**Prognostic and predictive impact of beta-2 adrenergic receptor expression in
HER2-positive breast cancer**

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Abstract

Background: Beta-2 adrenergic receptor (ADRB2) mediates proliferation and treatment resistance in preclinical models of HER2+ breast cancer. We evaluated *ADRB2* gene expression as a prognostic and predictive biomarker in HER2+ early breast cancer patients.

Methods: *ADRB2* expression was retrieved from HER2+ patients enrolled in the FinHer study (N=202), and 2 public datasets containing data from HER2+ early breast cancer patients: one including patients who did not receive systemic treatment (disease-free survival [DFS] dataset; N=175) and another including patients who received neoadjuvant treatment (pathologic complete response [pCR] dataset; N=207). Survival was estimated with Kaplan-Meier method and Cox regression was used for uni-multivariate analyses. *ADRB2* expression was correlated with several gene signatures.

Results: *ADRB2* high expression was associated with improved DFS rates in HER2+ patients (HR 0.52, 95% confidence interval [CI] 0.32-0.84, p=0.0068). No association between *ADRB2* expression and pCR was observed (OR 1.14, 95%CI 0.63-2.10, p=0.67). No association between *ADRB2* and relapse-free survival (RFS) was observed in HER2+ patients enrolled in the FinHer study (HR 0.93; 95% CI 0.69-1.25; p=0.61). *ADRB2* was associated with a low expression of angiogenesis-related (VEGF -0.38, p<0.001) and proliferation-related (AURKA -0.36, p<0.001; GGI -0.028, p <0.001; STAT3 -0.17, p<0.001) genes; and a high expression of immune-related genes (Perez +0.45, p <0.001; STAT1 +0.28, p<0.001; IRM +0.29, p<0.001).

Conclusions: Opposing our initial hypothesis, a high *ADRB2* expression may be a favourable prognostic factor in HER2+ early breast cancer patients. This association appears to be mediated by anti-proliferative, anti-angiogenic and immunogenic effects of *ADRB2*.

Keywords: “beta-2 adrenergic receptor”; “ADRB2”; “breast cancer”; “HER2”; “gene expression”.

Introduction

The beta-2 adrenergic receptor (ADRB2) is a G protein coupled receptor that mediates physiologic processes such as smooth muscle relaxation, chronotropism and inotropism.¹ ADRB2 also exerts a potent trophic effect on the cardiac muscle, inducing proliferation and differentiation of cardiac progenitor cells.² The expression of ADRB2 has been demonstrated in breast cancer cells using immunohistochemistry, although its function in these cells is unknown.³ Given the trophic stimuli exerted by ADRB2 on cardiac myocytes, this receptor may also stimulate proliferation and therefore function as an oncogene in breast cancer cells.⁴

Preclinical studies demonstrate a significant interaction between human epidermal growth factor receptor 2 (HER2) and ADRB2 pathways: in breast cancer cells that harbour an amplification or overexpression of HER2 (HER2+), the activation of HER2 induces epinephrine production and augments ADRB2 expression, whereas ADRB2 activation increases HER2 expression.⁵ The physiologic regulatory mechanism of receptor desensitization, which consists in ADRB2 degradation after its activation to prevent exaggerated adrenergic stimuli, is also lost in HER2+ breast cancer cells, allowing a permanent activation of the ADRB2 pathway.⁵ Moreover, ADRB2 and HER2 share intracellular effectors such as mitogen activated protein kinases (MAPK) and phosphoinositide 3-kinases (PI3K), providing ADRB2 the capacity to drive cell proliferation when HER2 is blocked, and thus to drive resistance to anti-HER2 treatments.⁶

In vitro, ADRB2 activation stimulates mitosis and angiogenesis in HER2+ breast cancer cells, whereas beta-blockers interrupt these effects, suggesting that ADRB2 regulates HER2+ breast cancer proliferation.^{5,6} Retrospective studies evaluated the correlation between beta-blocker use and outcomes in breast cancer patients, with most series observing an improved survival in patients treated with beta-blockers when compared to patients who did not receive these medications. Notably, in most of these studies the population was heterogeneous in terms of breast cancer subtypes and stages. Therefore, although definitive conclusions are precluded, the available data suggest that beta-blockers may improve the prognosis

of breast cancer patients, highlighting the role of *ADRB2* as a potential oncogene in this scenario.^{7–12}

In xenograft models of trastuzumab-resistant HER2+ breast cancer, beta-blockers restore trastuzumab sensitivity and induce tumor shrinkage, suggesting that *ADRB2* may also be a therapeutic target.⁶ Supporting this hypothesis, in a retrospective study of 83 HER2+ early breast cancer patients treated with neoadjuvant chemotherapy and trastuzumab, a high *ADRB2* expression (assessed with immunohistochemistry) was associated with lower pathologic complete response (pCR) rates (13% in *ADRB2* high expression group versus 77% in *ADRB2* low expression group, $p < 0.001$).⁶

In view of the preclinical studies showing that *ADRB2* stimulates proliferation and drives treatment resistance in HER2+ breast cancer, and the clinical data suggesting that beta-blockers improve the prognosis of breast cancer patients, the present study aims to evaluate the expression of the *ADRB2* gene as a predictive and prognostic biomarker in HER2+ early breast cancer patients.

Objectives

The main objectives of this study are to correlate the expression levels of the *ADRB2* gene with survival outcomes (disease-free survival [DFS]/relapse-free survival [RFS]) and pCR rates in HER2+ early breast-cancer patients.

The secondary objectives of this study are: to perform subgroup analyses for the outcomes DFS/RFS and pCR according to *ADRB2* expression (high vs. low) in estrogen-receptor (ER) negative and ER-positive patients; to evaluate *ADRB2* expression in different molecular subtypes defined by SCMGene classification in samples from the DFS and pCR datasets; to assess the correlation between *ADRB2* expression and gene expression signatures related to proliferation, invasiveness, immune activation and angiogenesis.

Methods

Study population – description of the 3 datasets used in the study

DFS dataset

Public available datasets were retrieved from Haibe-Kains et al. 2012 using the provided link (<http://compbio.dfci.harvard.edu/pubs/sbtpaper/>).¹³ To assess the role of *ADRB2* expression as a prognostic biomarker, data from HER2+ early breast cancer patients, with node-negative disease, who were treated with surgery alone and did not receive any systemic neoadjuvant or adjuvant treatment before the collection of the samples, and who had available information about DFS, RFS or distant metastasis-free survival (DMFS) were retrieved from this dataset. Patients were defined as HER2+ according to the data retrieved from the dataset. In this dataset, HER2+ classification was based either on immunohistochemistry or in situ hybridization (ISH). DFS was defined as the time between sample collection (surgery) and recurrence or death, whichever occurred first. In case DFS data was not available, DMFS or RFS data were used.

Probes were matched to their gene symbols; in case of multiple matches the one having the highest variance in the dataset under study was used. Kaplan Meier curves for DFS according to the categorized *ADRB2* expression level were drawn using *survcomp* package version 1.26 in R version 4.4.1. HRs and CIs were computed as univariate analysis with adjustment for datasets, using a Cox regression model. A multivariate analysis adjusted for datasets, age (≤ 50 versus > 50), tumour size in centimeters (≤ 2 versus > 2), histologic grade (1 and 2 versus 3) and ER status (negative vs positive), was also performed.

pCR dataset

Public available datasets were retrieved from Ignatiadis et al 2012.¹⁴ To assess the role of *ADRB2* expression as a predictive biomarker, data from HER2+ early breast cancer patients who received neoadjuvant treatment with anthracyclines $\pm$ taxanes, and for whom information regarding pathological response assessment after neoadjuvant treatment was available, were retrieved from this dataset. Patients were defined as HER2+ according to the data retrieved from the dataset. In this dataset, HER2+ classification was based either on immunohistochemistry or in situ

hybridization (ISH). In the studies included in this dataset, the administration of trastuzumab for HER2+ patients was not mandatory, and information regarding trastuzumab use was not available for all patients. pCR was defined as the absence of residual invasive disease in the breast in one study; and no residual invasive disease in the breast and axillary lymph nodes in the other seven studies of the dataset. Duplicates across studies have been managed as follows: (I) Spearman coefficients were computed for all patients across studies; (II) patients from MDACC and MAQCIII studies having a coefficient ≥ 0.90 of patients from another study were removed.

Probes were matched to their gene symbols; in case of multiple matches the one having the highest variance in the dataset under study was used. Cox regression models to assess the correlation between ADRB2 expression and pCR rates were performed, using *ADRB2* expression as a continuous variable. Odds ratios (OR) and CIs were computed as univariate analysis adjusted for datasets using a linear regression model. A multivariate analysis adjusted for tumor size datasets, treatment type (A versus AT), age (≤ 50 versus > 50), tumor size ($\leq T2$ versus $\geq T3$), nodal status (negative versus positive), histologic grade (1 and 2 versus 3) and ER status (negative versus positive) was also performed.

FinHer dataset

In the FinHer study, 232 women with HER2+ early breast cancer were randomized to receive adjuvant chemotherapy alone or combined with 9 weeks of trastuzumab (loading dose 4mg/kg followed by 8 weekly doses of 2 mg/kg).¹⁵ In the primary analysis of the study, after a median follow up of 36 months, 3-year RFS was significantly higher in patients who received trastuzumab versus those who did not receive trastuzumab (89% vs. 78%, respectively; hazard ratio [HR] 0.42; 95% confidence interval [CI], 0.21-0.83; $p=0.01$).¹⁵ After 62 months of follow-up, a sustained trend in favour of trastuzumab was observed in terms of distant DFS (HR = 0.65; 95% CI, 0.38-1.12; $p=0.12$).¹⁶

Public available gene expression data from the patients enrolled in the FinHer study were retrieved from the GEO portal under the accession GSE65095. HER2+ was defined according to the study protocol as a HER2 expression ≥ 2 on a scale from 0 to 3 in immunohistochemistry confirmed by a copy number gain in chromogenic in situ hybridization.^{15,17} All HER2+ patients with available data regarding the expression of the *ADRB2* gene were included. The definition of RFS was specified in the study protocol as the time from randomization to the detection of local or distant recurrence, contralateral invasive breast cancer or death, whichever occurred first.¹⁵ Kaplan Meier method was used to estimate RFS according to the categorized *ADRB2* expression level. HR and confidence intervals were computed as univariate analysis using a Cox regression model.

Definition of low and high expression of ADRB2

In all survival analyses, *ADRB2* expression was evaluated as a categorical variable (high versus low). The median expression of *ADRB2* was initially estimated in the overall samples from each dataset evaluated. Patients were classified as either “*ADRB2*-high” group, when *ADRB2* expression level in their samples was above the median value of their dataset; or as “*ADRB2*-low” group, when *ADRB2* expression level in their samples was below the median value of their dataset.

Additional analyses were performed to explore different cut-off points of *ADRB2* expression (25% and 75% of the samples) to define “*ADRB2*-high” and “*ADRB2*-low” groups.

ER and HER2 status

When available, ER and HER2 status of the patients were retrieved from the GeneSys Export (GSE) files. When missing, they were inferred using the function *bimod* from the *genefu* package version 2.6.0.¹⁸

Molecular classification of samples according to gene expression - SCMGENE

The SCMGENE, a breast cancer classification model based on the expression levels of 3 genes (*ER*, *HER2* and aurora kinase A [*AURKA*]) was used to categorize samples from the datasets as previously described.¹³ According to

SCMGene classification, 5 subgroups were identified: LumA, LumB, HER2+/ER+, HER2+/ER- and HER2-/ER- (TNBC).¹⁹ LumA and LumB differ by their proliferative rate, either low or high, respectively. To define LumA and LumB subgroups according to proliferation rates, the median expression levels of *AURKA* in the overall ER+/HER2- patients were used as a cut-off: *AURKA* expression levels lower than the median were defined as LumA, while levels higher than the median were defined as LumB. In some of the analyses performed, the HER2+/ER- and HER2+/ER+ groups were pooled to form the HER2+ subgroup, representing the overall population of HER2+ patients.

Correlation of ADRB2 expression with gene expression signatures

In the pooled samples from the DFS and the pCR datasets, the correlation of *ADRB2* expression with 9 gene signatures associated with the following characteristics was performed: *AURKA* (proliferation, 219 genes); *STAT3* (proliferation, 123 genes); *PLAU* (invasion, 68 genes); *VEGF* (angiogenesis, 14 genes); *GGI* (grading, 129 genes); *IRM* (immune, 7 genes); *STAT1* (immune, 95 genes); *PEREZ* (immune, 14 genes); *MAPK* (activation of the MAPK pathway, 315 genes),^{20–25}. The correlations were assessed by computing the Spearman coefficient between the 2 variables and the associated p-value was considered significant if ≤ 0.05 .

Statistics

All analyses were performed using the R software version 4.4.1 (<https://www.r-project.org/>) and p-values were corrected for multiple testing by false discovery rate (FDR).

Results

Patient's characteristics

Two datasets have been designed aggregating public available gene expression data reproducing Haibe-Kains et al. (N=1,258) and Ignatiadis et al. (N=1,013) to study DFS and pCR, respectively. These datasets comprise a total of

237 and 207 HER2+ early breast cancer patients respectively, of whom 175 (73.8%) and 207 (100%) had available data regarding *ADRB2* expression. The characteristics of the patients and the studies comprised in each dataset are presented in **Supplementary material Tables 1 and 2**.

From the FinHer study, data from 202 HER2+ early breast cancer patients was publically available (102 patients randomized to chemotherapy and trastuzumab and 100 patients randomized to chemotherapy alone). The characteristics of the patients included from FinHer study's dataset are displayed in **Supplementary material Table 3**.

ADRB2 expression according to breast cancer subtypes

Samples from the 2 datasets (DFS and pCR, N=382) were pooled and categorized into 5 subtypes (LumA, LumB, HER2+/ER+, HER2+/ER- and TNBC) using the SCMGENE signature, as previously described.¹³ The expression of *ADRB2* was then quantified in the 5 subtypes, being significantly higher in the LumA and the HER2+/ER- subtypes. **(Figure 1)**

Correlation of ADRB2 expression with DFS

In HER2+ patients from the DFS dataset (N=175), DFS was significantly improved in the *ADRB2*-high group in comparison to the *ADRB2*-low group (median 119 months versus 68 months; HR 0.52, 95% CI 0.32-0.84, p=0.0068). **(Figure 2)** When using different cut-off values of *ADRB2* expression to define *ADRB2*-high and low (25% and 75%), a trend for improved DFS in patients with *ADRB2*-high was observed in both scenarios, apparently with a more robust association observed with the 75% cut-off. (Supplementary Figure 1)

The prognostic impact of *ADRB2*-high was significant in the subgroup of HER2+/ER+ patients (N=117) (median DFS 131 months versus 102 months; HR 0.45, 95% CI 0.24-0.84, p=0.0097), whereas in HER2+/ER- patients (N=58), the difference was not statistically significant (median DFS 120 months versus 69 months; HR 0.68, 95% CI 0.29-1.58, p=0.37). **(Figure 3)**

A significant difference in terms of DFS was observed between *ADRB2*-high and *ADRB2*-low groups in univariate analysis (HR 0.54, 95% CI 0.34-0.87, $p=0.001$) although in multivariate analysis the difference was no longer significant (HR 0.67, 95% CI 0.36-1.23, $p=0.19$). **(Supplementary Table 4)** In the subgroup of HER2+/ER+ patients, a superior DFS was observed in *ADRB2*-high compared to the *ADRB2*-low group in univariate (HR 0.39, 95% CI 0.19-0.79, $p=0.008$) and multivariate analyses (HR 0.36, 95% CI 0.14–0.93, $p=0.035$). No significant interaction was observed between ER status and *ADRB2* expression in an independent Cox model using only these 2 variables ($p_{\text{interaction}}=0.42$). In the subgroup of HER2+/ER- patients, no significant difference was observed between *ADRB2*-high and *ADRB2*-low groups in univariate (HR 0.71, 95%CI 0.36-1.41, $p=0.33$) or multivariate analyses (HR 0.78, 95% CI 0.32-1.92, $p=0.59$). **(Supplementary Table 4)**

Correlation of ADRB2 expression with pCR rates

In the pCR dataset, 70 pCR events were recorded amongst 207 HER2+ patients (33%). No significant difference was observed in terms of pCR rates between *ADRB2-high* and *ADRB2-low* groups in univariate (OR 1.14, 95% CI 0.63-2.10, $p=0.67$) and multivariate analyses (OR 1.52, 95% CI 0.76- 3.16, $p=0.25$). **(Supplementary Table 5)**

In the subgroup of HER2+/ER+ patients (N=80), no significant difference in terms of pCR was observed between the *ADRB2-high* and *ADRB2-low* groups in univariate (OR 0.98, 95% CI 0.30-3.61, $p=0.98$) and multivariate analyses (OR 1.75, 95% CI 0.47-8.05, $p=0.43$).

In the subgroup of HER2+/ER- patients (N=127), no significant difference in terms of pCR was observed between the *ADRB2-high* and *ADRB2-low* groups in univariate (OR 1.06, 95% CI 0.49-2.28, $p=0.88$) and multivariate analyses (OR 1.41, 95% CI 0.56-3.72, $p=0.47$). **(Supplementary Table 5)**

RFS in the FinHer dataset

In the overall HER2+ patients enrolled in the FinHer trial (N=202), no significant difference in terms of RFS was observed between *ADRB2*-high and *ADRB2*-low groups (HR 0.93; 95% CI 0.69-1.25; p=0.61). **(Figure 4)**

No significant difference in terms of RFS was observed between *ADRB2*-high and *ADRB2*-low groups both in patients randomized to chemotherapy and trastuzumab (N=102; HR 0.73; 95% CI 0.46-1.15; p=0.17) and in patients randomized to chemotherapy alone (N=100; HR 1.07; 95% CI 0.67-1.71; p=0.78). **(Figure 5)**

No significant difference in terms of RFS was observed between *ADRB2*-high and *ADRB2*-low groups both in HER2+/ER+ (N=97; HR 0.82; 95% CI 0.54-1.26; p=0.37) and HER2+/ER- patients (N=105; HR 1.1; 95% CI 0.72-1.69; p=0.66). **(Figure 6)**

Correlation of ADRB2 with gene expression signatures

Samples from the DFS and the pCR datasets were pooled into a single dataset (N=382), that was used to assess the correlation between *ADRB2* expression and gene signatures associated with angiogenesis, proliferation, invasion and immune activation. *ADRB2* expression was significantly associated with low expression levels of angiogenesis-related (VEGF -0.38, p<0.001) and proliferation-related genes (AURKA -0.36, p<0.001; GGI -0.28, p<0.001; STAT3 -0.17, p<0.001); whereas the expression of genes related to immune activation was directly associated with *ADRB2* expression (Perez +0.45, p<0.001; STAT1 +0.28, p<0.001; IRM +0.29, p<0.001). A positive, although weak, correlation between *ADRB2* and MAPK (+0.14, p<0.001) and invasion-related genes (PLAU, +0.12, p<0.001) was also observed. **(Figure 7)**

An additional analysis restricted to HER2+/ER+ patients (N=116), the subgroup in which *ADRB2* high expression appeared to have a more pronounced prognostic effect, was performed. **(Supplementary Figure 2)** Similar to what was observed in the overall HER2+ population, *ADRB2* expression was significantly associated with a low expression of angiogenesis-related (VEGF -0.35, p<0.001)

and proliferation-related genes (AURKA -0.21, $p=0.004$; GGI -0.18, $p=0.01$); whereas the expression of genes related to immune activation (Perez +0.5, $p<0.001$; STAT1 +0.35, $p<0.001$; IRM +0.31, $p<0.001$) was directly associated with *ADRB2* expression.

Discussion

We observed a higher expression of *ADRB2* in the LumA and HER2+/ER- subtypes, suggesting that *ADRB2* is active in HER2+ breast cancer, as observed in preclinical studies that show a cross-talk between HER2 and ER pathways.^{5,26,27} Opposing our initial hypothesis, *ADRB2* high expression was associated with improved DFS in HER2+ patients from our DFS dataset, although in multivariate analysis this association was not sustained. The positive prognostic impact of *ADRB2* high expression was more pronounced in HER2+/ER+ patients, with a trend being also observed in the HER2+/ER- subgroup. Interestingly, *ADRB2* was associated with a low expression of genes involved in angiogenesis and proliferation, and a high expression of genes related to immune activation, which may justify the better prognosis observed in the *ADRB2* high expression group. No association between *ADRB2* expression and pCR was observed. In the FinHer dataset, no association between *ADRB2* expression and RFS was found both in patients who received chemotherapy and trastuzumab and in those who received chemotherapy alone. Therefore, the hypothesis that *ADRB2* mediates treatment resistance was not supported by our findings.

The practice of regular physical activity reduces the risk of recurrence and improves the prognosis of breast cancer patients.²⁸ In cell models of HER2-/ER+ breast cancer, serum samples obtained from both breast cancer patients and healthy women immediately after exercise induce the expression of the Hippo tumor suppressor pathway genes, impairing cancer cells' viability and survival. However, when the same cells are pre-treated with beta-blockers, the anti-proliferative effect of the post-exercise serum is not observed, suggesting that its anti-cancer effect is mediated by beta-adrenergic stimulation.²⁹ Physical exercise induces the expression of *ADRB2* in leukocytes.³⁰ Hypothetically, if *ADRB2* expression also increases in

breast cancer cells as a consequence of exercise, the better prognosis observed in the *ADRB2* high expression group in our study may be the result of an indirect effect, meaning that physical exercise might be more frequent in the *ADRB2* high expression group.

MCF-7 is a lineage of HER2-/ER+ breast cancer cells that are highly dependent on ER signalling, frequently used to reproduce *in vitro* the features of luminal breast cancer.³¹ Gargiulo et al. evaluated the effects of *ADRB2* activation in MCF-7 and in non-tumoral immature breast cells (MCF-10A). After *ADRB2* expression was induced by gene transfection into MCF-7 cells, treatment with epinephrine inhibited their proliferation and migration. Interestingly, isoproterenol (an *ADRB2* agonist) increased ER expression and induced the differentiation of MCF-10A cells into mature normal breast epithelial cells. The differentiation mediated by isoproterenol was not observed when either fulvestrant or tamoxifen were administered, demonstrating that the effect exerted by *ADRB2* depends on the ER pathway, supporting our findings of a significant prognostic impact of *ADRB2* high expression in HER2+/ER+ patients, and also the higher *ADRB2* expression levels observed in HER2+ and luminal tumors.³⁰

The proliferation of vascular smooth-muscle cells and the synthesis of vascular endothelial growth factor (VEGF) are key steps in angiogenesis, which is essential for tumor growth and metastases development.^{32,33} *ADRB2* expression was associated with a low expression of genes related to angiogenesis in our study. In a lineage of vascular smooth-muscle cells (A7r5) transfected with the ER gene, Walters et al. observed that estradiol stimulated proliferation, while isoproterenol inhibited the proliferative effects of estradiol. The anti-proliferative effects of isoproterenol were not observed in the presence of propranolol (beta-2 adrenergic blocker), suggesting that *ADRB2* activation may inhibit angiogenesis in ER+ tumors.³³ The effect of *ADRB2* to antagonize the pro-angiogenic effects of estradiol may also explain the positive prognostic impact of *ADRB2* high expression in HER2+/ER+ patients in our study.

Preclinical studies evaluating the interactions of *ADRB2* with the immune system present contradictory results. Wrobel et al. observed that propranolol increased the infiltration of cytotoxic lymphocytes and decreased the concentration of immunosuppressive myeloid cells into the stroma of melanoma in mice, suggesting that *ADRB2* activation may render the tumoral microenvironment less immunogenic.³⁴ On the other hand, Swanson et al. have demonstrated that *ADRB2* stimulation induces differentiation of immature T cells into T helper lymphocytes type 1, increasing the production of interferon-gamma, which enhances the activity of T cytotoxic lymphocytes.³⁵ We observed a significant association between the expression of *ADRB2* and genes involved in immune activation. The induction of an anti-tumor immune response by immunotherapy has proven to be an effective treatment in a variety of tumors, including breast cancer.^{36,37} Indeed, high levels of tumor infiltrating lymphocytes are associated with a good prognosis in HER2+ breast cancer, and immunotherapy presents promising results in this subgroup of patients.^{38,39} A potential action of *ADRB2* to render the tumoral microenvironment more immunogenic may explain its potential positive prognostic impacts. The role of *ADRB2* expression as a predictive biomarker for immunotherapy response and the use of *ADRB2* agonists with immune checkpoint inhibitors are thus potential strategies to be further explored.

In the pCR dataset, no association between *ADRB2* expression and pCR was observed. Notably, the information regarding trastuzumab use in this population could not be retrieved. Given the robust survival benefit yielded by trastuzumab in HER2+ early breast cancer patients, a potential imbalance between trastuzumab use in both groups (*ADRB2*-high versus *ADRB2*-low) could have influenced our results.⁴⁰ However, the analysis of the FinHer study also found no significant association between *ADRB2* expression and RFS both in patients who received and in those who did not receive trastuzumab, even though the 9-week duration of trastuzumab used in this trial is now considered sub-optimal.⁴¹

A study by Rivero et al. has evaluated the correlation between *ADRB2* expression and distant-metastasis-free survival in 1,924 early breast cancer patients,

demonstrating that a high expression of *ADRB2* was significantly correlated with a favourable prognosis in the overall study population ($p=0.006$). Notably, no significant association between *ADRB2* expression and prognosis was observed in the HER2+ subgroup ($p=0.412$), however, due to the limited number of HER2+ patients included ($N=50$), definitive conclusions are precluded on the role of *ADRB2* expression as a biomarker in the HER2+ subgroup. Interestingly, *ADRB2* expression was correlated with genes involved in T cell activation and inflammatory response in this study, corroborating our findings.⁴²

Potential limitations have to be considered when interpreting our findings. Data was extracted from public available datasets and not collected prospectively. *ADRB2* expression levels were not available for all patients in the DFS dataset, and the small size of the HER2+/ER- subgroup may have limited the analysis of this subset. Also, no data regarding the use of beta-blockers, beta-agonists, and patients' physical exercise habits were available, being these potential factors that could have influenced our results. However, given the encouraging preclinical evidence suggesting that *ADRB2* might be involved in treatment resistance in HER2+ breast cancer, this hypothesis warranted further investigation, and to our knowledge this is the first study evaluating *ADRB2* expression as a biomarker specifically in HER2+ breast cancer patients. Notably, the population of HER2+ patients who did not receive any systemic treatment in our DFS dataset created an interesting scenario to study *ADRB2* expression as a prognostic biomarker.

In conclusion, our study suggests that *ADRB2* high expression can be a favourable prognostic factor in HER2+ early breast cancer patients, especially in the HER2+/ER+ subgroup. This effect might be the result of a tumoral microenvironment that is more immunogenic and less favourable to angiogenesis and proliferation in *ADRB2*-high tumors. *ADRB2* arises as a promising biomarker and potential therapeutic target in HER2+ breast cancer patients.

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Compliance with Ethical Standards

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Author's contribution: RC, FR and EdA have conceived the project. FR and CS have performed the bioinformatics and statistical analysis. RC has written the manuscript. All authors have collaborated with their expertise and revised the final version of this manuscript before submission.

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Figure Legends

Figure 1 - ADRB2 expression according to breast cancer subtypes defined per SCMGENE in the pooled samples from the DFS and pCR datasets (N=382).

Figure 2 - Kaplan Meier curves of disease-free survival according to ADRB2 expression in the overall HER2+ population of the DFS dataset (N=175).

Figure 3 - Kaplan Meier curves of disease-free survival according to ADRB2 expression in HER2+/ER+ (N=117, A) and HER2+/ER- (N=58, B) subgroups of patients from the DFS dataset.

Figure 4 - Kaplan Meier curves of relapse-free survival according to ADRB2 expression in the overall HER2+ patients enrolled in the FinHer study (N=202).

Figure 5 - Kaplan Meier curves of relapse-free survival according to ADRB2 expression in patients who received chemotherapy and trastuzumab (N=102, A); and in those who received chemotherapy alone (N=100, B) in the FinHer study.

Figure 6 - Kaplan Meier curves of relapse-free survival according to ADRB2 expression in hormone-receptor positive (N=97, A); and hormone-receptor negative patients (N=105, B) in the FinHer study.

Figure 7 - Spearman correlation between ADRB2 expression in HER2+ patients from both datasets (N=382) and different gene signatures associated with proliferation (AURKA), invasion (PLAU), angiogenesis (VEGF), grading (GGI), immune (IRM, Perez and STAT1) and MAP kinase activation (MAPK). Only the associations that reached statistical significance are coloured, with blue meaning a positive correlation (high ADRB2 expression is associated with a high-expression of that signature) and pink meaning a negative correlation (high ADRB2 expression is associated with a low-expression of that signature).

Table 1 – Cox regression models for the correlation between *ADRB2* expression and disease-free survival in HER2+ (N=175), HER2+/ER+ (N=117) and HER2+/ER- patients (N=58), univariate and multivariate. Multivariate analysis was adjusted for age (≤ 50 vs > 50), tumor size (≤ 2 vs > 2), histologic grade (1, 2 vs 3) and ER status (negative vs positive).

	N	Hazard ratio and 95% confidence interval
Univariate analysis		
HER2+	175	0.54 (0.34-0.87; p=0.01)
HER2+/ER+	117	0.39 (0.19-0.79; p=0.008)
HER2+/ER-	58	0.71 (0.36-1.41; p=0.33)
Multivariate analysis		
HER2+	175	0.67 (0.36-1.23; p=0.19)
HER2+/ER+	117	0.36 (0.14-0.93; p=0.03)
HER2+/ER-	58	0.78 (0.32-1.92; p=0.59)

Figures

Prognostic and predictive impact of the beta-2 adrenergic receptor expression in HER2-positive breast cancer

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Figure 1 - *ADRB2* expression according to breast cancer subtypes defined per SCMGene in the pooled samples from the DFS and pCR datasets (N=382).

Figure 2 - Kaplan Meier curves with disease-free survival rates according to *ADRB2* expression in the overall HER2+ population of the DFS dataset (N=175).

A – HER2+/ER+

B – HER2+/ER-

Figure 3 - Kaplan Meier curves with disease-free survival rates according to *ADRB2* expression in the HER2+/ER+ (N=117, A) and the HER2+/ER- (N=58, B) subgroups of the DFS dataset.

Figure 4 - Kaplan Meier curves with RFS rates according to *ADRB2* expression in the overall population of the FinHer study (N=202).

A

B

Figure 5 - Kaplan Meier curves with RFS rates according to *ADRB2* expression in patients who received trastuzumab (N=102, A); in those who did not receive trastuzumab (N=100, B); in the FinHer study.

A

B

Figure 6 - Kaplan Meier curves with RFS rates according to *ADRB2* expression in hormone-receptor positive (N=97, A); and hormone-receptor negative patients (N=105, B) in the FinHer study.

Figure 7 - Spearman correlation between *ADRB2* expression in HER2+ patients from both datasets (N=382) and different gene signatures associated with proliferation (*AURKA*), invasion (*PLAU*), angiogenesis (*VEGF*), grading (*GGI*), immune (*IRM*, *Perez* and *STAT1*) and MAP kinase activation (*MAPK*). Only the associations that reached statistical significance are coloured, with blue meaning a positive correlation (high *ADRB2* expression is associated with a high-expression of that signature) and pink meaning a negative correlation (high *ADRB2* expression is associated with a low-expression of that signature).

Supplementary material

Prognostic and predictive impact of the beta-2 adrenergic receptor expression in HER2-positive breast cancer

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Supplementary Table 1 - Characteristics of patients and studies included in the dataset Haibe-Kains et al. (13). HER2+ early breast cancer patients included in the DFS analysis were retrieved from this dataset.

		N	CAL	DFHCC	EMC2	MAINZ	NCI	NKI	STNO2	TRANSBIG	UCSF	UNT	UPP	VDX
		1258	8	6	48	200	10	165	7	198	11	133	128	344
Age	≤ 50	567	4	0	0	51	3	137	4	142	6	61	30	129
	>50	585	4	6	0	149	7	28	3	56	5	72	98	157
	missing	106	0	0	48	0	0	0	0	0	0	0	0	58
Tumor size	≤2cm	788	5	3	0	112	5	98	7	102	8	83	87	278
	>2cm	364	3	3	0	88	5	67	0	96	3	50	41	8
	missing	106	0	0	48	0	0	0	0	0	0	0	0	58
ER	negative	367	4	5	4	38	5	42	5	64	4	44	17	135
	positive	891	4	1	44	162	5	123	2	134	7	89	111	209
HER2	negative	1014	8	5	28	164	9	136	6	154	2	115	104	283
	positive	237	0	1	20	36	1	29	1	44	2	18	24	61
	missing	7	0	0	0	0	0	0	0	0	7	0	0	0
Grade	1	186	2	0	0	29	2	36	0	30	1	32	47	7
	2	435	1	1	0	136	1	49	3	83	6	51	62	42
	3	418	5	5	0	35	7	80	4	83	4	29	18	148
	missing	219	0	0	48	0	0	0	0	2	0	21	1	147
SCMGENE	LumA	278	2	0	9	59	1	55	0	37	0	59	56	0
	LumB	279	2	0	17	78	4	46	2	72	1	24	33	0
	TNBC	277	4	5	2	27	4	32	4	45	1	32	15	106
	HER2+/ER-	88	0	0	2	11	1	10	1	19	1	12	2	29
	HER2+/ER+	149	0	1	18	25	0	19	0	25	1	6	22	32
	missing	187	0	0	0	0	0	3	0	0	7	0	0	177

Abbreviations: Datasets were retrieved from the author's or Institution's websites. Each dataset was assigned a short acronym and an instance number as follows: CAL = dataset of breast cancer patients from the University of California, San Francisco, and the California Pacific Medical Center (United States); DFHCC = Dana-Farber Harvard Cancer Center (United States); EMC = Erasmus Medical Center (the Netherlands); MAINZ = Mainz hospital (Germany); NCI = National Cancer Institute (United States); NKI = National Kanker Instituut (the Netherlands); STNO = Stanford/Norway (United States and Norway); TRANSBIG = dataset collected by the TransBIG consortium (Europe); UCSF = University of California, San Francisco (United States); UNT = cohort of untreated breast cancer patients from the Oxford Radcliffe (United Kingdom) and Karolinska (Sweden) hospitals; UPP = Uppsala Hospital (Sweden); VDX = Veridex (the Netherlands).

Supplementary Table 2 - Characteristics of patients and studies included in the dataset Ignatiadis et al. (14). HER2+ early breast cancer patients included in the pCR analysis were retrieved from this dataset.

			EORTC (A)	EORTC (AT)	ISPY (AT)	LBJ/IN/GEI (AT)	MDACCT (A)	MDACCT (AT)	TOP (A)	MDACC (AT)	MAQCIII (A)	USO (AT)
		1013	102	59	79	57	62	116	114	271	92	61
Age	≤50	540	38	30	51	30	35	65	69	137	50	35
	>50	435	28	29	28	27	27	51	45	133	41	26
	missing	38	36	0	0	0	0	0	0	1	1	0
Tumor size	T0	7	0	0	0	0	1	1	0	4	1	0
	T1	54	2	1	1	1	4	7	16	20	1	1
	T2	501	38	38	32	19	31	45	79	149	50	20
	T3	266	26	20	38	18	10	27	5	58	24	40
	T4	146	0	0	8	19	15	36	14	38	16	0
	missing	39	36	0	0	0	1	0	0	2	0	0
Nodal status	N0	331	27	22	25	16	23	36	52	88	21	21
	N1	422	34	32	46	25	21	50	57	113	12	32
	N2	119	5	5	6	15	15	23	3	32	10	5
	N3	56	0	0	2	1	2	7	2	37	2	3
	missing	85	36	0	0	0	1	0	0	1	47	0
ER	negative	559	65	59	36	21	29	51	114	105	50	29
	positive	454	37	0	43	36	33	65	0	166	42	32
HER2	negative	857	69	39	76	57	53	99	81	230	92	61
	positive	156	33	20	3	0	9	17	33	41	0	0
	missing	0	0	0	0	0	0	0	0	0	0	0
Grade	G1	45	2	0	6	5	3	12	2	11	3	1
	G2	307	21	16	24	19	24	37	20	98	29	19
	G3	515	32	38	27	23	25	47	87	152	47	37
	missing	131	47	5	22	10	10	20	5	10	13	4
pCR	NO	778	63	32	65	46	58	94	98	216	65	41
	YES	235	39	27	14	11	4	22	16	55	27	20
SCMGENE	LumA	205	19	0	14	11	21	39	12	71	16	2
	LumB	205	9	0	22	25	10	19	12	79	22	7
	TNBC	396	54	43	28	21	22	41	58	80	37	12
	HER2+/ER-	127	11	16	8	0	7	10	20	25	13	17
	HER2+/ER-	80	9	0	7	0	2	7	12	16	4	23

Abbreviations: A, anthracycline-based neoadjuvant chemotherapy; AT, anthracycline plus taxane-based neoadjuvant chemotherapy; EORTC, European Organisation for Research and Treatment of Cancer; ER, estrogen receptor; GEICAM, Grupo Español de Investigación en Cáncer de Mama; GEO, gene expression omnibus; HER2, human epidermal growth factor receptor 2; INEN, Instituto Nacional de Enfermedades Neoplásicas; I-SPY, Investigation of Serial Studies to Predict Your Therapeutic Response With Imaging and Molecular Analysis; LBJ, Lyndon B. Johnson Hospital; MAQCII, MicroArray Quality Control Consortium II; MDACC, MD Anderson Cancer Center; pCR, pathologic complete response; TOP, Trial of Principle; USO, US Oncology.

Supplementary Table 3 - Characteristics of the patients from the FinHer study.

		Chemotherapy	Chemotherapy and trastuzumab
Overall		100	102
Age	≤ 50	57	48
	>50	43	54
Tumor size	≤2cm	33	41
	>2cm	67	60
	missing	0	1
Nodal status	negative	20	9
	positive	80	93
ER	negative	54	51
	positive	46	51
HER2	negative	0	0
	positive	100	102
Grade	1	3	2
	2	29	35
	3	64	63
	missing	4	2

Supplementary Table 4 - Cox regression models for the correlation between *ADRB2* expression and disease-free survival in HER2+ (N=175), HER2+/ER+ (N=117) and HER2+/ER- patients (N=58), univariate and multivariate. Multivariate analysis was adjusted for age (≤ 50 vs > 50), tumor size (≤ 2 vs > 2), histologic grade (1, 2 vs 3) and ER status (negative vs positive).

	N	Hazard ratio and 95% confidence interval
Univariate analysis		
HER2+	175	0.54 (0.34-0.87; p=0.01)
HER2+/ER+	117	0.39 (0.19-0.79; p=0.008)
HER2+/ER-	58	0.71 (0.36-1.41; p=0.33)
Multivariate analysis		
HER2+	175	0.67 (0.36-1.23; p=0.19)
HER2+/ER+	117	0.36 (0.14-0.93; p=0.03)
HER2+/ER-	58	0.78 (0.32-1.92; p=0.59)

Supplementary Table 5 - Cox regression models for the correlation between *ADRB2* expression and pathologic complete response in HER2+ (N = 207), HER2+/ER+ (N = 80) and HER2+/ER- patients (N = 127), univariate and multivariate. Multivariate analysis was adjusted for age (≤ 50 vs >50), tumor size (≤ 2 vs >2), histologic grade (1, 2 vs 3) and ER status (negative vs positive).

	N	Hazard ratio and 95% confidence interval
Univariate analysis		
HER2+	207	1.14 (0.63-2.10; p=0.67)
HER2+/ER+	80	0.98 (0.30-3.61; p=0.98)
HER2+/ER-	127	1.06 (0.49-2.28; p=0.88)
Multivariate analysis		
HER2+	207	1.52 (0.76-3.16; p=0.25)
HER2+/ER+	80	1.75 (0.47-8.05; p=0.43)
HER2+/ER-	127	1.41 (0.56-3.72; p=0.47)

A **B**

Supplementary Figure 1 – Kaplan Meier curves of disease-free survival according to ADRB2 expression in the overall HER2+ population of the DFS dataset (N=175) using the alternative cut-off values of 25% (A) and 75% of the samples (B) to define ADRB2 expression as high. Patients were classified as either “ADRB2-high” group, when ADRB2 expression level in their samples was above the cut-off value of their dataset; or as “ADRB2-low” group, when ADRB2 expression level in their samples was below the cut-off value of their dataset.

Supplementary Figure 2 - Spearman correlation ADRB2 expression in HER2+/ER+ patients from both datasets (N=116) and different gene signatures associated with proliferation (AURKA), invasion (PLAU), angiogenesis (VEGF), grading (GGI), immune (IRM, Perez and STAT1) and MAP kinase activation (MAPK). Only the associations that reached statistical significance are coloured, with blue meaning a positive correlation (high ADRB2 expression is associated with a high-expression of that signature) and pink meaning a negative correlation (high ADRB2 expression is associated with a low-expression of that signature).