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

Amir Sonnenblick[1#*], Mali Salmon-Divon[2*], Roberto Salgado[3*], Efrat Dvash[1], Noam Pondé[4], Tamar Zahavi[5], Asher Salmon[5], Sibylle Loibl[6], Carsten Denkert[7], Heikki Joensuu[8], Ameye Lieveke[9], Gert Van den Eynden[10], Pirkko-Liisa Kellokumpu-Lehtinen[11], Amos Azaria[12], Sherene Loi[13] , Stefan Michiels[14], François Richard[15] and Christos Sotiriou[15].

1. Institute of Oncology, Tel Aviv Sourasky Medical Center, Tel Aviv, Israel; Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel.
2. Department of Molecular Biology, Adelson School of Medicine, Ariel University, Ariel, Israel.
3. Department of Pathology, GZA-ZNA, Antwerp, Belgium; Division of Research, Peter Mac Callum Cancer Center, Melbourne, Australia.
4. Institut Jules Bordet, Université Libre de Bruxelles, Brussels, Belgium and AC Camargo Cancer Center, Medical Oncology Department, São Paulo Brasil.
5. Sharett Institute of Oncology, Hadassah Hebrew University Medical Center, Jerusalem, Israel.
6. German Breast Group, Neu-Isenburg and Goethe University Frankfurt and Centre for Haematology and Oncology, Bethanien, Frankfurt Germany.
7. Institute of Pathology, Philipps-University Marburg and UKGM Marburg, Marburg, Germany.
8. Department of Oncology, Helsinki University Hospital and University of Helsinki, Helsinki, Finland.
9. Data Management Unit, Institut Jules Bordet, Université Libre de Bruxelles, Belgium.

10. Molecular Immunology Lab, Institut Jules Bordet, Université Libre de Bruxelles, Brussels, Belgium.
11. Department of Oncology, University of Tampere and Tampere University Hospital, B.O.X. 607, FIN 33101, Tampere, Finland.
12. Department of Computer Science, Ariel University, Israel.
13. Peter MacCallum Cancer Centre, University of Melbourne VIC, Australia.Service de Biostatistique et d'Epidémiologie, Gustave Roussy, CESP U108, University Paris-Sud, University Paris-Saclay, Villejuif, France.
14. Department of Oncology, Laboratory for Translational Breast Cancer Research, KU Leuven, , Leuven, Belgium
15. Institut Jules Bordet, Université Libre de Bruxelles, Belgium.

Corresponding author: Amir Sonnenblick, Institute of Oncology, Tel Aviv Sourasky Medical Center, Tel Aviv, Israel 6423906. Tel: 972-3-6972446, Fax: 972-3-6974789; email: amirsonn@gmail.com

*These authors contributed equally

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

List of abbreviations: extracellular matrix (ECM), carcinoma associated fibroblast (CAF) , distant disease-free survival (DDFS), tumor-infiltrating lymphocytes (TILs), stromal predominant breast cancer (SPBC)**,** epithelial-to-mesenchymal transition (EMT)

Novelty and Impact statement:

Given the increasing 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. We show that reactive

stroma is associated with resistance to trastuzumab therapy in HER2 positive breast cancer patients. Moreover we show that the expected benefit from trastuzumab in patients with high levels of TILs is abolished in tumors with reactive stroma.

Abstract

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; ≥50% stromal) and correlated with distant disease-free survival (DDFS).

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 (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.

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[1–5]. It has also been suggested that tumor-associated stroma contribute to cancer growth and progression by promoting stromal–epithelial paracrine signaling[6]. A few stromal signatures have been developed and were found to be prognostic, especially in the HER2 breast cancer subgroup[5,7–9]. 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[10].

Trastuzumab, a monoclonal antibody targeted against HER2, has dramatically improved clinical outcomes for patients with HER2-positive disease [11]. 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[12–14]. Given the increasing evidence of the significant contribution of tumor-associated stroma to trastuzumab efficacy[15], 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 [16]. 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 (REMARK) criteria were followed for reporting this study.

Patients

This study used samples and data from the FinHer trial, a multicenter, phase 3, randomized breast cancer trial in an adjuvant setting that enrolled 1010 patients (CONSORT diagram figure 1)[16]. 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[17], 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 with 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 [17].

Reactive stroma and tumor-infiltrating lymphocyte (TIL) pathologic assessment

Evaluation of reactive stroma was performed on full-face hematoxylin and eosin-stained (H&E) 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 (RS and GVdE) performed the readings and reached a consensus. TILs were assessed as described by Loi et al [18].

Statistical analysis

The primary pre-defined hypothesis was that higher levels of reactive stroma would be associated with trastuzumab 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)[5,7–10,19] (Table S1) were calculated for each patient as previously described [20]. PLAU, which was associated with prognosis in HER2 positive breast cancer subgroup[8]; DCN, defined as a list of genes correlated to decorin and was able to predict chemoresistance in patients treated with preoperative chemotherapy[7]; Yoshiara stromal signature which represents the fraction of "normal" stromal cells in tumor tissue[19]; CAFS1 defined by Costa et al, via the concomitant analysis of six fibroblast markers[10]; and CD10+ stroma signature which is able to predict chemoresistance in the HER2 positive subpopulation[9]. We computed the sum of the products of the gene coefficient (–1 or 1, depending upon down-regulation or up-regulation,

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 [20]. 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) versus T2 (>2 cm and ≤5 cm) and T3 (>5 cm)], histological grade (1, 2, vs 3), and age (≤50 years 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, i.e., 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 were evaluated separately for patients with low- and those with high-reactive stoma (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 -Table S1), we used a method called ProGENI[21], implemented as part of the KnowEnG analytical platform (www.knoweng.org)[22]. 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 [23], 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 [20] 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[20,24] . 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 modelling with the Limma R package[25] was used to detect genes responding to reactive stroma in ER-positive and in ER-negative patients ($p < 0.005$). Those genes were subjected to Ingenuity Pathway Analysis (IPA®) 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 which represent luminal, luminal progenitors, basal (mammary stem cell-enriched), and epithelial-to-mesenchymal transition (EMT) were obtained from the literature [26–29]. 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 reactivation score was performed using the cor.test R function.

Data Availability statement

Gene mRNA expression data are available at:

http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=wfermmkijzktzcb&acc=GSE65095

Results

The gene-expression analysis from the TCGA data[30] revealed that reactive stromal gene expression signatures were associated more with the luminal A cancer subtype than with the luminal B breast cancer subtype (figure 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[31]. Our analysis also revealed that other subtypes, such as HER2-positive, have differential reactive stromal expression (figure S1). Given that preclinical data suggested that reactive stroma may decrease trastuzumab efficacy[15], 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 with the original series (Table 1). Associations between reactive stroma and other clinical-pathological characteristics showed that reactive stroma was not significantly associated with any of the investigated characteristics (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]) (figure 2). The YOSHIARA signature [19] that represents normal stromal volume in the tumor specimen was not associated with trastuzumab resistance (figure 2). We then evaluated two signatures that represent specific stromal CAF subtypes (CAF-S1 and CD10) [9,10]. Both the CD10 signature and the CAF-S1 signature (which represent a subset of cells which are associated with immunosuppression) were non-significantly associated with trastuzumab resistance (Supplementary figure 2).

Reactive stroma signatures were associated with the stromal pathological score, TGF-β1 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% to 90%). Stromal genomic signatures scores were positively but weakly correlated with pathological stromal reactivity (DCN r=0.3*; p*=2.287^{e-05}; PLAU r=0.28 *p*=8.75^{e-05}) (figure 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 CI: 0.066–0.996; ER-negative/SPBC ≥50%; HR=0.66 CI: 0.22-1.93) (figure 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 (figure S3A) together with expression data to rank genes with respect to their relationship with pathological reactive stroma (see Methods). The top 70 genes (Table S1) were selected, and the correlation of their polygenic score with reactive stroma was 0.41 (p value = 3.364 $^{e-09}$). To validate the association of the HER2STROMA signature with pathological reactive stroma, we calculated the signatures' predictive ability in an independent dataset which had been previously evaluated for reactive stroma (Responsify) [20]. The area under the curve (AUC) resulting from the use of the HER2STROMA signature score as predictor to reactive stroma outcome was 0.78 (figure S3B), which was higher than the AUC from using DCN (0.68), PLAU (0.71),

and YOSHIARA (0.62) (Figure S3B). Ten out of the 70 genes in the HER2STROMA signature were shared by DCN and/or PLAU signatures (Figure S3C and Table S1), and the main biological process that had been enriched in the HER2STROMA gene signature (by GO analysis) was organization of the extracellular matrix (false discovery rate (FDR) =2.54^{e-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 (figure S4). Caveolin1 (CAV1) and PDGFRβ (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) (figure 2). In an effort to show that reactive stroma assessment can be performed on breast cancer biopsies in the neo-adjuvant setting we analyzed gene expression data from neo-adjuvant studies[32] and show that HER2STROMA signature has trend towards worse outcome in HER2 positive patients (odds ratio=0.30; 0.09–1.01 *p*=0.052).

Next, we used IPA® software to uncover pathways associated with reactive stromal tumors. A few pathways were identified as being significantly associated with reactive stroma (figure 3B, *Z* score≥2). The TGF-β1 pathway was found to be up-regulated in both stromal reactive ER-positive as well as 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 [26–29] (figure 3C). The expression profile of the reactive stromal tumors showed a significant but low correlation with EMT, suggesting

that the TGF-β1 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 [18], we tested whether reactive stroma is positively or negatively correlated with TILs. Pathological evaluations of reactive stroma did not correlate with mean intra-tumoral 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) (figure 5).

Discussion

Given the increasing pre-clinical 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 this 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 [12,33,34]. 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 is still clinically relevant, since 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 extracellular matrix [35,36]. Specifically, integrin and collagen can lead to resistance to anti-HER2 treatment by activating the PI3K/Akt or the ERK pathway [37].

Another mechanism through which stroma support tumor cells and drug resistance is through secretion of cytokines and growth factors [38–40]. The secretion of cytokines, such as TGF-β1 and stromal-derived factor 1, promotes the transition of normal fibroblasts to CAFs, which can impact tumor progression and response to therapy [41,42]. Notably, there is evidence for cross-talk between the HER2 and the TGF-β1 signaling pathways, and evidence that TGF-β1 activation may be associated with trastuzumab resistance [43]. Indeed, we were able to demonstrate in a clinical setting that the TGF-β1 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-β1[44].

Several studies have shown an association between increasing TILs and increased benefit from trastuzumab, highlighting the role of immunity in the efficacy of trastuzumab [18,45]. 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 [45]. 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 [10,15]. 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 IHC staining was performed. In addition, four carcinoma associated fibroblast (CAF) with six concomitant analysis of six fibroblast markers[10] accumulate differently in breast cancer, making any such analysis more difficult[10]. 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 neo-adjuvant 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)[46].

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

Acknowledgments

We would like thank Amin Emad (http://www.ece.mcgill.ca/~aemad2) for his assistance with ProGENI.

Funding

AS is supported by a Clinical Research Career Development Award from the Israel Cancer Research Fund grants (16-116-CRCDA) and from the Israeli Cancer Research Association (2017-0140). The Parasol Center for Women's Cancer Research, The Parasol Foundation. CS is supported by the Breast Research Cancer Foundation (BCRF). RS is supported by a grant from the Breast Cancer Research Foundation (BCRF 17-194)

Disclosures

AS reports consulting or advisory role from: Eli Lilly, Pfizer, Novartis. Travel, accommodations expenses: Neopharm, Celgene, Speakers bureau: Teva, Roche, Pfizer. RS reports research funding from Merck, Roche and Puma, as well as travel funds from Astra Zeneca, Roche, Merck and an advisory role for BMS outside the scope of this work. SL 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 and Roche-Genentech.

References

1. Ma X-J, Dahiya S, Richardson E, Erlander M, Sgroi DC. Gene expression profiling of the tumor microenvironment during breast cancer progression. *Breast Cancer Res* 2009;11:R7.

2. Bauer M, Su G, Casper C, He R, Rehrauer W, Friedl A. Heterogeneity of gene expression in stromal fibroblasts of human breast carcinomas and normal breast. *Oncogene* 2010;29:1732–40.

3. Allinen M, Beroukhim R, Cai L, Brennan C, Lahti-Domenici J, Huang H, Porter D, Hu M, Chin L, Richardson A, Schnitt S, Sellers WR, et al. Molecular characterization of the tumor microenvironment in breast cancer. *Cancer Cell* 2004;6:17–32.

4. Casey T, Bond J, Tighe S, Hunter T, Lintault L, Patel O, Eneman J, Crocker A, White J, Tessitore J, Stanley M, Harlow S, et al. Molecular signatures suggest a major role for stromal cells in development of invasive breast cancer. *Breast Cancer Res Treat* 2009;114:47–62.

5. Finak G, Bertos N, Pepin F, Sadekova S, Souleimanova M, Zhao H, Chen H, Omeroglu G, Meterissian S, Omeroglu A, Hallett M, Park M. Stromal gene expression predicts clinical outcome in breast cancer. *Nat Med* 2008;14:518–27.

6. Mao Y, Keller ET, Garfield DH, Shen K, Wang J. Stromal cells in tumor microenvironment and breast cancer. *Cancer Metastasis Rev* 2013;32:303–15.

7. Farmer P, Bonnefoi H, Anderle P, Cameron D, Wirapati P, Becette V, André S, Piccart M, Campone M, Brain E, MacGrogan G, Petit T, et al. A stroma-related gene signature predicts resistance to neoadjuvant chemotherapy in breast cancer. *Nat Med* 2009;15:68–74.

8. Desmedt C, Haibe-Kains B, Wirapati P, Buyse M, Larsimont D, Bontempi G, Delorenzi M, Piccart M, Sotiriou C. Biological Processes Associated with Breast Cancer Clinical Outcome Depend on the Molecular Subtypes. *Clin Cancer Res* 2008;14:5158–65.

9. Desmedt C, Majjaj S, Kheddoumi N, Singhal SK, Haibe-Kains B, El Ouriaghli F, Chaboteaux C, Michiels S, Lallemand F, Journe F, Duvillier H, Loi S, et al. Characterization and Clinical Evaluation of CD10+ Stroma Cells in the Breast Cancer Microenvironment. *Clin Cancer Res* 2012;18:1004–14.

10. Costa A, Kieffer Y, Scholer-Dahirel A, Pelon F, Bourachot B, Cardon M, Sirven P, Magagna I, Fuhrmann L, Bernard C, Bonneau C, Kondratova M, et al. Fibroblast Heterogeneity and Immunosuppressive Environment in Human Breast Cancer. *Cancer Cell* 2018;33:463–479.e10.

11. Hudis CA. Trastuzumab — Mechanism of Action and Use in Clinical Practice. *N Engl J Med* 2007;357:39–51.

12. von Minckwitz G, Procter M, de Azambuja E, Zardavas D, Benyunes M, Viale G, Suter T, Arahmani A, Rouchet N, Clark E, Knott A, Lang I, et al. Adjuvant Pertuzumab and Trastuzumab in Early HER2-Positive Breast Cancer. *N Engl J Med* 2017;377:122–31.

13. von Minckwitz G, Huang C-S, Mano MS, Loibl S, Mamounas EP, Untch M, Wolmark N, Rastogi P, Schneeweiss A, Redondo A, Fischer HH, Jacot W, et al. Trastuzumab Emtansine for Residual Invasive HER2-Positive Breast Cancer. *N Engl J Med* 2018;

14. Chan A, Delaloge S, Holmes FA, Moy B, Iwata H, Harvey VJ, Robert NJ, Silovski T, Gokmen E, von Minckwitz G, Ejlertsen B, Chia SKL, et al. Neratinib after trastuzumab-based adjuvant therapy in patients with HER2-positive breast cancer (ExteNET): a multicentre, randomised, double-blind, placebo-controlled, phase 3 trial. *Lancet Oncol* 2016;17:367–77.

15. Nguyen M, De Ninno A, Mencattini A, Mermet-Meillon F, Fornabaio G, Evans SS, Cossutta M, Khira Y, Han W, Sirven P, Pelon F, Di Giuseppe D, et al. Dissecting Effects of Anti-cancer Drugs and Cancer-Associated Fibroblasts by On-Chip Reconstitution of Immunocompetent Tumor Microenvironments. *Cell Rep* 2018;25:3884–3893.e3.

16. Joensuu H, Kellokumpu-Lehtinen P-L, Bono P, Alanko T, Kataja V, Asola R, Utriainen T, Kokko R, Hemminki A, Tarkkanen M, Turpeenniemi-Hujanen T, Jyrkkiö S, et al. Adjuvant docetaxel or vinorelbine with or without trastuzumab for breast cancer. *N Engl J Med* 2006;354:809–20.

17. Joensuu H, Bono P, Kataja V, Alanko T, Kokko R, Asola R, Utriainen T, Turpeenniemi-Hujanen T, Jyrkkiö S, Möykkynen K, Helle L, Ingalsuo S, et al. Fluorouracil, epirubicin, and cyclophosphamide with either docetaxel or vinorelbine, with or without trastuzumab, as adjuvant treatments of breast cancer: final results of the FinHer Trial. *J Clin Oncol Off J Am Soc Clin Oncol* 2009;27:5685–92.

18. Loi S, Michiels S, Salgado R, Sirtaine N, Jose V, Fumagalli D, Kellokumpu-Lehtinen P-L, Bono P, Kataja V, Desmedt C, Piccart MJ, Loibl S, et al. Tumor infiltrating lymphocytes are prognostic in triple negative breast cancer and predictive for trastuzumab benefit in early breast cancer: results from the FinHER trial. *Ann Oncol Off J Eur Soc Med Oncol* 2014;25:1544–50.

19. Yoshihara K, Shahmoradgoli M, Martínez E, Vegesna R, Kim H, Torres-Garcia W, Treviño V, Shen H, Laird PW, Levine DA, Carter SL, Getz G, et al. Inferring tumour purity and stromal and immune cell admixture from expression data. *Nat Commun* 2013;4:2612.

20. Sonnenblick A, Brohée S, Fumagalli D, Vincent D, Venet D, Ignatiadis M, Salgado R, Van den Eynden G, Rothé F, Desmedt C, Neven P, Loibl S, et al. Constitutive phosphorylated STAT3-associated gene signature is predictive for trastuzumab resistance in primary HER2-positive breast cancer. *BMC Med [Internet]* 2015 [cited 2015 Sep 2];13. Available from: http://www.biomedcentral.com/1741-7015/13/177

21. Emad A, Cairns J, Kalari KR, Wang L, Sinha S. Knowledge-guided gene prioritization reveals new insights into the mechanisms of chemoresistance. *Genome Biol* 2017;18:153.

22. Sinha S, Song J, Weinshilboum R, Jongeneel V, Han J. KnowEnG: a knowledge engine for genomics. *J Am Med Inform Assoc JAMIA* 2015;22:1115–9.

23. Fabregat A, Sidiropoulos K, Garapati P, Gillespie M, Hausmann K, Haw R, Jassal B, Jupe S, Korninger F, McKay S, Matthews L, May B, et al. The Reactome pathway Knowledgebase. *Nucleic Acids Res* 2016;44:D481-487.

24. Sonnenblick A, Brohée S, Fumagalli D, Rothé F, Vincent D, Ignatiadis M, Desmedt C, Salgado R, Sirtaine N, Loi S, Neven P, Loibl S, et al. Integrative proteomic and gene expression analysis identify potential biomarkers for adjuvant trastuzumab resistance: analysis from the Fin-her phase III randomized trial. *Oncotarget* 2015;6:30306–16.

25. Ritchie ME, Phipson B, Wu D, Hu Y, Law CW, Shi W, Smyth GK. limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Res* 2015;43:e47.

26. Prat A, Perou CM. Deconstructing the molecular portraits of breast cancer. *Mol Oncol* 2011;5:5–23.

27. Ye X, Tam WL, Shibue T, Kaygusuz Y, Reinhardt F, Ng Eaton E, Weinberg RA. Distinct EMT programs control normal mammary stem cells and tumour-initiating cells. *Nature* 2015;525:256–60.

28. Gupta PB, Fillmore CM, Jiang G, Shapira SD, Tao K, Kuperwasser C, Lander ES. Stochastic state transitions give rise to phenotypic equilibrium in populations of cancer cells. *Cell* 2011;146:633–44.

29. Taube JH, Herschkowitz JI, Komurov K, Zhou AY, Gupta S, Yang J, Hartwell K, Onder TT, Gupta PB, Evans KW, Hollier BG, Ram PT, et al. Core epithelial-to-mesenchymal transition interactome gene-expression signature is associated with claudin-low and metaplastic breast cancer subtypes. *Proc Natl Acad Sci U S A* 2010;107:15449–54.

30. Koboldt DC, Fulton RS, McLellan MD, Schmidt H, Kalicki-Veizer J, McMichael JF, Fulton LL, Dooling DJ, Ding L, Mardis ER, Wilson RK, Ally A, et al. Comprehensive molecular portraits of human breast tumours. *Nature* 2012;490:61–70.

31. Dennison JB, Shahmoradgoli M, Liu W, Ju Z, Meric-Bernstam F, Perou CM, Sahin AA, Welm A, Oesterreich S, Sikora MJ, Brown RE, Mills GB. High Intratumoral Stromal Content Defines Reactive Breast Cancer as a Low-risk Breast Cancer Subtype. *Clin Cancer Res Off J Am Assoc Cancer Res* 2016;22:5068–78.

32. Ignatiadis M, Singhal SK, Desmedt C, Haibe-Kains B, Criscitiello C, Andre F, Loi S, Piccart M, Michiels S, Sotiriou C. Gene modules and response to neoadjuvant chemotherapy in breast cancer subtypes: a pooled analysis. *J Clin Oncol Off J Am Soc Clin Oncol* 2012;30:1996–2004.

33. de Azambuja E, Holmes AP, Piccart-Gebhart M, Holmes E, Di Cosimo S, Swaby RF, Untch M, Jackisch C, Lang I, Smith I, Boyle F, Xu B, 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;15:1137–46.

34. Martin M, Holmes FA, Ejlertsen B, Delaloge S, Moy B, Iwata H, von Minckwitz G, Chia SKL, Mansi J, Barrios CH, Gnant M, Tomašević Z, et al. Neratinib after trastuzumab-based adjuvant therapy in HER2-positive breast cancer (ExteNET): 5-year analysis of a randomised, double-blind, placebo-controlled, phase 3 trial. *Lancet Oncol* 2017;18:1688–700.

35. Dittmer J, Leyh B. The impact of tumor stroma on drug response in breast cancer. *Semin Cancer Biol* 2015;31:3–15.

36. Hanker AB, Estrada MV, Bianchini G, Moore PD, Zhao J, Cheng F, Koch JP, Gianni L, Tyson DR, Sánchez V, Rexer BN, Sanders ME, et al. Extracellular Matrix/Integrin Signaling Promotes Resistance to Combined Inhibition of HER2 and PI3K in HER2+ Breast Cancer. *Cancer Res* 2017;77:3280–92.

37. Huang C, Park CC, Hilsenbeck SG, Ward R, Rimawi MF, Wang Y-C, Shou J, Bissell MJ, Osborne CK, Schiff R. β1 integrin mediates an alternative survival pathway in breast cancer cells resistant to lapatinib. *Breast Cancer Res BCR* 2011;13:R84.

38. Moskovits N, Kalinkovich A, Bar J, Lapidot T, Oren M. p53 Attenuates cancer cell migration and invasion through repression of SDF-1/CXCL12 expression in stromal fibroblasts. *Cancer Res* 2006;66:10671–6.

39. Orimo A, Gupta PB, Sgroi DC, Arenzana-Seisdedos F, Delaunay T, Naeem R, Carey VJ, Richardson AL, Weinberg RA. Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion. *Cell* 2005;121:335–48.

40. Straussman R, Morikawa T, Shee K, Barzily-Rokni M, Qian ZR, Du J, Davis A, Mongare MM, Gould J, Frederick DT, Cooper ZA, Chapman PB, et al. Tumour micro-environment elicits innate resistance to RAF inhibitors through HGF secretion. *Nature* 2012;487:500–4.

41. Pickup M, Novitskiy S, Moses HL. The roles of TGFβ in the tumour microenvironment. *Nat Rev Cancer* 2013;13:788–99.

42. Kojima Y, Acar A, Eaton EN, Mellody KT, Scheel C, Ben-Porath I, Onder TT, Wang ZC, Richardson AL, Weinberg RA, Orimo A. Autocrine TGF-beta and stromal cell-derived factor-1 (SDF-1) signaling drives the evolution of tumor-promoting mammary stromal myofibroblasts. *Proc Natl Acad Sci U S A* 2010;107:20009–14.

43. Nahta R. Pharmacological strategies to overcome HER2 cross-talk and Trastuzumab resistance. *Curr Med Chem* 2012;19:1065–75.

44. Principe DR, Doll JA, Bauer J, Jung B, Munshi HG, Bartholin L, Pasche B, Lee C, Grippo PJ. TGF-β: duality of function between tumor prevention and carcinogenesis. *J Natl Cancer Inst* 2014;106:djt369.

45. Arnould L, Gelly M, Penault-Llorca F, Benoit L, Bonnetain F, Migeon C, Cabaret V, Fermeaux V, Bertheau P, Garnier J, Jeannin J-F, Coudert B. Trastuzumab-based treatment of HER2-positive breast cancer: an antibody-dependent cellular cytotoxicity mechanism? *Br J Cancer* 2006;94:259–67.

46. Yang L, Pang Y, Moses HL. TGF-beta and immune cells: an important regulatory axis in the tumor microenvironment and progression. *Trends Immunol* 2010;31:220–7.

47. Fumagalli D, Venet D, Ignatiadis M, Azim HA, Maetens M, Rothé F, Salgado R, Bradbury I, Pusztai L, Harbeck N, Gomez H, Chang T-W, et al. RNA Sequencing to Predict Response to Neoadjuvant Anti-HER2 Therapy: A Secondary Analysis of the NeoALTTO Randomized Clinical Trial. *JAMA Oncol* 2017;3:227–34.

Figure legends

Figure 1: CONSORT diagram.

Figure 2: Forest plots according to different reactive stroma signature statuses in all patients, in ER-negative only patients, or in ER-positive only patients. The plots indicate Cox regression hazard ratios, 95% confidence intervals, and p-values for trastuzumab benefit for DDFS, as well as p-values of the interaction (Int pval) between reactive stromal signatures and trastuzumab treatment.

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®) of pathways associated with stromal reactivation. A Z score ≥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). * $p \leq 0.05$

Figure 4: Kaplan–Meier curves of stromal predominant breast cancer (SPBC) status assessed in the FinHer dataset. Patients with a low SPBC level (<50%) and a high SPBC level (≥50%) according to trastuzumab treatment in the ER-negative group (up) or in the ER-positive group (down).

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 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).

Supplementary material

Figure S1: Reactive stroma gene expression signatures in the TCGA.

Figure S2: Forest plots according to CAFS1 and CD10 stroma signature status in all patients, ER-negative only patients, or ER-positive only patients. The plots indicate Cox regression hazard ratios, 95% confidence intervals, and p-values for trastuzumab benefit for DDFS, as well as p-values of the interaction between the signature and trastuzumab treatment.

Figure S3: HER2-reactive stromal signature (HER2STROMA). A. Protein-protein interaction from REACTOME used for the gene prioritization method in the HER2STROMA signature. B. The AUC derived from using the different reactive stromal signatures as predictor of reactive stromal outcome in an independent dataset. C. Venn diagram of shared genes between the HER2STROMA signature and the DCN and/or PLAU signatures.

Figure S4: Correlation of HER2STROMA gene signature and potential proteomic markers of reactive stroma in the Responsify data set.