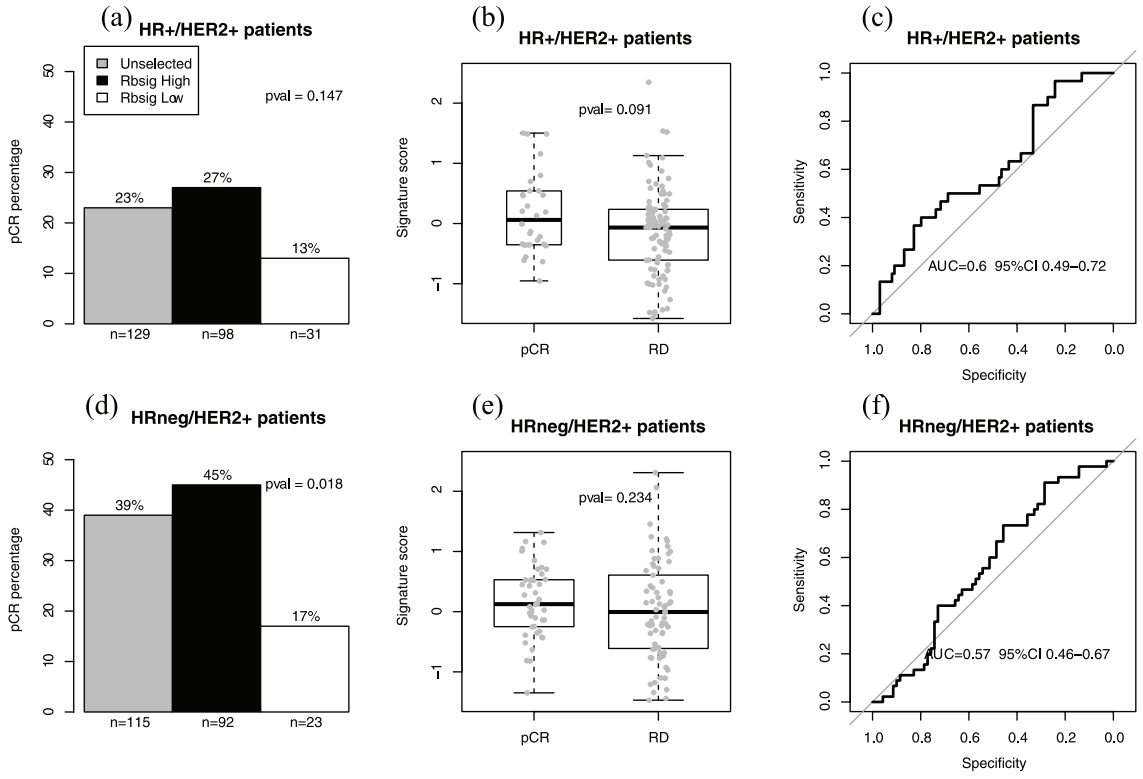

Figure 4. Bar graphs showing the frequency of pathological complete response (pCR) in patients unselected for RBsig expression, RBsig High and RBsig Low, within HR+/HER2+ breast cancer patients (a) and within HR–/HER2+ breast cancer patients (d).
Box plots representing RBsig expression value as a function of pCR *versus* residual disease (RD) and receiver-operating characteristic (ROC) analysis of RBsig within HR+/HER2+ (b,c) and HR–/HER2+ breast cancer patients (e,f).

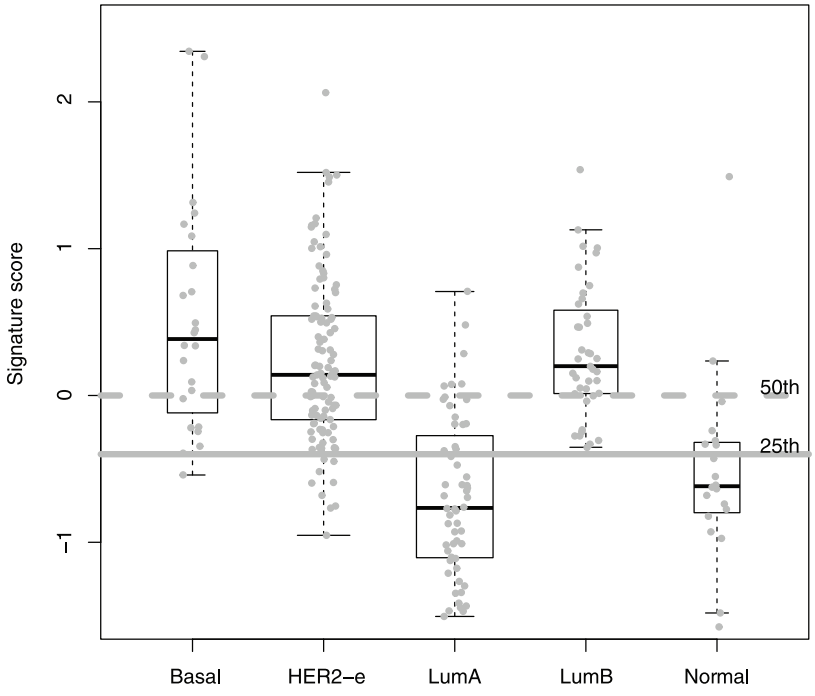

Figure 5. Box plots representing RBsig distribution within PAM50 molecular subtypes in the overall population.
25th, 25th percentile cutoff; 50th, 50th percentile cutoff; Basal, basal subtype; HER2-e, HER2-enriched subtype; LumA, luminal-A subtype; LumB, luminal-B subtype; normal, normal subtype.

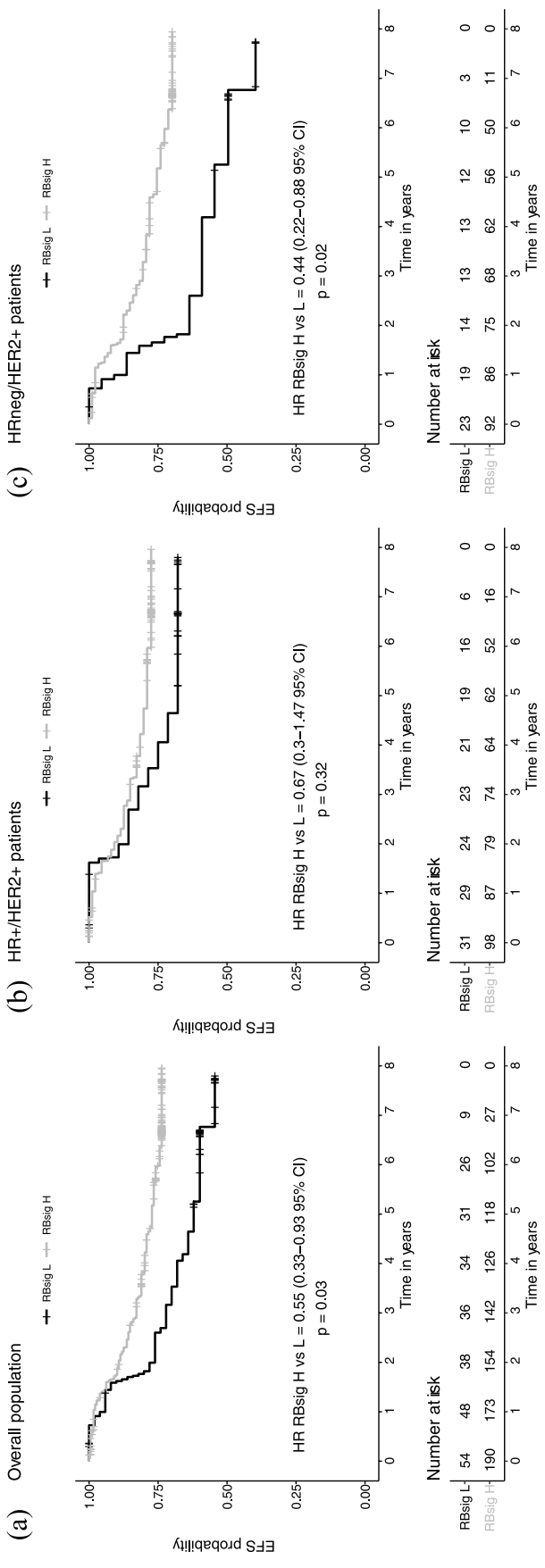

Figure 6. Event-free survival (EFS) in the overall population (a); in the HR+ subgroup (b); and in the HR− subgroup (c).

only (RBsig cont $p = 0.016$; RBsig High/Low 25th percentile cutoff $p = 0.044$) (Supplementary Figure 2). No marker was independently associated with EFS at multivariate analysis adjusted for clinicopathological parameters and treatment arm (not shown).

Discussion

The NeoALTTO trial showed that neoadjuvant CT plus dual HER2 inhibition was superior to single-agent anti-HER2 therapy plus CT. However, approximately 50% of patients treated with dual therapy did not achieve pCR, which subsequently translated into a lower survival benefit. The percentage of patients with RD after CT plus double HER2 blockade further increases to almost 60% if we consider only the HR+/HER2+ patients, a group known to respond less favourably to CT in combination with H.[3,4] Various biomarkers have been unsuccessful in subselecting within HER2+ tumours in order to identify patients who are less likely to respond to CT + H, and who might be suitable for alternative treatments.[19–25] In this study, we present RBsig, a biomarker of potential future interest, that appears to be predictive of response to CT plus H in HER2+ patients enrolled in the NeoALTTO trial. We found that the functional loss of RB1, as expressed by RBsig High levels, was significantly associated with higher response to treatment. This was consistent with prior observations that have shown that RB pathway alterations, most specifically cyclin D1 amplification and CDK4 gains, occur frequently in HER2+ breast cancers.[26] Loss of RB1 function is also associated with increased response to CT.[27–29]

The observed correlation between RBsig expression and response to CT + H in the NeoALTTO population is in line with a previous analysis performed by our group on a meta-dataset of 10 neoadjuvant clinical trials, wherein RBsig was predictive of response to CT with or without H in HR+/HER2+ patients.[31] Similarly, in the current study, we found that only 11% of patients with HR+/HER2+/RBsig Low tumours achieved pCR. This percentage is remarkably low if viewed in the context of the pCR rates generally seen in patients receiving neoadjuvant ET in combination with H for HR+/HER2+ BC. For instance, in the TBCRC023 trial, patients with early breast cancer treated with combined letrozole, trastuzumab and lapatinib for 24 weeks obtained a pCR rate of 33%.[11] In the NA-PHER2 trial, a pCR rate of

27% was achieved in this subgroup following 20 weeks of combined fulvestrant, trastuzumab, pertuzumab and palbociclib.[33] A direct comparison between CT and ET in combination with H is required to more clearly define the optimal regimen in HR+/HER2+ patients.

This substudy also assessed the distribution of RBsig across PAM50 molecular subtypes. In line with previous analyses, RBsig levels varied considerably across molecular subtypes.[30,31] Normal and lum-A tumours were mostly RBsig Low, while RBsig High levels were predominant in HER2-e, lum-B and basal subtypes. HER2-e represented more than 40% of the overall population, and the majority of pCRs obtained belonged to this subtype. Several studies support the use of PAM50 classification to subselect breast cancers, and to refine the use of molecularly targeted treatments. The five molecular subtypes are characterized by different response to CT + H. In the NOAH[18] and CALGB40601[8] trials, pCR following neoadjuvant CT + single or double HER2 blockade was higher among HER2+/HER2-e tumours than any other subtype. Furthermore, data from the PAMELA study showed that the HER2-e subtype was a strong predictor of sensitivity to neoadjuvant HER2 dual inhibition in the absence of CT.[12] In our analysis of NeoALTTO, HR+/RBsig Low tumours were represented only in HER2-e, LumA and normal subtypes. Although based on small numbers, we showed that, within such molecular subtypes, patients with HR+/RBsig Low tumours achieved lower pCR rates than HR+/RBsig High, suggesting that RBsig may have some potential to refine PAM50 subtyping.

Our findings showed that the increased pCR rate obtained in the RBsig High subgroup translated into an EFS advantage only within women with HR– tumours. This is in line with findings of several studies showing that patients who obtain a pCR have improved survival, especially those with aggressive BCs, like HR–/HER2+ subtype, while pCR is not prognostic in HR+/HER2+ patients.[34]

This substudy had various strengths. The analysis was performed within a randomised phase III trial on prospectively collected frozen tissue samples; all tumour samples were centrally analysed, and an accredited technique was utilized to evaluate gene expression. All patients received the same CT backbone. For the purposes of the current analysis, we decided to combine the three NeoALTTO arms together. The resulting heterogeneity in treatment received was justified by the fact that, on the basis of the previous meta-dataset analysis,[31] RBsig was expected to be predictive of response to CT independent of the anti-HER2 regimen received. Limitations of this substudy were that it included analysis of only 244 of the 455 patients originally recruited to NeoALTTO, and the correlations made between RBsig, pCR and EFS were partly influenced by low sample numbers, which may explain the inconsistent results found in the univariate and multivariate analyses. However, despite these shortcomings, we were able to provide additional support to the original hypothesis that RBsig may be predictive of response to CT in combination with H.

Our results indicate that RBsig could add valuable information to HER2 and HR expression, which may in turn inform treatment choices. HR+/HER2+/RBsig Low breast cancers exhibited the poorest pathological response following CT plus H. Accordingly, we hypothesize that in such patients, ET in combination with H and, potentially, a CDK4/6 inhibitor, could be a more effective treatment. The use of CDK4/6 inhibitors in RBsig Low patients is supported by previous preclinical findings reported by our group, showing that RBsig in breast cancer cell lines was predictive of response to the CDK4/6 inhibitor palbociclib.[30] In order to validate these results, we are now undertaking a prospective randomised trial designed to explore the interaction between RBsig status (High or Low) and treatment activity, assessed by pCR, of neoadjuvant palbociclib plus letrozole *versus* paclitaxel when given with trastuzumab plus pertuzumab in elderly women with HR+/HER2+ primary breast cancer.

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Conflict of interest statement

Hilbers Florentine:

research funding: Novartis, Pfizer, Genentech

Huober Jens:
Honoraria: Roche, Novartis
ADBoard: Roche, Novartis
Travel support: Roche, Novartis

Sotiriou Christos: has patents on gene expression and methylation signatures. He has served on advisory boards, and/or speaker at meetings, and/or recipient of travel support for participation in medical meetings for/from (in alphabetical order) Amgen, Astellas, AstraZeneca, Bayer, Celgene, Nanostring Technologies, Novartis, Pfizer, Roche.

Biganzoli Laura:
Grant/research support: Celgene, Genomic Health, Novartis
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Supplemental material

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References

1. Slamon DJ, Godolphin W, Jones LA, *et al.* Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. *Science* 1989; 244: 707–712.
2. Curigliano G, Burstein HJ, P Winer E, *et al.* De-escalating and escalating treatments for early-stage breast cancer: the St. Gallen International Expert Consensus Conference on the Primary Therapy of Early Breast Cancer 2017. *Ann Oncol* 2017; 28: 1700–1712.
3. Baselga J, Bradbury I, Eidtmann H, *et al.* Lapatinib with trastuzumab for HER2-positive early breast cancer (NeoALTTO): a randomised, open-label, multicentre, phase 3 trial. *Lancet* 2012; 379: 633–640.
4. De Azambuja E, Holmes AP, Piccart-Gebhart M, *et al.* Lapatinib with trastuzumab for HER2-positive early breast cancer (NeoALTTO): survival outcomes of a randomised, open-label, multicentre, phase 3 trial and their association with pathological complete response. *Lancet Oncol* 2014; 15: 1137–1146.
5. Gianni L, Eiermann W, Semiglazov V, *et al.* Neoadjuvant chemotherapy with trastuzumab followed by adjuvant trastuzumab versus neoadjuvant chemotherapy alone, in patients with HER2-positive locally advanced breast cancer (the NOAH trial): a randomised controlled superiority trial with a parallel HER2-negative cohort. *Lancet* 2010; 375: 377–834.
6. Untch M, Loibl S, Bischoff J, *et al.* Lapatinib versus trastuzumab in combination with neoadjuvant anthracycline-taxane based chemotherapy (GeparQuinto, GBG 44): a randomised phase 3 trial. *Lancet Oncol* 2012; 13: 135–144.
7. Gianni L, Pienkowski T, Im YH, *et al.* Efficacy and safety of neoadjuvant pertuzumab and trastuzumab in women with locally advanced, inflammatory, or early HER2-positive breast cancer (NeoSphere): a randomised multicentre, open-label, phase 2 trial. *Lancet Oncol* 2011; 13: 25–32.
8. Carey LA, Berry DA, Cirrincione CT, *et al.* Molecular heterogeneity and response to neoadjuvant human epidermal growth factor receptor 2 targeting in CALGB 40601, a randomized phase III trial of paclitaxel plus trastuzumab with or without lapatinib. *J Clin Oncol* 2016; 34: 542–549.
9. Arpino G, Wiechmann L, Osborne C, *et al.* Crosstalk between the estrogen receptor and the HER tyrosine kinase receptor family: molecular mechanism and clinical implications for

endocrine therapy resistance. *Endocr Rev* 2008; 29: 217–233.

10. Rimawi MF, Mayer IA, Forero A, *et al.* Multicenter phase II study of neoadjuvant lapatinib and trastuzumab with hormonal therapy and without chemotherapy in patients with human epidermal growth factor receptor 2-overexpressing breast cancer: TBCRC 006. *J Clin Oncol* 2013; 31: 1726–1731.
11. Rimawi MF, Niravath PA, Wang T, *et al.* TBCRC023: a randomized multicenter phase II neoadjuvant trial of lapatinib plus trastuzumab, with endocrine therapy and without chemotherapy, for 12 vs. 24 weeks in patients with HER2 overexpressing breast cancer. *Cancer Res* 2015; 75: abstract S6-02.
12. Llombart-Cussac A, Cortés J, Paré L, *et al.* HER2-enriched subtype as a predictor of pathological complete response following trastuzumab and lapatinib without chemotherapy in early-stage HER2-positive breast cancer (PAMELA): an open-label, single-group, multicentre, phase 2 trial. *Lancet Oncol* 2017; 18: 545–554.
13. Johnston S, Pippen J Jr, Pivot X, *et al.* Lapatinib combined with letrozole versus letrozole and placebo as first-line therapy for postmenopausal hormone receptor-positive metastatic breast cancer. *J Clin Oncol* 2009; 27: 5538–5546.
14. Kaufman B, Mackey JR, Clemens MR, *et al.* Trastuzumab plus anastrozole versus anastrozole alone for the treatment of postmenopausal women with human epidermal growth factor receptor 2-positive, hormone receptor-positive metastatic breast cancer: results from the randomized phase III TAnDEM study. *J Clin Oncol* 2009; 27: 5529–5537.
15. Marcom PK, Isaacs C, Harris L, *et al.* The combination of letrozole and trastuzumab as first or second-line biological therapy produces durable responses in a subset of HER2 positive and ER positive advanced breast cancers. *Breast Cancer Res Treat* 2007; 102: 43–49.
16. Huober J, Fasching PA, Barsoum M, *et al.* Higher efficacy of letrozole in combination with trastuzumab compared to letrozole monotherapy as first-line treatment in patients with HER2-positive, hormone-receptor-positive metastatic breast cancer: results of the eLEcTRA trial. *Breast* 2012; 21: 27–33.
17. Rimawi M, Ferrero JM, de la Haba-Rodriguez J, *et al.* First-line trastuzumab plus an aromatase inhibitor, with or without pertuzumab, in human epidermal growth factor receptor 2-positive and hormone receptor-positive metastatic or locally advanced breast cancer (PERTAIN): a randomized, open-label phase II trial. *J Clin Oncol*. Epub ahead of print 8 September 2018. DOI: 10.1200/JCO.2017.76.7863.
18. Prat A, Bianchini G, Thomas M, *et al.* Research-based PAM50 subtype predictor identifies higher responses and improved survival outcomes in HER2-positive breast cancer in the NOAH study. *Clin Cancer Res* 2014; 20: 511–521.
19. Azim HA Jr, Rothé F, Aura CM, *et al.* Circulating tumor cells and response to neoadjuvant paclitaxel and HER2-targeted therapy: a sub-study from the NeoALTTO phase III trial. *Breast* 2013; 22: 1060–1065.
20. Majewski IJ, Nuciforo P, Mittempergher L, *et al.* PIK3CA mutations are associated with decreased benefit to neoadjuvant human epidermal growth factor receptor 2-targeted therapies in breast cancer. *J Clin Oncol* 2015; 33: 1334–1339.
21. Salgado R, Denkert C, Campbell C, *et al.* Tumor-infiltrating lymphocytes and associations with pathological complete response and event-free survival in HER2-positive early-stage breast cancer treated with lapatinib and trastuzumab: a secondary analysis of the NeoALTTO trial. *JAMA Oncol* 2015; 1: 448–454.
22. Scaltriti M, Nuciforo P, Bradbury I, *et al.* High HER2 expression correlates with response to the combination of lapatinib and trastuzumab. *Clin Cancer Res* 2015; 21: 569–576.
23. Fumagalli D, Venet D, Ignatiadis M, *et al.* RNA sequencing to predict response to neoadjuvant anti-HER2 therapy: a secondary analysis of the NeoALTTO randomized clinical trial. *JAMA Oncol* 2016; 3: 227–234.
24. Shi W, Jiang T, Nuciforo P, *et al.* Pathway level alterations rather than mutations in single genes predict response to HER2-targeted therapies in the neo-ALTTO trial. *Ann Oncol* 2017; 28: 128–135.
25. Powles RL, Redmond D, Sotiriou C, *et al.* Association of T-cell receptor repertoire use with response to combined trastuzumab–lapatinib treatment of HER2-positive breast cancer: secondary analysis of the NeoALTTO randomized clinical trial. *JAMA Oncol* 2018; 4: e181564.
26. Cancer Genome Atlas Network. Comprehensive molecular portraits of human breast tumours. *Nature* 2012; 490: 61–70.
27. Herschkowitz JI, He X, Fan C, *et al.* The functional loss of the retinoblastoma tumour suppressor is a common event in basal-like and luminal B breast carcinomas. *Breast Cancer Res* 2008; 10: R75.

28. Ertel A, Dean JL, Rui H, *et al*. RB-pathway disruption in breast cancer: differential association with disease subtypes, disease-specific prognosis and therapeutic response. *Cell Cycle* 2010; 9: 4153–4163.

29. Witkiewicz AK, Ertel A, McFalls J, *et al*. RB-pathway disruption is associated with improved response to neoadjuvant chemotherapy in breast cancer. *Clin Cancer Res* 2012; 18: 5110–5122.

30. Malorni L, Piazza S, Ciani Y, *et al*. A gene expression signature of retinoblastoma loss-of-function is a predictive biomarker of resistance to palbociclib in breast cancer cell lines and is prognostic in patients with ER positive early breast cancer. *Oncotarget* 2016; 7: 68012–68022.

31. Risi E, Grilli A, Migliaccio I, *et al*. A gene expression signature of retinoblastoma loss-of-function predicts resistance to neoadjuvant chemotherapy in ER-positive/HER2-positive breast cancer patients. *Breast Cancer Res Treat* 2018; 170: 329–341.

32. Pearce DA, Nirmal AJ, Freeman TC, *et al*. Continuous biomarker assessment by exhaustive survival analysis. *bioRxiv*. Epub ahead of print 25 October 2018. DOI: 10.1101/208660.

33. Gianni L, Bisagni G, Colleoni M, *et al*. Neoadjuvant treatment with trastuzumab and pertuzumab plus palbociclib and fulvestrant in HER2-positive, ER-positive breast cancer (NA-PHER2): an exploratory, open-label, phase 2 study. *Lancet Oncol* 2018; 19: 249–256.

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