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CLINICAL TRIAL

A gene expression signature of Retinoblastoma loss-of-function predicts resistance to neoadjuvant chemotherapy in ER-positive/HER2-positive breast cancer patients

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Abstract

Purpose HER2-positive (HER2+) breast cancers show heterogeneous response to chemotherapy, with the ER-positive (ER+) subgroup deriving less benefit. Loss of retinoblastoma tumor suppressor gene (RB1) function has been suggested as a cardinal feature of breast cancers that are more sensitive to chemotherapy and conversely resistant to CDK4/6 inhibitors. We performed a retrospective analysis exploring RBsig, a gene signature of RB loss, as a potential predictive marker of response to neoadjuvant chemotherapy in ER+/HER2+ breast cancer patients.

Methods We selected clinical trials of neoadjuvant chemotherapy ± anti-HER2 therapy in HER2+ breast cancer patients with available information on gene expression data, hormone receptor status, and pathological complete response (pCR) rates. RBsig expression was computed in silico and correlated with pCR.

Results Ten studies fulfilled the inclusion criteria and were included in the analysis (514 patients). Overall, of 211 ER+/HER2+ breast cancer patients, 49 achieved pCR (23%). The pCR rate following chemotherapy ± anti-HER2 drugs in patients with RBsig low expression was significantly lower compared to patients with RBsig high expression (16% vs. 30%, respectively; Fisher's exact test $p = 0.015$). The area under the ROC curve (AUC) was 0.62 ($p = 0.005$). In the 303 ER-negative (ER−)/HER2+ patients treated with chemotherapy ± anti-HER2 drugs, the pCR rate was 43%. No correlation was found between RBsig expression and pCR rate in this group.

Conclusions Low expression of RBsig identifies a subset of ER+/HER2+ patients with low pCR rates following neoadjuvant chemotherapy ± anti-HER2 therapy. These patients may potentially be spared chemotherapy in favor of anti-HER2, endocrine therapy, and CDK 4/6 inhibitor combinations.

Keywords Gene expression profiling · RB pathway · HER2+ breast cancer · Neoadjuvant chemotherapy · Predictive marker

Abbreviations

A-based	Anthracycline-based chemotherapy
AUC	Area under the curve
BC	Breast cancer
CDK	Cyclin-dependent kinase

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CT	Chemotherapy
ER	Estrogen receptor
ER+	Estrogen receptor positive
ER−	Estrogen receptor negative
ET	Endocrine therapy
GE	Gene expression
H	Anti-HER2 drugs
HER2	Human epidermal growth factor receptor-2
HER2 +	Human epidermal growth factor receptor-2 positive
HR	Hormone receptors
N	Lymph node status
pCR	Pathological complete response
PIK3CA	Phosphoinositide-3-kinase catalytic alpha polypeptide gene
PFS	Progression-free survival
PR	Progesterone receptor
RB1	Retinoblastoma tumor suppressor gene
RBsig	RB1 loss-of-function gene signature
RD	Residual disease
ROC	Receiver-operating characteristic
T	Tumor status
T-based	Taxane-based chemotherapy
T + A-based	Taxane–anthracycline-based chemotherapy or ixabepilone–anthracycline-based chemotherapy
T + A-based + H	Taxane–anthracycline-based chemotherapy plus anti-HER2 drugs

Introduction

The human epidermal growth factor receptor-2 (HER2) is over-expressed and/or amplified in about 20% of all invasive breast cancers (BC) [1]. HER2+ BCs are clinically and biologically heterogeneous [2]. One important element of this heterogeneity dwells in the co-expression of the estrogen receptor (ER), with half of HER2+ tumors also being ER+. Additionally, gene expression profiling of breast tumors by PAM50 has shown that all the intrinsic molecular subtypes (Luminal A, Luminal B, HER2-enriched, and Basal-like) are represented in HER2+ BC, with a different subtype distribution between hormone receptors (HR) negative and positive tumors [3–5]. Notwithstanding this heterogeneity, HER2+ BC patients are generally treated with chemotherapy (CT) and anti-HER2 therapy (H), despite the potential suitability of such patients for alternatives such as endocrine therapy (ET) in combination with biological agents [6]. There is increasing evidence suggesting that ER+/HER2+ and ER−/HER2+ tumors demonstrate characteristically different responses to CT combined with H [7]. Data from several neoadjuvant clinical trials [3, 4, 8–10] show that ER+/HER2+ BC treated with CT plus H achieve lower pCR rates than ER−/HER2+ tumors.

Preclinical data suggest that the ER and HER2 pathways are closely connected by bidirectional crosstalk, and that optimal blockade of both pathways simultaneously may be a superior therapeutic alternative to single agent therapy [11]. Clinical trials have examined the addition of H to ET in patients with early and advanced ER+/HER2+ disease, showing a significant benefit from the combination [5, 12–17]. On the basis of these data, it can be hypothesized that, in a subgroup of patients with ER+/HER2+ tumors, CT could be avoided in favor of less toxic treatments. In this context, the use of predictive markers allowing the identification of the subgroup of patients who will less likely respond to CT is becoming increasingly relevant.

Inactivation of the RB pathway occurs in approximately 20–35% of all BCs, and has been associated with poor disease outcome [18, 19]. The ability of gene expression studies to measure RB deficiency has been previously demonstrated by our group and others. Two gene expression signatures reflecting loss of RB function, RB LOH [20] and Rb loss [21], were shown to have a strong prognostic value across BC subtypes. We have recently developed a gene signature of RB1 loss-of-function (RBsig) including 87 E2F1/E2F2-associated genes.

RBsig was strongly prognostic in ER+ luminal A-like and luminal B-like BC, with patients displaying high RBsig expression showing a poor prognosis independently of the treatment received. We have also shown that RBsig has a potential role in predicting response to the CDK4/6 inhibitor palbociclib in BC cell lines [22], while other signatures of Rb deficiency have been shown to potentially predict response to neoadjuvant CT [20, 21]. However, none of these signatures have, as yet, been extensively studied in the context of ER+/HER2+ tumors.

We hypothesized that ER+/HER2+ tumors displaying high expression levels of RBsig (RBsig High), a condition reflecting loss of RB function, would achieve comparatively higher pCR rates after neoadjuvant CT ± H. Conversely, we hypothesized that tumors with low expression of RBsig (RBsig Low), reflective of intact RB signaling, would show reduced sensitivity to neoadjuvant CT ± H, and might potentially benefit from alternative treatments (e.g., CDK4/6 inhibitors).

Here we report the results of a retrospective in silico analysis of RBsig in ER+/HER2+ tumors aiming to investigate its role as a potential predictive marker of response to neoadjuvant CT, with or without H.

Materials and methods

Neoadjuvant breast cancer studies

A search of PubMed, GEO, and array express was performed to identify clinical trials of neoadjuvant CT with or without H in HER2+ BC patients. This search included studies published up to March 2016. Selection was limited to studies published in peer-reviewed journals with publicly available data on gene expression (GE), HR, and HER2 status derived from pre-treatment primary tumor biopsies, as well as pathological response rates. Neoadjuvant trials of pertuzumab in combination with CT were not included in this analysis, due to the absence of publicly available GE data. In order to be able to combine and homogenize datasets deriving from different microarray platforms, studies that employed Affymetrix platforms were selectively chosen. pCR was defined as the absence of invasive tumor cells in the breast and in the axillary lymph nodes at the time of surgery (ypT0/is ypN0).

The neoadjuvant CT regimens were categorized as: anthracycline-based CT (A-based), taxane-based CT (T-based), taxane–anthracycline-based CT, or ixabepilone–anthracycline-based CT (T + A-based); neoadjuvant anti-HER2 agents trastuzumab and/or lapatinib were analyzed together (H).

Dataset creation and normalization

Expression data from 10 datasets profiled with 3 different Affymetrix platforms (HG-U133 Plus2, HG-U133A, HG-U133A2) were collected, for a total of 514 samples. Distribution of GE data was consistent among the platforms, as shown in supplemental Figs. 1 and 2. Expression data were downloaded as raw CEL files with the GEOquery package (v. 2.40) in R (v. 3.3). The raw intensity signals were extracted from CEL files and normalized using the justRMA function of the affy package (v. 1.52). Fluorescence intensities were background-adjusted and normalized using quantile normalization; log2 expression values were calculated separately for each platform version, using the median polish summarization and custom Brain Array chip definition files for Human Affymetrix arrays based on Entrez genes (HGU-133Plus2_Hs_ENTREZG, HGU133A_Hs_ENTREZG, and HGU133A2_Hs_ENTREZG v. 20). Only common probe sets (n. 12,079) across the 3 platforms were retained for further analyses. Expression data were then corrected for batch effect using the combat function from the sva package (v. 3.22), with the GEO dataset set as batch.

Prediction of response to neoadjuvant chemotherapy

A retrospective in silico analysis was performed to examine correlation between RBsig expression and pCR using several statistical tests. The analysis was performed according to the REporting recommendations for tumor MARKer prognostic

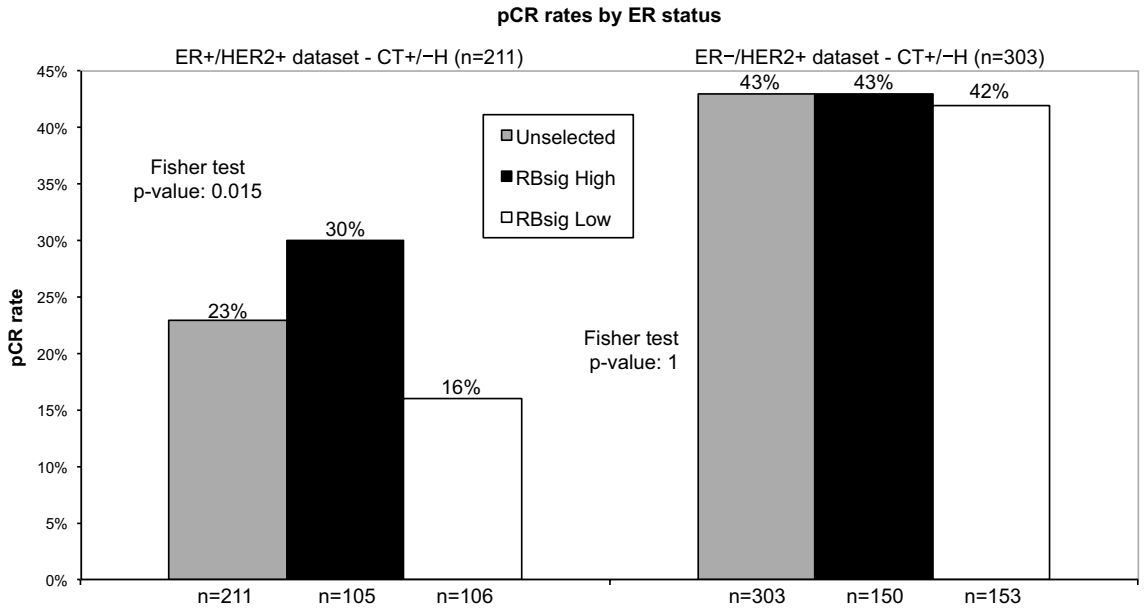

Fig. 1 Bar graphs showing the frequency of pCR in patients unselected for RBsig expression, RBsig high, and RBsig low, within ER+/HER2+ BC patients treated with CT ± H (left) and within ER−/HER2+ BC patients treated with CT ± H (right)

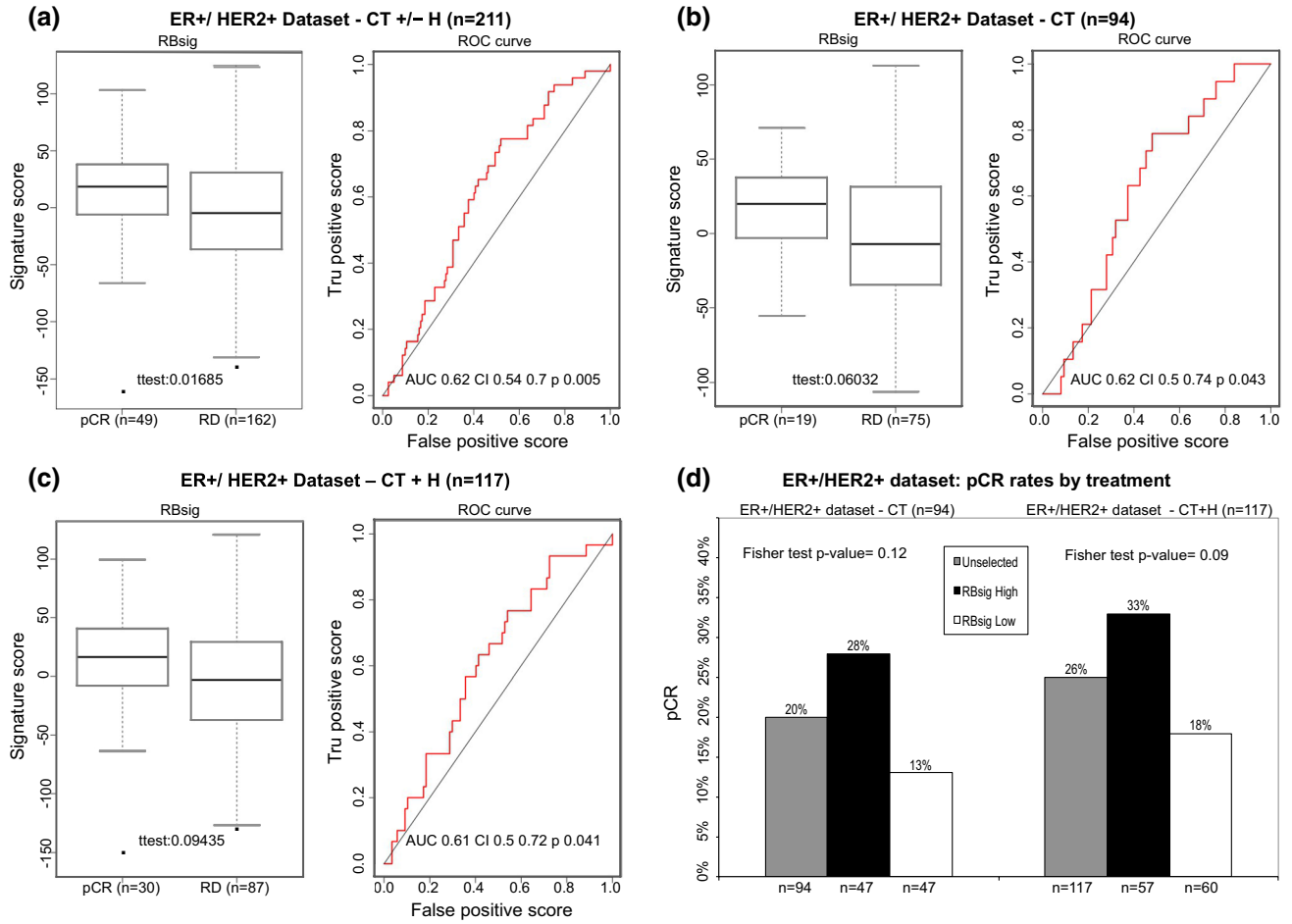

Fig. 2 RBsig is associated with response to neoadjuvant CT in ER+/HER2+ BC patients. Box plots representing RBsig expression value as a function of pCR versus RD (left) and ROC analysis of RBsig (right) in ER+/HER2+ patients treated with CT ± H (**a**), in ER+/HER2+ patients treated with CT (**b**), and in ER+/HER2+ patients treated with CT + H (**c**). Bar graphs showing the frequency of pCR in patients unselected for RBsig expression, RBsig High, and RBsig Low, within ER+/HER2+ BC patients treated with CT (left) and ER+/HER2+ BC patients treated with CT + H (right) (**d**)

studies (REMARK) criteria on reporting of biomarkers [23]. Tumors were categorized as ER+/HER2+ or ER−/HER2+ and analyzed on the basis of treatment received and RBsig expression.

To identify two groups of tumors with either high or low levels of the RBsig expression, a previously described classifier [24] was applied separately for the ER−/HER2+ and ER+/HER2+ datasets. Expression data of the genes included in the RBsig was extracted according to the annotations as described above; this expression data were available for 73 out of 87 RBsig genes. Subsequently, a classification rule was defined based on summarizing the standardized expression levels of the 73 genes into a combined score with zero mean. Additionally, samples were classified as RBsig "Low" if their combined score was negative or RBsig "High" if positive (about 50% of samples for each group). This classification was applied to the log2 expression values on the two metadatasets described above. Welch's *t* test by *t* test function in R was used to evaluate differences in the RBsig score distribution of patients achieving a pCR and those with residual disease (RD) after neoadjuvant CT. Differences in the frequency of pCR events in the High and Low RBsig subgroups were evaluated by Fisher's exact test using the fisher.test function in R. 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 analysis was performed using the ROCR (v. 1.07) and survcomp (v. 1.24) packages in R. The RBsig score value was determined as a function of pCR versus RD, using box plots and 2-sides, 2 sample *t* test. The RBsig score was tested as independent predictor of response by multivariate analysis using the glm function of the stats package in R. Age, tumor (T) and node (N) status, tumor grade, progesterone receptor (PR), and Ki67 expression were added to the model and tested on samples for which above information were available. In this specific analysis,

both RBsig and Ki67 expression were scaled as z-scores (0 mean, 1 sd) to make their ORs comparable. PAM50 subtypes were defined using the genefu (v. 2.6) package in R with the pam50 model. Differences in the RBsig score distribution among PAM50 subtypes were calculated by the anova function in R.

Results

Metadataset, patient characteristics, and pCR rates

Out of 16 identified studies, 10 fulfilled the inclusion criteria and were selected for the analysis [3, 25–33] (Table 1). Of the 514 HER2+ patients included, 211 were ER+ and 303 were ER−. In both subgroups, patients received neoadjuvant chemotherapy alone (CT) or in combination with trastuzumab and/or lapatinib (CT + H) (Table 2). The two groups were well balanced in terms of critical pathological factors, with the exception of PR, which was more frequently and not unexpectedly negative in the ER− cohort. The treatment distribution was also well balanced. In the ER+/HER2+ population, 55% of patients were treated with CT + H versus 45% treated with CT alone, while in the ER−/HER2+ subgroup, 52% of patients received CT + H, and 48% received CT. pCR rates in the two groups were in line with previous data. In the ER +/HER2+ cohort, pCR was 26% with CT + H, and 20% with CT alone. Higher rates of pCR were achieved in the ER−/HER2+ population, reaching 51% with CT + H and 31% with CT alone.

Table 1 Clinical trials included in the analysis; type of treatment received, number of patients for treatment arm, and gene expression omnibus (GEO) accession number are reported

Clinical trials	Type of treatment	*n*	GEO
USO-02103, Shen et al. [25]	T + A-based +H	24	GSE42822
	T + A-based	10	
Korde et al. [26]	T-based	5	GSE18728
Liu et al. [27]	T + A-based +H	47	GSE37946
TRANS-NOAH, Prat et al. [3]	T + A-based +H	63	GSE50948
	T + A-based	51	
CHERLOB, Guarneri et al. [28]	T + A-based +H	23	GSE66305
	T + A-based +H	31	
	T + A-based +H	34	
MDACC trial, Tabchy et al. [29]	T + A-based	16	GSE20271
	A-based	10	
MAQCII, Popovici et al. [30]	T + A-based	59	GSE20194
REMAGUS-02, Valet et al. [31]	T + A-based +H	42	GSE26639
	T + A-based	38	
Miyake et al. [32]	T + A-based	34	GSE32646
NCT00455533, Horak et al. [33]	T + A-based	11	GSE41998
	T + A-based	16	

Table 2 Patient characteristics in ER+/HER2+ and ER−/HER2+ datasets

	ER+/HER2+ ($n = 211$) *n* (%)	ER−/HER2+ ($n = 303$) *n* (%)
Age, y		
Median	51	51
Range	24–79	26–80
Unknown *n* (%)	99 (47)	74 (24)
T		
0	1 (0)	0 (0)
1	3 (1)	5 (2)
2	33 (16)	45 (15)
3	16 (8)	22 (7)
4	10 (5)	19 (6)
Unknown	148 (70)	212 (70)
N		
Negative	26 (12)	17 (6)
Positive	35 (17)	75 (24)
Unknown	150 (71)	211 (70)
Grade		
1/2	64 (30)	75 (25)
3	63 (30)	130 (43)
Unknown	84 (40)	98 (32)
PR status		
Negative	62 (29)	243 (80)
Positive	99 (47)	22 (7)
Unknown	50 (24)	38 (13)
Neoadjuvant therapy		
A-based	2 (1)	8 (3)
T-based	12 (6)	22 (7)
T + A-based	80 (38)	115 (38)
T + A-based +H	117 (55)	158 (52)

Correlation between pCR and RBsig in patients with ER+/HER2+ tumors

Of 211 patients with ER+/HER2+ disease, 49 obtained a pCR following CT with or without trastuzumab and/or lapatinib (CT ± H) (pCR rate = 23%). The classifier identified 106 patients with RBsig Low tumors, and 105 RBsig High. pCR rates were significantly higher in patients with RBsig High tumors compared to those with RBsig Low (30% vs. 16%, respectively; Fisher's exact test $p = 0.015$) (Fig. 1, left).

The RBsig distribution significantly differed between patients who achieved pCR and those with RD (Welch's *t* test $p = 0.01685$, Fig. 2a, left). The ROC curve AUC for the RBsig was 0.62 (95% CI 0.54–0.7 $p = 0.005$, Fig. 2a, right).

Correlation analysis was repeated with patients delineated according to the type of treatment received. The predictive value of RBsig was confirmed in both CT and CT + H subgroups (CT + H AUC = 0.61, 95% CI 0.5–0.72, $p = 0.041$; CT AUC = 0.62, 95% CI 0.5–0.74, $p = 0.043$) (Fig. 2b–d).

Correlation between pCR and RBsig in patients with ER−/HER2+ tumors

Of the 303 patients with ER−/HER2+ disease included in the metadataset, 129 obtained a pCR after CT ± H (pCR rate = 43%). The classifier identified 153 and 150 patients with RBsig Low and High expression, respectively. No difference was observed in pCR rates when tumors were analyzed according to RBsig status (43% vs. 42% in RBsig High vs. RBsig Low tumors, respectively; Fisher's exact test $p = 1$) (Fig. 1, right). Similarly, ROC curve analysis suggested that RBsig was not predictive of response to neoadjuvant treatment in this subpopulation (CT ± H AUC = 0.5, 95% CI 0.43–0.56, $p = 0.973$; CT + H AUC = 0.51, 95% CI 0.42–0.6, $p = 0.821$; CT AUC = 0.5, 95% CI 0.41–0.59, $p = 0.993$) (Fig. 3a–c).

Univariate and multivariate analyses

RBsig expression and the value of conventional clinical, biological, and histological parameters in predicting pCR were examined by univariate and multivariate regression analyses. In patients with ER+/HER2+ tumors, only Ki67-mRNA ($p \leq 0.017$) and RBsig ($p \leq 0.028$) were significantly associated with pCR at univariate analysis, whether they were considered as continuous (Ki67 expression and RBsig score) or categorical (high/low) variables. However, none of these factors were significantly associated with pCR at a multivariate analysis, taking into account age, T and N status, tumor grade, PR status, RBsig, and

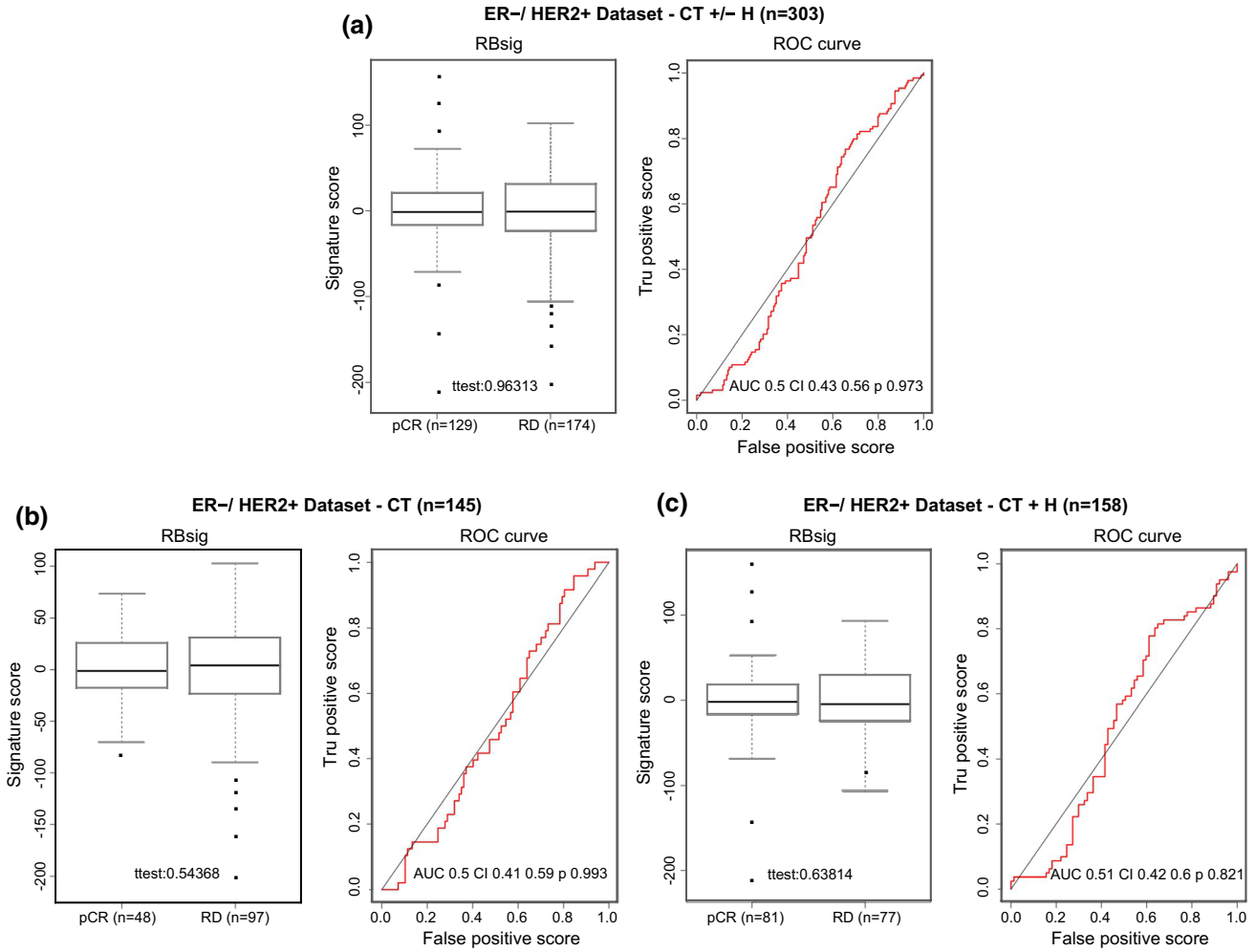

Fig. 3 RBsig is not associated with response to neoadjuvant CT in ER−/HER2+ BC patients. Box plots representing RBsig expression value as a function of pCR versus RD (left) and ROC analysis of RBsig (right) in ER−/HER2+ patients treated with CT ± H (**a**), ER−/HER2+ patients treated with CT (**b**), ER−/HER2+ patients treated with CT + H (**c**)

Ki67-mRNA as continuous variable (analysis performed on 55 samples) (Table 3). A non-significant association with pCR was found when multivariate analysis was performed using only Ki67-mRNA and RBsig variables (analysis performed on 211 samples; $p = 0.224$ and 0.695 for Ki67-mRNA and RBsig, respectively). Similar results in multivariate analysis were obtained when patients were classified by either Ki67 and RBsig as High or Low group (data not shown). In ER−/HER2+ tumors, none of the variables analyzed were significantly associated with pCR, both at univariate and multivariate analyses.

Table 3 Univariate and Multivariate analyses in ER+/HER2+ BC patients

	n	OR	95% CI		*p*-value univariate	*p*-value multivariate
Age						0.407
<= 50	54	1				
> 50	58	0.672	0.273	1.603	0.374	
T						0.297
0/1/2	37	1				
3/4	26	2.840	0.762	13.803	0.145	
N						0.369
0	26	1				
1/2/3	35	1.450	0.400	5.274	0.565	
Grade						0.651
1/2	64	1				
3	63	1.223	0.486	3.134	0.669	
PR						0.588
−	62	1				
+	99	1.591	0.748	3.374	0.225	
Type of treatment						0.597
CT	94	1				
CT + single H	97	0.815	0.407	1.618	0.560	
CT + dual H	20	0.470	0.167	1.398	0.158	
GEO dataset						/
GSE66305	50	1				
GSE18728	4	0.117	0.006	1.004	0.074	
GSE20194	23	2.342	0.659	11.089	0.223	
GSE20271	9	2.811	0.450	54.731	0.351	
GSE26639	45	2.284	0.812	7.083	0.129	
GSE32646	16	1.523	0.408	7.400	0.558	
GSE37946	18	0.439	0.141	1.370	0.151	
GSE41998	8	2.459	0.383	48.264	0.420	
GSE42822	11	0.937	0.230	4.761	0.931	
GSE50948	27	0.834	0.297	2.430	0.733	
Ki67 expression[a]	211	0.644	0.446	0.906	*0.014*	0.182
RBsig score[a]	211	0.680	0.477	0.951	*0.028*	0.968
Ki67 class			1.165	4.428	*0.017*	/
High	105	1				
Low	106	2.238				
RBsig class			1.194	4.540	*0.014*	/
High	105	1				
Low	106	2.295				

Significant *p* values (≤ 0.05) were italised

[a]z-score normalized (0 mean, 1 SD)

Correlation between pCR and Ki67-mRNA expression level

We investigated the role of Ki67 in predicting response to CT ± H, by correlating Ki67-mRNA expression with pCR rate in the ER+/HER2+ and ER−/HER2+ subgroups. Cases were divided into high and low Ki67 based on the median mRNA expression value. In ER+/HER2+ tumors, Ki67-mRNA was significantly associated with pCR (CT ± H *t* test $p = 0.007$), and distinguished between patients who achieved a pCR and those with RD (CT ± H AUC = 0.62, 95% CI 0.54–0.7, $p = 0.005$) (Fig. 4a–c). Conversely, in ER−/HER2+ tumors, Ki67 expression was not associated with pCR rates and showed no predictive value (CT ± H *t* test $p = 0.402$; CT ± H AUC = 0.52, 95% CI 0.45–0.58, $p = 0.589$) (Fig. 4d).

Correlation between pCR and the PAM50 subtype predictor

We tested the association between the PAM50 subtype predictor and pCR within the ER+/HER2+ subpopulation. In contrast to RBsig, PAM50 was not significantly associated with pCR rates (p = 0.155 CT ± H; $p = 0.797$ CT; $p = 0.104$ CT + H). Next, the distribution of RBsig expression within PAM50 subtypes was evaluated. RBsig levels significantly varied within molecular subtypes. The lowest scores were observed in the luminal A and normal-like subtypes, while the highest scores were found in the basal-like, HER2-enriched, and luminal B subtypes (Fig. 5). The ability of RBsig to identify patients achieving pCR within each subtype was tested, but results were significant for the luminal A subtype only (Fig. 6). Interestingly, we found that within the ER+/HER2+ dataset, 51 of the 69 luminal B patients

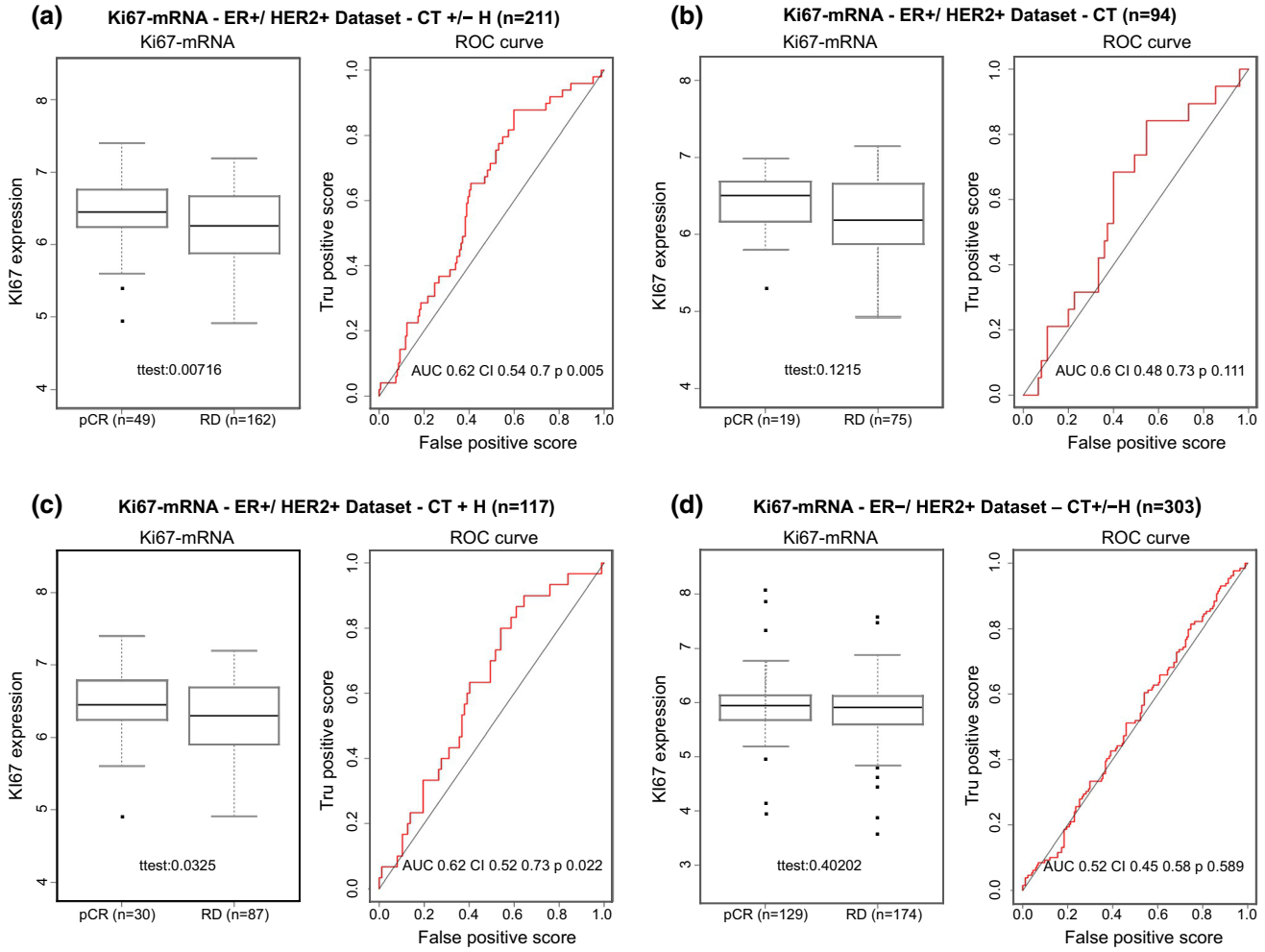

Fig. 4 Ki67 is associated with response to neoadjuvant CT in ER+/HER2+ BC patients. Box plots representing Ki67-mRNA expression value as a function of pCR versus RD (left) and ROC analysis of Ki67-mRNA (right) in ER+/HER2+ patients treated with CT ± H (**a**), ER+/HER2+ patients treated with CT (**b**), ER+/HER2+ patients treated with CT + H (**c**), ER−/HER2+ patients treated with CT ± H (**d**)

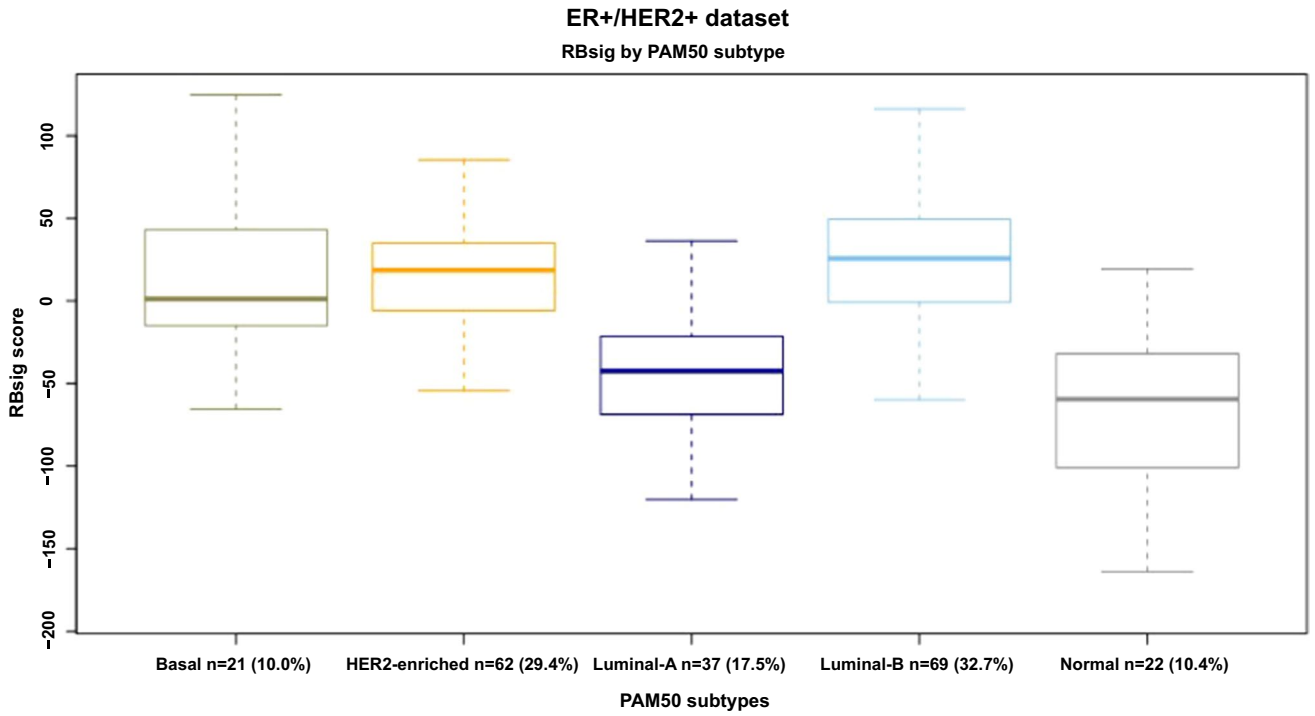

Fig. 5 Box plots representing the distribution of RBsig expression within PAM50 molecular subtypes in the ER+/HER2+ dataset

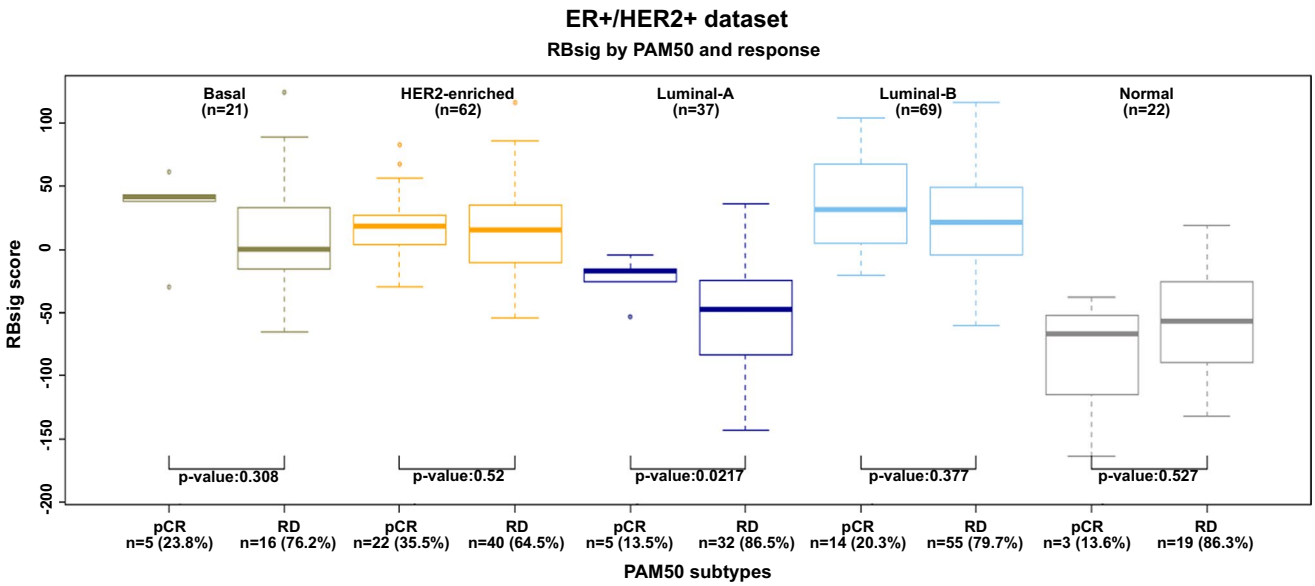

Fig. 6 Box plots representing the distribution of RBsig expression within PAM50 molecular subtypes. RBsig is significantly associated with response in the Luminal A subtype

(74%), were also RBsig High, while 18 of 69 (26%) were RBsig low (Table 4).

Discussion

HER2+ amplification or over-expression classically predicts a more aggressive course in BC, but its prognosis has dramatically improved following the introduction of

Table 4 Number of patients RBsig Low and RBsig High by PAM50 subtypes

	Basal *n* (%)	Her2-enriched *n* (%)	Luminal A *n* (%)	Luminal B *n* (%)	Normal *n* (%)	Total *n* (%)
RBsig low	8 (38%)	23 (37%)	36 (97%)	18 (26%)	21 (95%)	106 (50%)
RBsig high	13 (62%)	39 (63%)	1 (3%)	51 (74%)	1 (5%)	105 (50%)
Total	21	62	37	69	22	211

anti-HER2 agents [34].

In the neoadjuvant setting, anti-HER2 agents are generally used in combination with CT, regardless of HR status. HER2 over-expression or amplification is widely accepted as a biomarker of response to H; however, there are no validated biomarkers to identify patients who will benefit from CT. This is particularly significant for the modest percentage of ER+/HER2+ BCs who do not achieve a pCR following neoadjuvant CT, despite having been exposed to serious and unnecessary side effects [35], that could potentially have been mitigated by replacing chemotherapy with alternative agents.

Loss-of-function of the tumor suppressor RB1 and alterations in the RB pathway have been linked to higher sensitivity to CT in BC [20, 21, 36]. However, data on the predictive role of any signature of RB loss-of-function in ER+/HER2+ patients are lacking. Our results suggest that RBsig is able to select a subset of ER+/HER2+ BCs who are less likely to respond to neoadjuvant CT. Interestingly, the correlation between RBsig and response to CT has been confirmed, regardless of whether RBsig was considered as a categorical (high/low ascertained using the 50th percentile as a threshold) or a continuous variable (RBsig score). This is clearly shown by the ROC curve analysis and the univariate analysis.

The predictive value of the RBsig for pCR was shown to be independent of H, with RBsig being significantly associated with pCR in ER+/HER2+ patients treated with CT alone, as well in those treated with CT in combination with trastuzumab and/or lapatinib. This highlights the possibility that RBsig might predominantly reflect the chemosensitivity of the tumor rather than the relative effect of H. The correlation between loss of RB function and response to CT could be explained by the central role that RB1 plays in cell-cycle control [37]. Indeed, RB-deficient tumor cells are unable to arrest following CT-induced cytotoxic and genotoxic damage which leads to enhanced CT-induced apoptosis and tumor response.

We did not find a significant correlation between RBsig and pCR in ER−/HER2+ patients. A plausible explanation may be attributed to the known greater chemosensitivity of ER−/HER2+ tumors [7]. Using RBsig to predict which tumors are likely to be especially chemosensitive is of little clinical utility in ER−/HER2+ patients, as this population already has a recognized overall susceptibility to cytotoxic treatment. Moreover, RB pathway does not seem to play a relevant role in controlling the cell cycle in ER− tumors [21]. RB loss, the signature described by Ertel et al., was shown to be associated with improved response to multiple CT regimens in both ER+ and ER− tumors [21, 36]. That study included more than 900 patients but of these, only 49 were HER2+, and within that small subset, RB loss was not associated with response independent of ER status. The small number of HER2+ patients tested for RB loss, and the fact that ER+ tumors were not analyzed separately, might explain the discrepancy between those results and the findings of our study.

In our study, the association between pCR, RBsig, and conventional clinical, biological, and histological parameters was examined via univariate analysis. In ER+/HER2+ tumors, a significant association was found only for RBsig and Ki67-mRNA. However, these two factors were shown to be strictly dependent on a multivariate analysis. This is not surprising, as RBsig is constructed based on the selection of genes that correlated with E2F1 and E2F2, two transcription factors that play a crucial role in mediating progression through the G1–S phase of the cell cycle. Succinctly, RBsig includes genes involved in proliferation. Notably, MKI67, the gene that encodes for Ki67, is one of the genes of the RBsig [22]. The association between Ki67 and response to neoadjuvant CT has been previously studied, achieving discordant results. Denkert et al. [38] suggested Ki67 was a significant predictive marker in most ER+ and ER− subtypes, but not in HER2+ disease, whereas Fashing et al. [39] found Ki67 to be an independent predictor for pCR in all patients across all subtypes. Alba et al. [40], showed the predictive value of Ki67 to be especially relevant in ER−/HER2− and ER−/HER2+ patients. Further to this, separate studies by Jones et al. [41] and Tordai et al. [42] demonstrated no association between Ki67 and pCR, in both ER+ and ER− tumors. Our study evaluated whether KI67-mRNA could be a more valuable predictive marker than RBsig. KI67-mRNA, as with RBsig, was predictive of response in ER+/HER2+ patients, but not in ER−/HER2+ patients. We correlated pCR with Ki67 gene expression level (KI67-mRNA), rather than Ki67 determined by IHC, the latter being the standard measurement approach in neoadjuvant trials. This was due to the fact that IHC data for Ki67 were available only for a small number of the selected studies. Limited data directly compare RNA and IHC-based Ki67

measurement in the same BC samples [43]; however, there could be an incomplete concordance between the two parameters due to post-transcriptional mechanisms or intratumoral heterogeneity. Furthermore, data from the PALOMA-2 trial have shown that Ki67 does not seem to predict response to letrozole and the CDK4/6 inhibitor palbociclib in ER + BCs [44]. These data, together with the difficulties in assessing and interpreting Ki67 in a clinical context, indicate that Ki67 should not be considered a fully reliable predictive marker in ER+/HER2+ tumors.

Previous studies [3, 4] have shown that the distribution of the intrinsic subtypes identified by the PAM50 classifier, differs between ER+/HER2+ and ER−/HER2+ tumors. ER− tumors are predominantly classified as HER2-enriched, and are associated with higher pCR rates in response to CT given in combination with trastuzumab and lapatinib [4, 45]. Conversely, luminal subtypes predominate among ER+ tumors and are less responsive to CT plus trastuzumab and lapatinib. Therefore, we aimed to assess the distribution of RBsig across the PAM50 intrinsic subtypes. As expected, and in accordance with previous observations [22], RBsig levels varied considerably across molecular subtypes. Additionally, HER2-enriched and luminal B subtypes could be further subdivided into RBsig High and Low, indicating that RBsig may provide additional, supplementary information to molecular subtypes.

This study showed RBsig to be a compelling predictor of response to CT in ER+/HER2+ BCs. Despite the positive results, this metadataset analysis has some limitations. The studies included in the metadataset are heterogeneous, patients were treated with different CT regimens, and three different platforms were used for GE analysis. Nevertheless, to the best of our knowledge, this is the first time that a genomic signature analyzing the RB pathway has been tested for correlation with pCR rate in HER2+ patients.

Our group has previously shown that RBsig appears to be also predictive of response to the CDK4/6 inhibitor, palbociclib, in BC cell lines [22]. Recently, a growing body of evidence has identified CDK4/6 as potential crucial targets in HER2+ BC [46, 47]. Therefore, it could be hypothesized that RBsig might identify a cohort of ER+/HER2+ tumors with low RBsig status, that are resistant to CT but sensitive to the combination of ET + H + CDK4/6 inhibitors. If so, this subpopulation, consisting of about 25% of all the HER2+ patients, could potentially be spared CT.

In order to validate the results observed in this study, we are now retrospectively testing the predictive value of RBsig in the NeoALTTO trial [48], a completed multicenter, randomized study of neoadjuvant CT in combination with trastuzumab, lapatinib, or both, in HER2+ BC patients. In addition, we are undertaking a prospective randomized neoadjuvant trial designed to explore the interaction between RBsig status and treatment activity. In this trial, ER+/HER2+ early BC patients will be randomized to either chemotherapy or letrozole plus palbociclib, both arms in combination with trastuzumab and pertuzumab. The trial will recruit patients from several European BC centers, in conjunction with the International Breast Cancer Study Group (IBCSG) and Breast International Group (BIG).

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Compliance with ethical standards

Conflicts of interest A Di Leo is a consultant/advisory board member for AstraZeneca, Bayer, Eisai, Genomic Health, Ipsen, Lilly, Novartis, Pfizer, and Pierre Fabre. L. Malorni is a consultant for AstraZeneca and Pfizer. No potential conflicts of interest were disclosed by the other authors.

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