

# Reactive stroma and trastuzumab resistance in HER2-positive early breast cancer

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We investigated the value of reactive stroma as a predictor for trastuzumab resistance in patients with early HER2-positive breast cancer receiving adjuvant therapy. The pathological reactive stroma and the mRNA gene signatures that reflect reactive stroma in 209 HER2-positive breast cancer samples from the FinHer adjuvant trial were evaluated. Levels of stromal gene signatures were determined as a continuous parameter, and pathological reactive stromal findings were defined as stromal predominant breast cancer (SPBC;  $\geq 50\%$  stromal) and correlated with distant disease-free survival. Gene signatures associated with reactive stroma in HER2-positive early breast cancer ( $N = 209$ ) were significantly associated with trastuzumab resistance in estrogen receptor (ER)-negative tumors (hazard ratio [HR] = 1.27  $p$  interaction = 0.014 [DCN], HR = 1.58,  $p$  interaction = 0.027 [PLAU], HR = 1.71,  $p$  interaction = 0.019 [HER2STROMA, novel HER2 stromal signature]), but not in ER-positive tumors (HR = 0.73  $p$  interaction = 0.47 [DCN], HR = 0.71,  $p$  interaction = 0.73 [PLAU], HR = 0.84;  $p$  interaction = 0.36 [HER2STROMA]). Pathological evaluation of HER2-positive/ER-negative tumors suggested an association between SPBC and trastuzumab resistance. Reactive stroma did not correlate with tumor-infiltrating lymphocytes (TILs), and the expected benefit from trastuzumab in patients with high levels of TILs was pronounced only in tumors with low stromal reactivity (SPBC  $< 50\%$ ). In conclusion, reactive stroma in HER2-positive/ER-negative early breast cancer tumors may predict resistance to adjuvant trastuzumab therapy.

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**Additional Supporting Information** may be found in the online version of this article.

**Key words:** HER2, breast cancer, reactive stroma, CAF, TILs

**Abbreviations:** AUC: area under the curve; ECM: extracellular matrix; ER: estrogen receptor; CAF: carcinoma-associated fibroblast; CI: confidence interval; DDFS: distant disease-free survival; EMT: epithelial-to-mesenchymal transition; H&E: hematoxylin and eosin; pCR: pathological complete response; SPBC: stromal predominant breast cancer; TGF- $\beta$ 1: transforming growth factor; TILs: tumor-infiltrating lymphocytes

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**What's new?**

Tumor cells can interact with the surrounding microenvironment, to alter gene expression, suppress immune function, and enhance their own growth. While trastuzumab has dramatically improved clinical outcomes for patients with HER2-positive breast cancer, not all tumors respond to treatment. In this study, the authors found that one mechanism of trastuzumab resistance may be due to altered gene expression representing “reactive” stromal cells surrounding the tumor, even when high levels of tumor-infiltrating lymphocytes (TILs) are present. These “reactive” genetic signatures may provide valuable biomarkers for predicting treatment response.

**Introduction**

Cancer cells are surrounded by a tumor microenvironment, which is composed of the extracellular matrix (ECM) and various cell types, such as fibroblasts, endothelial cells, (myo) fibroblasts and leukocytes. There is growing evidence to show that interaction of stromal cells with tumor cells is pivotal in breast cancer progression and response to therapy. Several studies have provided insight on the molecular characteristics differentiating tumor-associated stroma from normal stroma.<sup>1–5</sup> It has also been suggested that tumor-associated stroma contribute to cancer growth and progression by promoting stromal–epithelial paracrine signaling.<sup>6</sup> A few stromal signatures have been developed and were found to be prognostic, especially in the HER2 breast cancer subgroup.<sup>5,7–9</sup> Four carcinoma-associated fibroblast (CAF) subsets that accumulated differently in breast cancer subtypes were recently identified, of which the CAF-S1 subset was associated with an immunosuppressive microenvironment.<sup>10</sup>

Trastuzumab, a monoclonal antibody targeted against HER2, has markedly improved clinical outcomes for patients with HER2-positive disease.<sup>11</sup> However, despite significant research efforts, there are currently no clinically useful biomarkers that can identify which of the patients are resistant to trastuzumab. The development of predictive biomarkers becomes an increasingly important issue in an era of a growing array of effective anti-HER2 agents available for clinical use and which add both to cost and toxicity.<sup>12–14</sup>

Given the increasing evidence of the significant contribution of tumor-associated stroma to trastuzumab efficacy,<sup>15</sup> we decided to evaluate biomarkers capable of identifying the patients who are resistant to trastuzumab in the context of a large Phase III adjuvant clinical trial in which patients were randomly assigned to anti-HER2 treatments.

**Materials and Methods**

The original FinHer trial was reported in depth elsewhere.<sup>16</sup> The clinicopathological characteristics of the original cohort and the cohort assessed for reactive stroma are given in Table 1. The Reporting Recommendations for Tumor Marker Prognostic Studies criteria were followed for reporting our study.

**Patients**

Our study used samples and data from the FinHer trial (the trial identifier is ISRCTN76560285), a multicenter, Phase 3, randomized breast cancer trial in an adjuvant setting that enrolled 1,010 patients (CONSORT diagram; Fig. 1).<sup>16</sup> A total of 232 (23.0%) of those patients had HER2-positive cancer. One patient with HER2-positive cancer who had overt distant metastases at the time of randomization was excluded from the analyses,<sup>17</sup> and 209 (90.5%) of the remaining 231 HER2-positive tumors had tissue slides that were available for the current study. The clinicopathological characteristics of the patients with HER2-positive cancer who also had available reactive stroma data ( $n = 209$ ) were compared to the HER2-positive subgroup of the FinHer series ( $n = 231$ ). The patients with reactive stroma evaluation findings were representative of the entire population, with no substantial differences in patient and tumor characteristics having been identified between the two groups (Table 1). Study participants provided written informed consent to allow further research analyses to be carried out on their tumor tissue. Profiling of the breast tumor samples was approved by the institutional review board (permission HUS 177/13/03/02/2011). The primary endpoint of the FinHer study, distant disease-free survival (DDFS), was reported to be superior for the trastuzumab-containing arms after a follow-up of 62 months.<sup>17</sup>

**Reactive stroma and tumor-infiltrating lymphocyte pathologic assessment**

Evaluation of reactive stroma was performed on full-face hematoxylin and eosin (H&E)-stained sections. The full stromal area of the tumor was taken as 100% (excluding the tumor cell nests), and the percentage of non-normal reactive stroma of the whole area was estimated. Reactive stroma was defined as scar-like desmoplastic tissue containing a higher proportion of reactive myofibroblasts compared to the normal stroma of the breast, which does not contain any reactive myofibroblasts. Two pathologists (R.S. and G.V.d.E.) performed the readings and reached a consensus. Tumor-infiltrating lymphocytes (TILs) were assessed as described by Loi *et al.*<sup>18</sup>

**Statistical analysis**

The primary predefined hypothesis was that higher levels of reactive stroma would be associated with trastuzumab

**Table 1.** Differences between patients with and without stromal evaluation

		All (N = 231)		Stromal evaluation				p Value
				No (N = 22)		Yes (N = 209)		
Age (in years)	<50	108	47%	10	45%	98	47%	0.90
	≥50	123	53%	12	55%	111	53%	
Tumor size (in mm)	≤20	81	35%	4	19%	77	37%	0.10
	21–50	135	59%	15	71%	120	57%	
	>50	14	6%	2	10%	12	6%	
	Missing	1		1				
Positive nodes	0	37	16%	5	23%	32	15%	0.63
	1–3	121	52%	10	45%	111	53%	
	≥4	73	32%	7	32%	66	32%	
Grade	1	5	2%	-	-	5	2%	0.77
	2	72	32%	8	38%	64	31%	
	3	148	66%	13	62%	135	66%	
	Missing	6		1		5		
ER status	Negative	122	53%	13	59%	109	52%	0.65
	Positive	109	47%	9	41%	100	48%	
Histology	Ductal	208	90%	21	95%	187	89%	0.71
	Lobular	21	9%	1	5%	20	10%	
	Other	2	1%	-	-	2	1%	

resistance. For the survival analyses, the primary endpoint was DDFS, which was defined by the time interval between the date of randomization and the date of first cancer recurrence outside of the ipsilateral locoregional region or to death, whenever death occurred before distant recurrence. Associations between reactive stroma and clinicopathological characteristics were investigated. A two-sided *p* value less than 0.05 was considered significant. No adjustment for multiple testing was made. Five stromal gene signature scores (PLAU, DCN, Yoshiara, CAFS1 and CD10)<sup>5,7–10,19</sup> (Supporting Information Table S1) were calculated for each patient as previously described.<sup>20</sup> PLAU, which was associated with prognosis in HER2-positive breast cancer subgroup<sup>8</sup>; DCN, defined as a list of genes correlated to decorin and was able to predict chemoresistance in patients treated with preoperative chemotherapy<sup>7</sup>; Yoshiara stromal signature which represents the fraction of “normal” stromal cells in tumor tissue<sup>19</sup>; CAFS1 defined by Costa *et al.*, via the concomitant analysis of six fibroblast markers<sup>10</sup>; and CD10+ stroma signature which is able to predict chemoresistance in the HER2-positive subpopulation.<sup>9</sup> We computed the sum of the products of the gene coefficient (−1 or 1, depending upon downregulation or upregulation, respectively) by the corresponding *z*-normalized gene expression value. Patients were divided into two groups, those with a high and those with a low stromal gene signature score, using the median as the dichotomous cutoff.<sup>20</sup> Multivariate Cox proportional hazards regression models were then applied to each group separately to test the association between trastuzumab treatment and patient survival. A possible interaction between survival with trastuzumab treatment was tested

using a Wald test after adding the gene score variable together with trastuzumab as main effects and a product interaction term in the Cox model. The following clinicopathological characteristics were included as covariates in all multivariate analyses: tumor size (T1 [≤2 cm] vs. T2 [≥2 and ≤5 cm] and T3 [≥5 cm]), histological grade (1, 2, vs. 3) and age (≤50 vs. >50 years). A total of 202 out of 209 patients for whom there was a pathology stroma value had expression arrays, and 195 of them had the grade, tumor size and age values required in the multivariate model. Kaplan-Meier survival curves were produced for visualization purposes, and they defined the groups as being high- and low-reactive stroma according to a predefined variable, that is, stromal predominant breast cancer (SPBC; ≥50% stromal). Interaction effects were displayed using forest plots. Stromal TILs were evaluated as a continuous variable (per increasing 10% increments). Associations between TILs and SPBC were investigated with Spearman’s rank correlation. To detect whether the benefit from trastuzumab in patients with high levels of TILs is dependent upon reactive stroma, the interaction between TILs and trastuzumab treatment was evaluated separately for patients with low-reactive stroma and those with high-reactive stroma (SPBC). The analyses were performed using the survival and forest-plot R software packages and SPSS (Chicago, IL).

#### Development of the HER2-reactive stromal signature

To develop a gene signature corresponding to reactive stroma in the FinHer HER2-positive cohort (HER2STROMA—Supporting Information Table S1), we used a method called

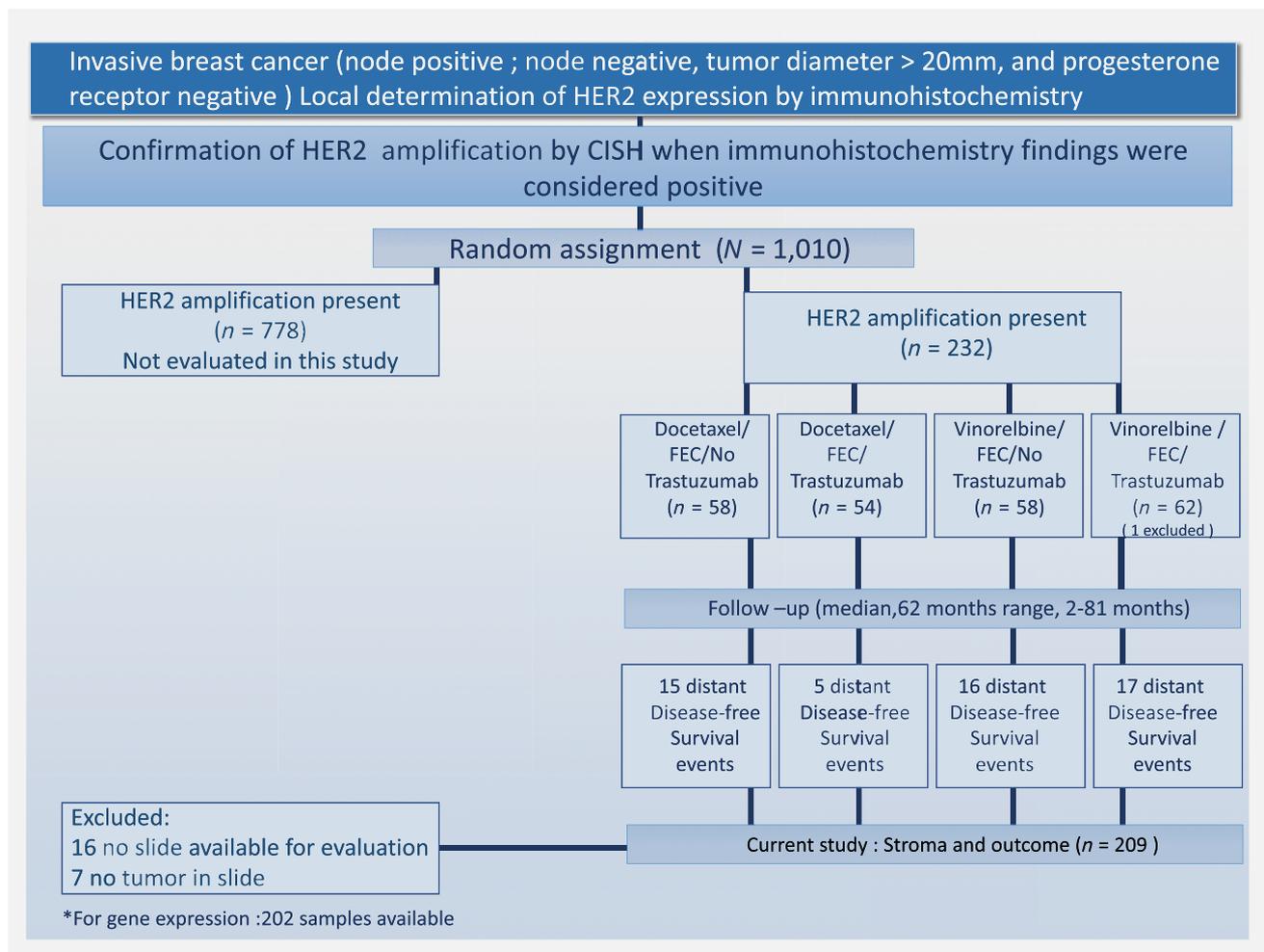


Figure 1. CONSORT diagram. [Color figure can be viewed at wileyonlinelibrary.com]

ProGENI,<sup>21</sup> implemented as part of the KnowEnG analytical platform ([www.knoweng.org](http://www.knoweng.org)).<sup>22</sup> ProGENI is a gene prioritization method that combines information on “omics” profiles of samples with a network of gene–gene interactions to improve the accuracy of prioritization. For this task, we used the *z*-normalized gene expression values and the pathological reactive stroma scores in the “Feature prioritization” pipeline of KnowEnG. For the gene interaction network, we used the protein–protein interaction from REACTOME,<sup>23</sup> which is readily available as an option in KnowEnG (“Reactome PPI reaction partners”). We selected the “Number of response-correlated features” as 10, and did not use any bootstrap sampling. The other parameters were kept as default. Out of the top 10, 20, 50, 70, and 100 ranked by ProGENI genes, the top 70 were best associated with reactive stroma and used as the gene signature. To obtain a polygenic score for each sample, we calculated a weighted average of the *z*-normalized expression of the 70 genes for each sample, in which the weights were equal to the Pearson correlation of the expression of that gene and the reactive

stroma score across all samples. The pROC R package<sup>20</sup> was applied for the receiver operating characteristic (ROC) analysis and for deriving the area under the curve (AUC) using the polygenic score as predictor, and the previously reported “Responsify” reactive stroma scoring level coded as 1 for low level and 3 for high level (medium levels were not included).

#### Gene expression analysis

Gene mRNA expression data were produced from 202 of the FinHer HER2 samples as described at <http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=wfermmkijzktzcb&acc=GSE65095>.<sup>20,24</sup> Before gene expression was done, all samples were reevaluated by pathologist to ensure tumor was present in the tissue sample. We computed the average value when multiple probe sets were mapped to the same official gene symbol. Linear modeling with the Limma R package<sup>25</sup> was used to detect genes responding to reactive stroma in ER-positive and ER-negative patients ( $p < 0.005$ ). Those genes were subjected to Ingenuity

Pathway Analysis (IPA<sup>®</sup>) software to detect functional gene networks associated with reactive stroma.

To calculate the distribution of stromal gene signature across breast cancer subtypes, we used the downloaded TCGA gene expression data of 514 breast cancer patients together with their subtype information as detected by the PAM50 test (98 basal like, 58 HER2, 127 Luminal B, and 231 Luminal A). Stromal metagene scores were calculated for each patient as described above, and the distribution of those scores across different breast cancer subtypes was visualized using boxplots.

### Correlation between differentiation states and reactive stroma

Gene sets that represent luminal, luminal progenitors, basal (mammary stem cell-enriched), and epithelial-to-mesenchymal transition (EMT) were obtained from the literature.<sup>26–29</sup> The metagene scores of these signatures were calculated for each patient using the same method described above. The Pearson correlation between these signatures and the stromal reactivity score was performed using the `cor.test` R function.

### Data availability

Gene mRNA expression data are available at <http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=wfermmkijzktcb&acc=GSE65095>.

### Results

The gene expression analysis from the TCGA data<sup>30</sup> revealed that reactive stromal gene expression signatures were associated more with the Luminal A cancer subtype than with the Luminal B breast cancer subtype (Supporting Information Fig. S1). This is in agreement with Dennison *et al.*'s study which showed that high intratumoral stromal content defines reactive breast cancer as a low-risk subtype.<sup>31</sup> Our analysis also revealed that other subtypes, such as HER2 positive, have differential reactive stromal expression (Supporting Information Fig. S1). Given that preclinical data suggested that reactive stroma may decrease trastuzumab efficacy,<sup>15</sup> we investigated this hypothesis in HER2-positive breast cancer.

### FinHer baseline patient characteristics

There were no significant differences in the characteristics of the patients of the stromal evaluation series ( $n = 209$ ) compared to the original series (Table 1). Associations between reactive stroma and other clinicopathological characteristics showed that reactive stroma was not significantly associated with any of the investigated characteristics (Supporting Information Table S2).

### Association between reactive stroma and trastuzumab resistance

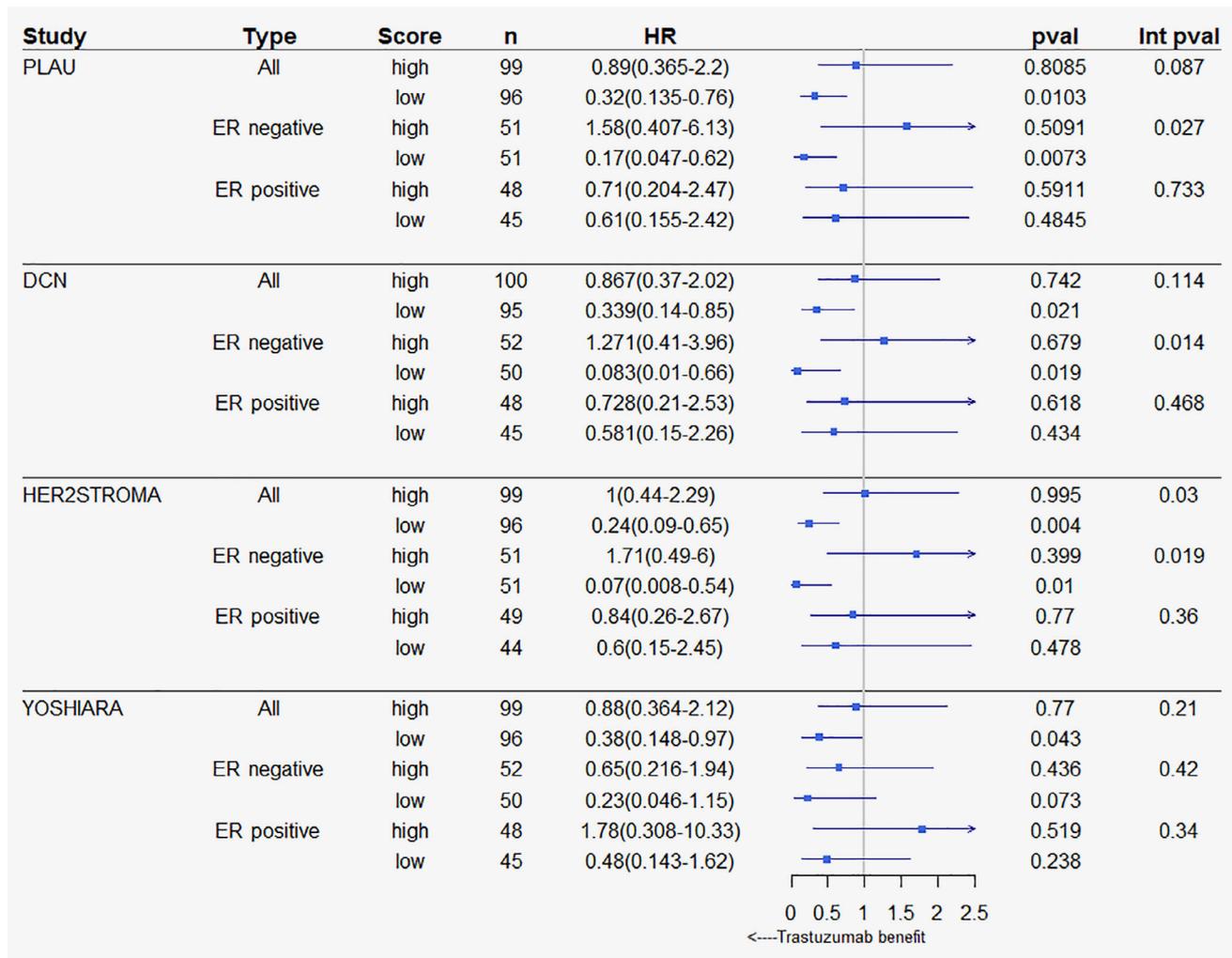
We evaluated the benefit of trastuzumab therapy according to the stromal signature score in the FinHer HER2-positive population. There was a statistically significant interaction between the

reactive stroma signature scores and trastuzumab resistance for two stromal reactive signatures (DCN and PLAU) in the ER-negative tumors (HR = 1.27  $p$  interaction = 0.014 [DCN], HR = 1.58,  $p$  interaction = 0.027 [PLAU]), but not in the ER-positive tumors (HR = 0.73  $p$  interaction = 0.47 [DCN]; HR = 0.71,  $p$  interaction = 0.73 [PLAU]) (Fig. 2). The YOSHIARA signature<sup>19</sup> that represents normal stromal volume in the tumor specimen was not associated with trastuzumab resistance (Fig. 2). We then evaluated two signatures that represent specific stromal CAF subtypes (CAF-S1 and CD10).<sup>9,10</sup> Both the CD10 signature and the CAF-S1 signature (which represent a subset of cells which are associated with immunosuppression) were nonsignificantly associated with trastuzumab resistance (Supporting Information Fig. S2).

### Reactive stroma signatures were associated with the stromal pathological score, TGF- $\beta$ 1 pathway activation and EMT

We next evaluated reactive stroma at the histological level on full-face H&E-stained sections from the FinHer samples by two independent pathologists who reached scoring consensus (Methods section). The mean pathological stromal reactivity level was 48% (SD 24.3%; range 5–90%). Stromal genomic signatures scores were positively but weakly correlated with pathological stromal reactivity (DCN,  $r = 0.3$ ;  $p = 2.287 \times 10^{-5}$ ; PLAU,  $r = 0.28$ ,  $p = 8.75 \times 10^{-5}$ ) (Fig. 3a). The pathological evaluation of HER2-positive/ER-negative tumors suggested that SPBC was associated with the lack of any benefit from trastuzumab (in contrast to non-SPBC) (ER negative/SPBC <50%: HR = 0.257, confidence interval [CI]: 0.066–0.996; ER-negative/SPBC  $\geq$ 50%: HR = 0.66, CI: 0.22–1.93) (Fig. 4). These results suggest that pathological evaluation of reactive stroma in HER2-positive/ER-negative early breast cancer tumors may predict resistance to adjuvant trastuzumab therapy.

In order to develop a gene signature corresponding to reactive stroma specifically in the FinHer HER2-positive population taken from the FinHer study (HER2STROMA signature), we used protein–protein interaction knowledge (Supporting Information Fig. S3A) together with expression data to rank genes with respect to their relationship with pathological reactive stroma (see Methods). The top 70 genes (Supporting Information Table S1) were selected, and the correlation of their polygenic score with reactive stroma was 0.41 ( $p$  value =  $3.364 \times 10^{-9}$ ). To validate the association of the HER2STROMA signature with pathological reactive stroma, we calculated the signatures' predictive ability in an independent data set which had been previously evaluated for reactive stroma (Responsify).<sup>20</sup> The AUC resulting from the use of the HER2STROMA signature score as predictor to reactive stroma outcome was 0.78 (Supporting Information Fig. S3B), which was higher than the AUC from using DCN (0.68), PLAU (0.71), and YOSHIARA (0.62) (Supporting Information Fig. S3B). Ten out of the 70 genes in the HER2STROMA signature were shared by DCN and/or PLAU signatures (Supporting



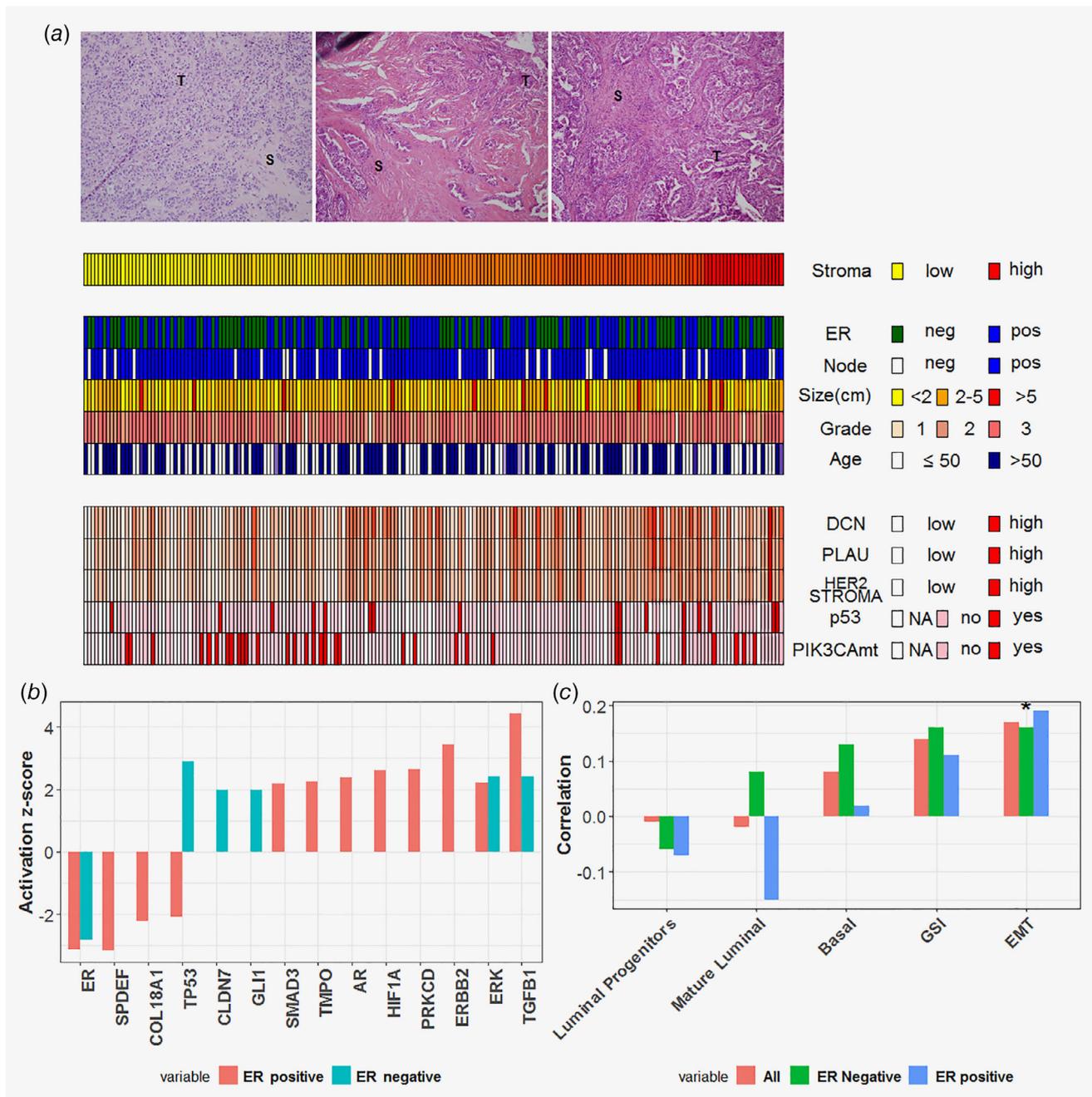
**Figure 2.** Forest plots according to different reactive stroma signature statuses in all patients, in ER-negative only patients, or in estrogen receptor (ER)-positive only patients. The plots indicate Cox regression hazard ratios, 95% confidence intervals, and *p* values for trastuzumab benefit for distant disease-free survival (DDFS), as well as *p* values of the interaction (Int *p*val) between reactive stromal signatures and trastuzumab treatment. [Color figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

Information Fig. S3C and Table S1), and the main biological process that had been enriched in the HER2STROMA gene signature (by gene ontology [GO] analysis) was organization of the ECM (false discovery rate =  $2.54 \times 10^{-12}$ ). In the Responsify data set, HER2STROMA gene signature positively correlated with CD29 protein (a potential marker of reactive stroma) in ER-negative tumors but not in ER-positive tumors (Supporting Information Fig. S4). Caveolin1 (CAV1) and PDGFR $\beta$  (other potential markers of reactive stroma) were not correlated with HER2STROMA gene signature. There was a statistically significant interaction between the HER2STROMA signature and trastuzumab resistance in the ER-negative tumors (HR = 1.71; *p* interaction = 0.019), but not in the ER-positive tumors (HR = 0.84; *p* interaction = 0.36) (Fig. 2). In an effort to show that reactive stroma assessment can be performed on breast cancer biopsies in the neoadjuvant setting, we analyzed gene

expression data from neoadjuvant studies<sup>32</sup> and show that HER2STROMA signature has trend toward worse outcome in HER2-positive patients (odds ratio = 0.30; 0.09–1.01 CI, *p* = 0.052).

Next, we used IPA<sup>®</sup> software to uncover pathways associated with reactive stromal tumors. A few pathways were identified as being significantly associated with reactive stroma (Figs. 3b Z score  $\geq 2$ ). The TGF- $\beta$ 1 pathway was found to be upregulated in both stromal reactive ER-positive and ER-negative tumors.

We further assessed whether HER2 tumors with reactive stroma are associated with distinguishable differentiation states. We analyzed the correlation of their transcription profiles with previously established signatures that represent luminal, luminal progenitors, basal (mammary stem cell enriched), and EMT<sup>26–29</sup> (Fig. 3c). The expression profile of



**Figure 3.** Correlation of stromal genomic signature scores with the evaluation of pathological reactive stroma. (a). Histological sections showing breast tumors containing low (right), intermediate, and high (left) reactive stroma. Heatmap showing the association of reactive stromal content with clinical pathological parameters and correlation with different gene signatures and mutations. (b). Ingenuity Pathway Analysis (IPA<sup>®</sup>) of pathways associated with stromal reactivation. A Z score  $\geq 2$  is considered significant. (c). Correlation of transcription profiles of stromal tumors with established signatures which represent luminal, luminal progenitor, basal (mammary stem cell enriched), and epithelial-to-mesenchymal transition (EMT). \* $p \leq 0.05$ .

the reactive stromal tumors showed a significant but low correlation with EMT, suggesting that the TGF- $\beta$ 1 pathway and EMT may be associated with trastuzumab resistance in tumors with reactive stroma.

### Stromal reactivation and TILs

Given that TILs are associated with benefit from trastuzumab,<sup>18</sup> we tested whether reactive stroma is positively or negatively correlated with TILs. Pathological evaluations of reactive

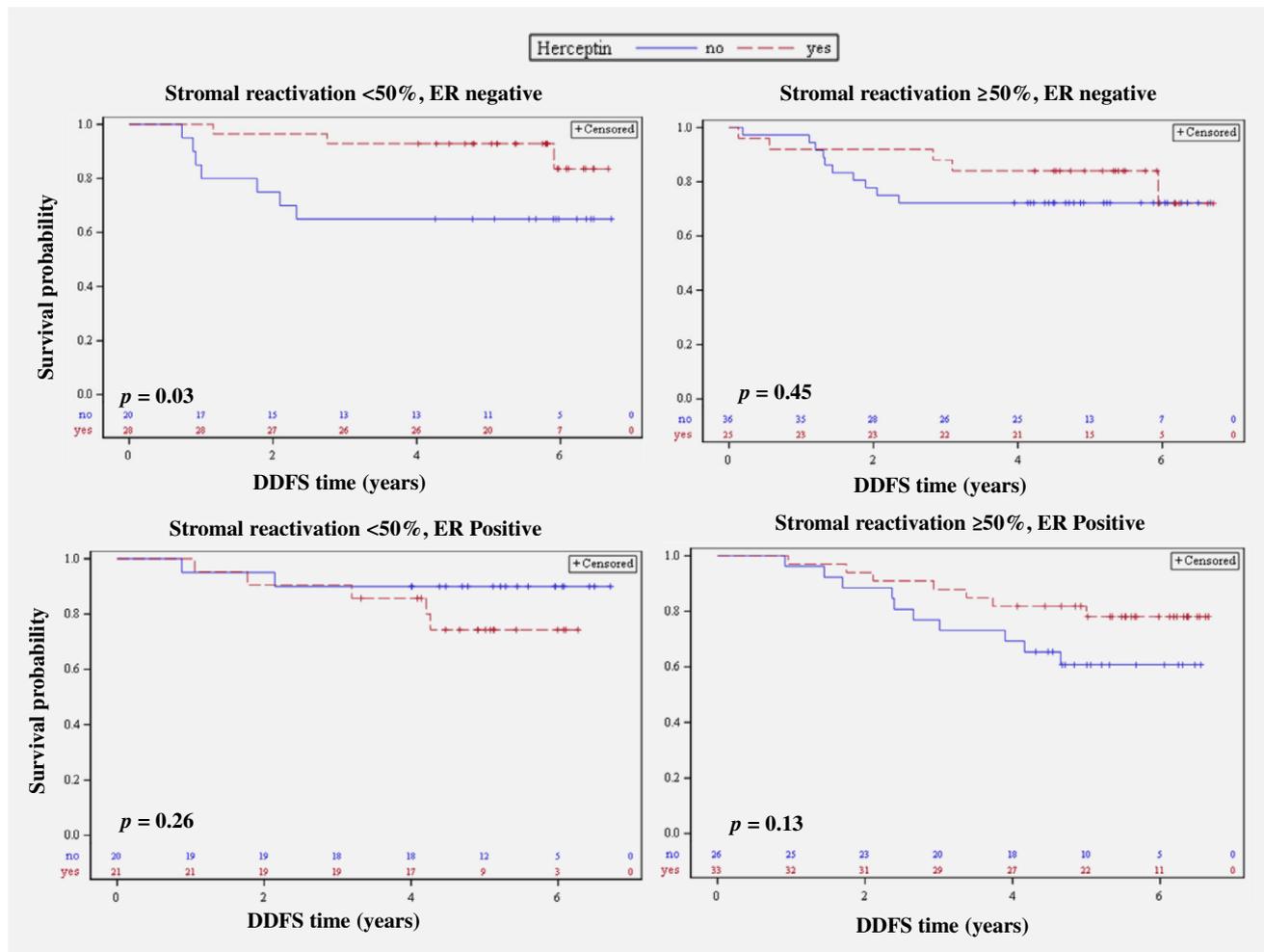


Figure 4. Kaplan-Meier curves of stromal predominant breast cancer (SPBC) status assessed in the FinHer data set. Patients with a low SPBC level (<50%) and a high SPBC level (≥50%) according to trastuzumab treatment in the estrogen receptor (ER)-negative group (up) or in the ER-positive group (down). [Color figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

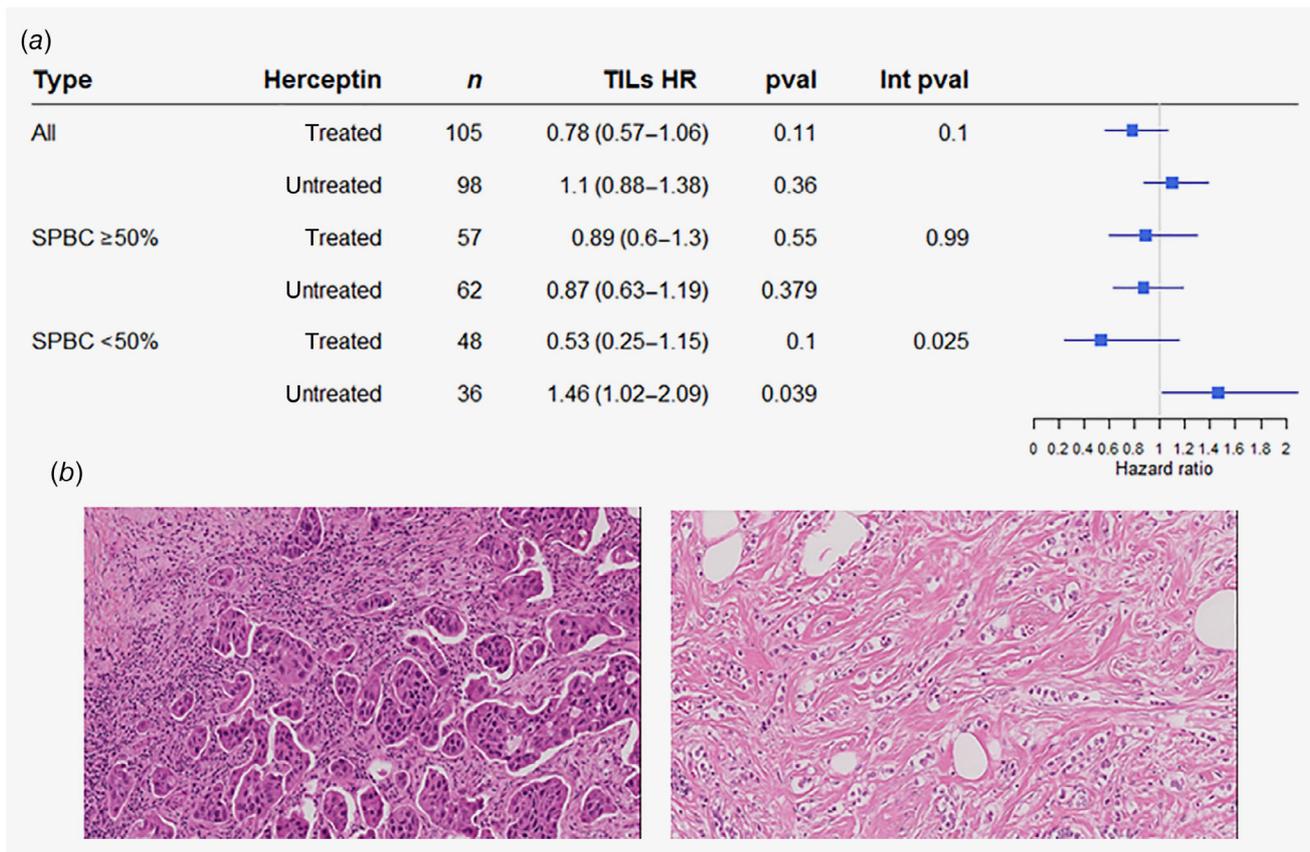
stroma did not correlate with mean intratumoral lymphocyte infiltration (Spearman correlation: 0.02,  $p = 0.80$ ) or with mean stromal lymphocyte infiltration (Spearman correlation: 0.03,  $p = 0.69$ ). The expected benefit from trastuzumab in patients with high levels of TILs was observed only in tumors with SPBC <50% (DDFS  $p$  interaction = 0.025), and not in tumors with SPBC ≥50% (DDFS  $p$  interaction = 0.99) (Fig. 5).

## Discussion

Given the increasing preclinical evidence of the significant contribution of tumor-associated stroma to trastuzumab efficacy, we decided to evaluate these hypotheses in the context of a Phase III clinical trial randomly assigning patients to anti-HER2 treatments. The results of our study reveal that reactive stroma may be associated with trastuzumab resistance in HER2-positive/ER-negative early breast cancer. Increased effectiveness of dual anti-HER2 treatments (pertuzumab + trastuzumab, trastuzumab + lapatinib and trastuzumab → neratinib) over single blockade (trastuzumab

alone) has been recently reported in clinical studies in HER2-positive breast cancer.<sup>12,33,34</sup> However, the actual benefit is relatively small (2–4% in terms of disease-free survival in the adjuvant setting), and there are currently no clinically useful biomarkers that can identify the patients who are resistant to trastuzumab and will benefit from a dual blockade. Therefore, while our finding may not change current management of HER2-positive patients, it remains clinically relevant, because stromal reactivity may be used as a stratification factor in clinical trials and be useful in identifying those patients that may benefit from a dual blockade.

There are a number of mechanisms by which reactive stroma can support cancer cells and contribute to drug resistance. Several studies have suggested that stromal drug resistance in breast cancer is partly induced by integrin signaling and an ECM.<sup>35,36</sup> Specifically, integrin and collagen can lead to resistance to anti-HER2 treatment by activating the phosphatidylinositol 3-kinases (PI3K/Akt) or the ERK pathway.<sup>37</sup>



**Figure 5.** Interactions between stromal tumor-infiltrating lymphocytes (TILs) and trastuzumab benefit in HER2-positive disease. (a). Forest plots indicating Cox regression hazard ratios, 95% confidence intervals, and *p*-values for trastuzumab benefit for distant disease-free survival (DDFS), as well as *p* values of the interaction between tumor-infiltrating lymphocytes (TILs) and trastuzumab treatment. An analysis based on patients with stromal predominant breast cancer (SPBC) of low levels (<50%) and SPBC of high levels (≥50%) is shown. (b). Histological sections showing breast tumors containing a high SPBC level (≥50%), high TIL levels (≥50%) (left side) and a low SPBC/low TIL level (right).

Another mechanism through which stroma support tumor cells and drug resistance is through secretion of cytokines and growth factors.<sup>38–40</sup> The secretion of cytokines, such as TGF- $\beta$ 1 and Stromal-derived factor 1, promotes the transition of normal fibroblasts to CAFs, which can impact tumor progression and response to therapy.<sup>41,42</sup> Notably, there is evidence for cross talk between the HER2 and the TGF- $\beta$ 1 signaling pathways, and evidence that TGF- $\beta$ 1 activation may be associated with trastuzumab resistance.<sup>43</sup> Indeed, we were able to demonstrate in a clinical setting that the TGF- $\beta$ 1 pathway is activated and associated with trastuzumab resistance in tumors with reactive stroma. Moreover in our study, the predictive role of reactive stroma was confined to the ER-negative group while the ER-positive group showed an opposite pattern, although not significant. A possible explanation is that in the ER-positive group, different signal transduction pathways are activated by TGF- $\beta$ 1.<sup>44</sup>

Several studies have shown an association between increasing TILs and increased benefit from trastuzumab, highlighting

the role of immunity in the efficacy of trastuzumab.<sup>18,45</sup> We now showed that reactive stroma is neither positively nor negatively correlated with the presence of TILs. More importantly, we demonstrated that the expected benefit from trastuzumab in patients with high levels of TILs was abolished in tumors with reactive stroma. Therefore, if TILs indicate the presence of an immune response, it may be possible that the role of immune surveillance for tumor control is relatively ineffective when using trastuzumab due to stromal reactivity which exerts immune suppression.<sup>46</sup> In support of this possibility are the findings of several recent preclinical studies that demonstrated that CAFs have immunosuppressive effects which may affect the response to trastuzumab.<sup>10,15</sup> The application of data from a prospective, rigorously conducted randomized clinical trial strengthens the relevance of our observation and highlights the importance of the role of the tumor microenvironment in the efficacy of trastuzumab.

Our study has limitations. While the evaluation of reactive stroma was done by gene expression profiling and pathological

evaluation, no specific immunohistochemistry (IHC) staining was performed. In addition, four CAFs with six concomitant analyses of six fibroblast markers<sup>10</sup> accumulate differently in breast cancer, making any such analysis more difficult.<sup>10</sup> Another limitation of our study is that reactive stroma was evaluated using full-face H&E-stained sections, in contrast to the practical more routine use of biopsy in the neoadjuvant setting. Of note, two stromal gene expression signatures were evaluated on the neoadjuvant Neo-ALTTO trial and showed opposite roles in modulating the response to trastuzumab as they predicted higher pCR rates in the single arms (trastuzumab or lapatinib) but lower pCR rates in the combination arm (trastuzumab + lapatinib).<sup>47</sup>

In conclusion, our study provides clinical evidence that reactive stroma is associated with resistance to trastuzumab therapy in HER2-positive early breast cancer patients.

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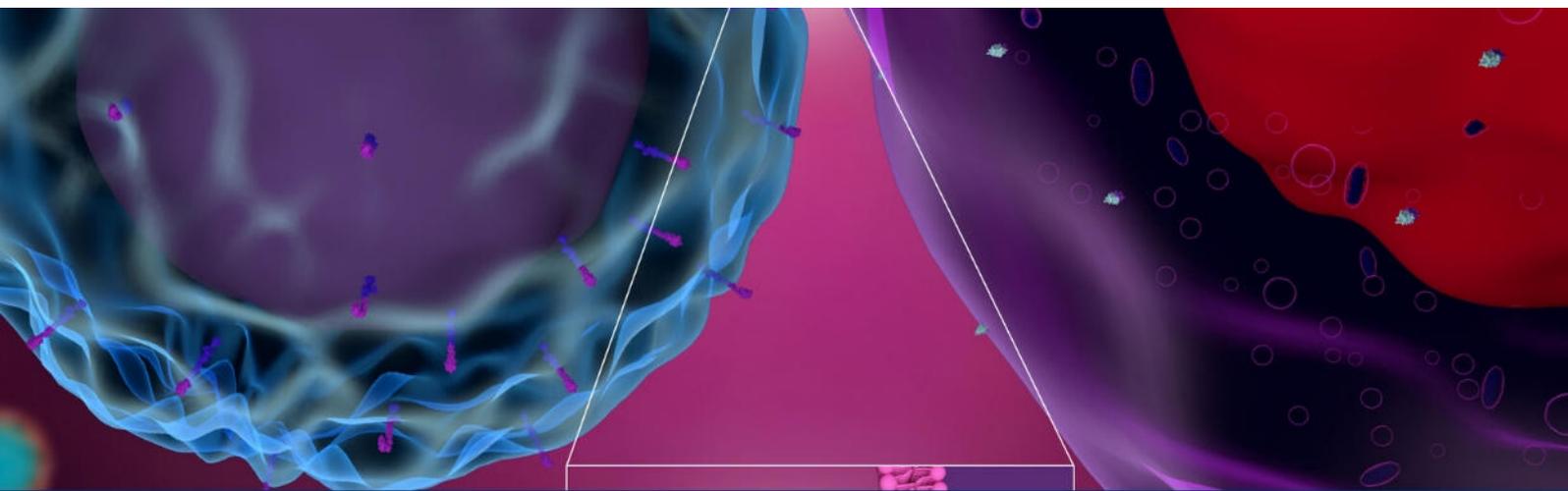
### Conflict of Interest

A.S. reports consulting or advisory role from: Eli Lilly, Pfizer, Novartis. Travel, accommodations expenses: Neopharm, Celgene, Speakers bureau: Teva, Roche, Novartis. Si.L. reports consulting or advisory role from: Abbvie, Amgen, Astra Zenca, Celgene, Pfizer, Roche, Seattle Genetics, Teva, Daiichi, Liily, Samsung, Novartis, Prime/Medscape, Vifor and Eitgenix. C.D. reports Honoria from: Teva, Novartis, Pfizer, Roche, Amgen, Advisory role at: MSD, Amgen, Daiichi. Ownership: Sividon. H.J. reports coemployment at Orion pharma, fees from Neutron Therapeutics, own stocks of Orion Pharma and Sartar therapeutics. Sh.L. receives research funding to her institution from Novartis, Bristol Meyers Squibb, Merck, Roche-Genentech, Puma Biotechnology, Pfizer and Eli Lilly. She has acted as consultant (not compensated) to Seattle Genetics, Pfizer, Novartis, BMS, Merck, AstraZeneca and Roche-Genentech. She has acted as consultant (paid to her institution) to Aduro Biotech.

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