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TUMOR MARKERS AND SIGNATURES

STAT3 activation in HER2-positive breast cancers: Analysis of data from a large prospective trial

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Abstract

The JAK/STAT3 signaling pathway may be aberrantly activated and have various and conflicting roles in breast cancer. The current study explored prognostic implications of activated STAT3 in human epidermal growth factor receptor 2 (HER2)-positive primary breast cancers in the context of a large prospective study (ALTTO). Activated STAT3 was determined by immunohistochemical analysis of STAT3 phosphorylation (Y705) performed on the primary tumors. This analysis evaluated whether patients with activated STAT3 had disease-free survival (DFS) and overall survival (OS) different from patients without activated STAT3. A total of 5694 patients out of the 8381 patients enrolled in ALTTO were included in this analysis (67.9%), and 2634 of them (46%) had evidence of STAT3 activation (minimum tumor Allred score ≥2). The median follow-up was 6.93 years (6.85-6.97 years), at the end of which 1035 (18.18%) and 520 (9.13%) patients experienced DFS and OS events, respectively. Patients with STAT3 activation experienced improved DFS compared to those without it (multivariable hazard ratio [HR], 0.84; 95% confidence interval [CI] 0.74-0.95; P = .006). There were no group differences in OS (multivariable HR, 0.92; 95% CI 0.78-1.10; P = .37). This effect was limited to ER-positive tumors. In conclusion, these

Abbreviations: DFS, disease-free survival; HER2, human epidermal growth factor receptor 2; HR, hazard ratios; IHC, immunohistochemistry; IL6, interleukin 6; JAK, Janus kinase; OS, overall survival; STAT, signal transducer and activator of transcription; TMA, tissue microarray.

findings support the role of STAT3 activation as a marker of favorable outcome in ER-positive/HER2-positive breast cancer patients.

KEYWORDS
ALTTO, breast cancer, estrogen receptor, HER2, STAT3

1 | INTRODUCTION

Human epidermal growth factor receptor 2 (HER2) gene amplification occurs in breast cancers and is associated with an aggressive clinical phenotype.[1] Trastuzumab and pertuzumab, both humanized monoclonal antibodies that target HER2, have shown efficacy in the treatment of HER2-positive breast cancer.[2-6] The signal transducer and activator of transcription (STAT) pathway was discovered through the study of transcriptional activation in response to interferon and interleukin 6 (IL6).[7,8] The IL6 Janus kinase (JAK) and STAT3 were found to be crucial for tumor progression with direct effect on proliferation and survival as well as a nondirect effect on the stromal and immune environment surrounding the tumor.[9-13] Although preclinical data imply that the IL6-JAK-STAT3 pathway is an important target for cancer therapy, the information on its clinical utility remains limited.[14,15]

STAT3 was shown in numerous studies to have a role in the development, progression and aggressiveness of breast cancer.[9,10,16-19] However, studies that evaluated the prognostic role of STAT3 have reported conflicting results, with some studies showing improved outcome in tumors with an activated STAT3 pathway.[20-24] STAT3 phosphorylation in HER2 positive breast cancers is associated with a distinct gene expression signature which was associated with trastuzumab resistance.[25] Other preclinical studies proposed a mechanism involving an IL6/STAT3 inflammatory loop that expands stemness and promotes a epithelial-mesenchymal transition.[16,26-28]

The present study aimed to evaluate the prognostic role of phosphorylated-Tyr705-STAT3 in HER2 positive breast cancer patients in the adjuvant clinical setting.

What's new?

The JAK/STAT3 signaling pathway is suspected of influencing tumor progression, with effects on tumor cell proliferation and survival. However, in breast cancer, evidence suggests that STAT3 in particular may have various and conflicting effects. In the present study, the prognostic implications of activated STAT3 were investigated in patients with HER2-positive primary breast cancer. In patients with estrogen receptor (ER)-positive/HER2-positive tumors, STAT3 activation was associated with better disease-free survival compared to patients without activated STAT3. No differences were detected in overall survival. The findings suggest that STAT3 activation is indicative of superior outcome in ER-positive/HER2-positive patients.

2 | MATERIALS AND METHODS

2.1 | Patients and study design

The ALTTO trial is an international, open-label, Phase III randomized trial that assigned patients with HER2-positive early breast cancer to receive different anti HER2 treatments.[29] As per study protocol, HER2, ER and PR were centrally tested for all patients before random assignment in one of the three central laboratories: The 2007 American Society of Clinical Oncology/College of American Pathologists guidelines were used to define HER2 positivity. Tumors with ≥1% tumor cells expressing ER and/or PR receptors were defined as HR positive.

2.2 | Tissue microarray (TMA) construction, immunohistochemistry (IHC) and evaluation of IHC staining

Primary tumors were collected from patients as part of additional translational research and TMAs were constructed for 6796 patients (most with four cores). For pSTAT3 IHC, TMAs were deparaffinized and processed automatically. Immunodetection of pSTAT3 was carried out as previously described.[30] In brief, the tissue microarray sections slides were incubated with primary antibody (p-STAT3 antibody; cat. no. 9145-D3A7-XP; Cell Signaling Technology, Beverly, MA) followed by incubation with the secondary antibody. Staining was visualized using DAB. Each run contained around 30 TMAs. Controls for the procedure were HeLa cells with and without interferon-α (cell signaling©) on one slide that served as positive and negative controls, respectively. Additionally, we used as controls, FFPE samples from previous cohorts that had previously demonstrated pSTAT3 negativity/positivity in multiple examinations.[30] All TMAs were scanned with a scanner zoomer and saved on an external driver.

2.3 | Evaluation of IHC staining

The TMA images were analyzed with QuPath software[31] in an automated manner employing a script that performed the "de-arraying" of the slide image to detect individual cores, which were manually validated and adjusted when needed. Cells and nuclei were detected

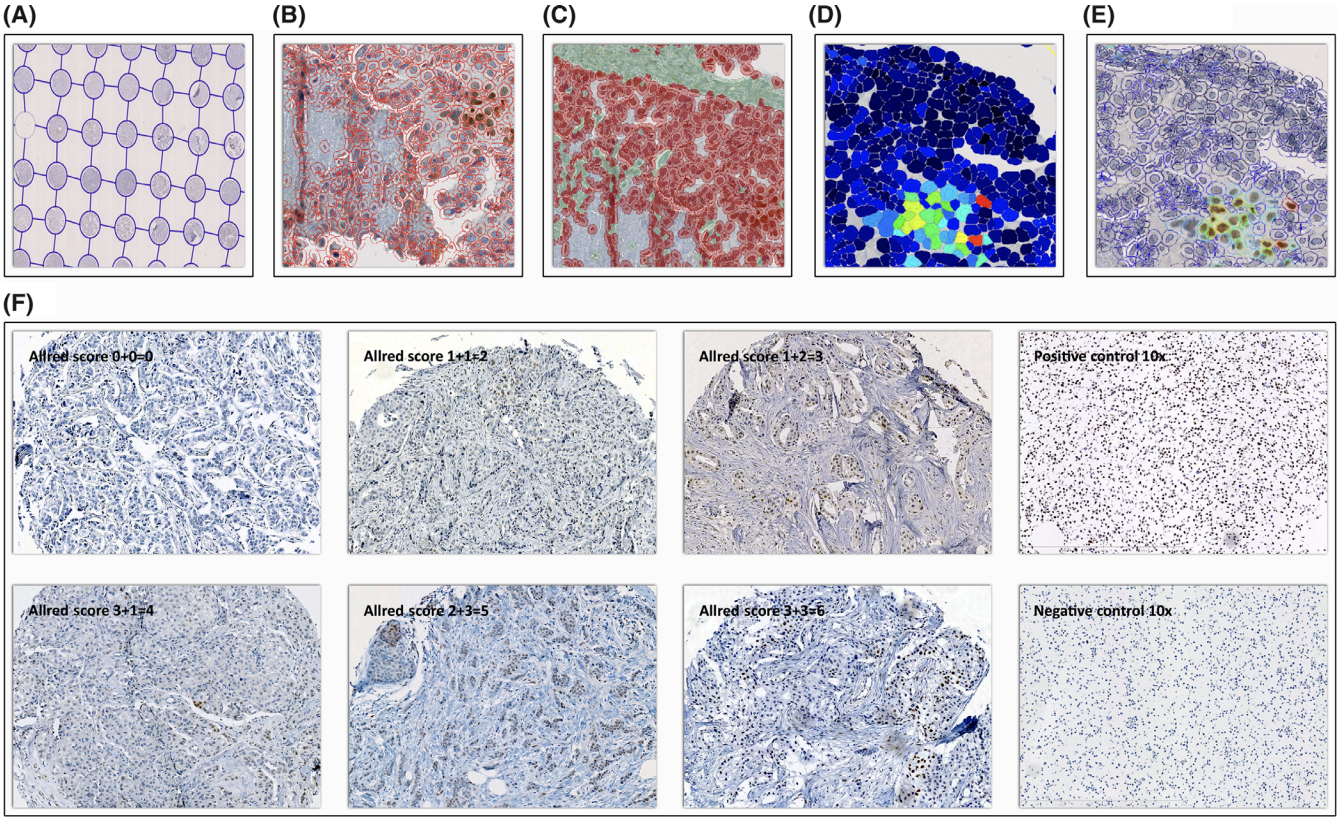

FIGURE 1 pSTAT3 immunohistochemistry (IHC) staining and scoring. The TMA images were analyzed in an automated manner (A). Cells and nuclei were detected based on hematoxylin optical density levels at a resolution of 1 μm (B). After cell detection, the cells were segregated into stromal and tumor cells by means of a classifier that was trained on five random core samples (C), and DAB mean intensity was measured for each nucleus and used to calculate the Allred score for each core and tissue type (stroma/tumor) (D,E). The staining score was determined according to the pSTAT3 Allred score (in each slide the first number represent %intensity and the second %positivity) (F)

FIGURE 2 CONSORT diagram

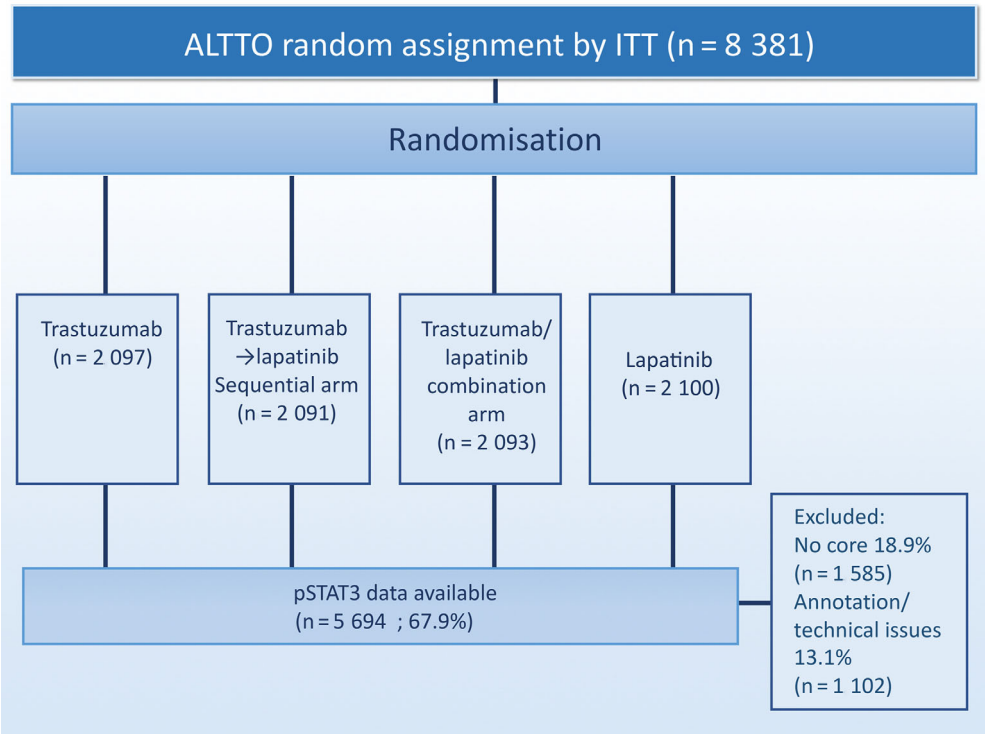

TABLE 1 Patients and tumors characteristics

Patients/tumors characteristics	Minimum tumor Allred score = 0 (N = 3060) N (%)	Minimum tumor Allred score ≥2 (N = 2634) N (%)	*P* value
Age (year) categorized			.556
≤50	1454 (48)	1231 (47)	
>50	1606 (52)	1403 (53)	
Hormone receptor status			.129
Positive	1769 (58)	1575 (60)	
Negative	1291 (42)	1059 (40)	
Timing of chemotherapy			<.001
Sequential	1656 (54)	1569 (60)	
Concurrent	1404 (46)	1065 (40)	
Lymph node status			<.001
Not applicable	114 (4)	202 (8)	
Node negative	1261 (41)	1074 (41)	
Node positive	1685 (55)	1358 (52)	
Pathological tumor size			<.001
No applicable (neo-adjuvant chemotherapy)	114 (4)	202 (8)	
≤2 cm	1209 (40)	1115 (42)	
>2 cm	1729 (57)	1306 (50)	
Missing	8 (<1)	11 (<1)	
Histologic grade			.248
GX: Differentiation cannot be assessed	78 (3)	79 (3)	
G1: Well differentiated	68 (2)	74 (3)	
G2: Moderately differentiated	1100 (36)	986 (37)	
G3: Poorly differentiated/undifferentiated	1802 (59)	1485 (56)	
Others/missing	12 (<1)	10 (<1)	
Randomized arm			.939
Trastuzumab+lapatinib	785 (26)	662 (25)	
Trastuzumab->lapatinib	767 (25)	652 (25)	
Lapatinib	754 (25)	659 (25)	
Trastuzumab	754 (25)	661 (25)	

according to the "watershed cell detection" method based on hematoxylin optical density levels at a resolution of 1 μm. After cell detection, the cells were segregated into stromal and tumor cells by means of a classifier that was trained on five random core samples, and DAB mean intensity was measured for each nucleus and used to calculate the Allred score for each core and tissue type (stroma/tumor) (Figure 1). Three staining thresholds were defined based on positive and negative controls. Only samples with a threshold of >500 tumor cells per core were included in order to minimize scoring bias. The staining score was determined according to the Allred score (Figure 1F). A score of not applicable (N/A) was given to specimens that were uninterruptable or did not contain >500 tumor cells. Scoring of the TMA was carried out by researcher blinded to the clinicopathological data (RG). Definition of a tumor as being pSTAT3-positive required a minimum cutoff point of ≥2 score in all available samples for a specific patient. That cutoff was chosen based on previous studies to improve signal-to-noise ratios.[21,30] Analyses based on mean score and for the stroma compartment were also performed.

2.4 | Statistical analysis

Patients, tumors and treatment characteristics at baseline were accessed by a dichotomized minimum tumor Allred score (score = 0 vs score ≥ 2). The stability of baseline characteristics across a minimum tumor Allred score (score = 0 vs score ≥ 2) was ascertained using chi-square tests, and the *P*-values of the tests were reported. The median follow-up duration was derived using the reverse Kaplan-Meier method. The prognostic impact of the tumor pSTAT3 Allred score on disease-free survival (DFS) and overall survival (OS) was assessed by comparing the time to DFS/OS for patients with a pSTAT3-positive tumor (score ≥ 2) to the time to DFS/OS for patients without a

TABLE 2 Disease-free survival (DFS) and overall survival (OS) comparing patients with positive pSTAT3 or not

	Events (%)	N	Univariate HR (95% CI)	Univariate *P* value	Multivariate[a] HR (95% CI)	Multivariate *P* value
DFS	1035 (18.18%)	5694				
pSTAT3 negative -all	589 (19.25%)	3060	—	—	—	—
HR positive	325 (18.37%)	1769	—	—	—	—
HR negative	264 (20.45%)	1291	—	—	—	—
pSTAT3 positive [b]-all	446 (16.93%)	2634	0.86 (0.76-0.97)	.018	0.84 (0.74-0.95)	.006
HR positive	241 (15.30%)	1575	0.81 (0.69-0.96)	.014	0.80 (0.67-0.94)	.008
HR negative	205 (19.36%)	1059	0.94 (0.78-1.13)	.499	0.91 (0.76-1.09)	.311
OS	520 (9.13%)	5694				
pSTAT3 negative -all	285 (9.31%)	3060	—	—	—	—
HR positive	132 (7.46%)	1769	—	—	—	—
HR negative	153 (11.85%)	1291	—	—	—	—
pSTAT3 positive -all	235 (8.92%)	2634	0.94 (0.79-1.12)	.495	0.92 (0.78-1.10)	.374
HR positive	115 (7.30%)	1575	0.96 (0.75-1.23)	.733	0.93 (0.72-1.20)	.572
HR negative	120 (11.33%)	1059	0.95 (0.74-1.20)	.645	0.94 (0.74-1.19)	.603

[a]Multivariate models adjusted for significant covariates (timing of chemotherapy, lymph node status and pathological tumor size).
[b]Minimum tumor Allred score ≥2.

pSTAT3-positive tumor (score = 0). Log-rank tests were used to perform the comparison, and the *P*-values of the tests were reported. The Cox proportional hazard model was used in the multivariate analysis to generate hazard ratios (HR), while adjusting for covariates deemed clinically important and unbalanced with baseline assessments of patients and tumors characteristics. Sample size considerations in the ALTTO design focused on the comparison between the combination arms vs the trastuzumab-alone arm. The current analysis was based on 1035 (18.18%) DFS events and 520 (9.13%) OS events at a median follow-up of 6.93 years (range 6.85-6.97 years). *P*-values <.05 were considered significant. The data of this tumor marker study were analyzed according the essential elements of "reporting recommendations for tumor marker prognostic studies (REMARK)."[32]

3 | RESULTS

3.1 | Characteristics of the patient's pSTAT3 status

Of the 8381 patients enrolled in the ALTTO trial, 5694 patients (67.9%) were interpretable for STAT3 staining and they were included in the current analysis (Figure 2). A total of 2634 patients (46%) had evidence of STAT3 activation (minimum tumor Allred score ≥2 in all samples). The pSTAT3-positive and the pSTAT3-negative groups were compared by the chi-square test (Table 1).

3.2 | Relation between pSTAT3 status and outcome

The pSTAT3 expression was analyzed as a function of DFS and OS of the 5694 patients with complete available data. The median follow-up was 6.93 years (range 6.85-6.97 years), during which 1035 (18.18%) and 520 (9.13%) patients experienced DFS and OS events, respectively. Positive pSTAT3 staining was associated with improved DFS (HR 0.84, 95% CI 0.74-0.95; *P* = .006) in multivariate models adjusted for significant covariates (timing of chemotherapy, lymph node status and pathological tumor size) (Table 2; Supplementary Table 1). Of note, improved DFS in patients with positive pSTAT3 staining was limited to the hormone receptor-positive cohort (HR 0.80, 95% CI 0.67-0.94; *P* = .008), whereas positive pSTAT3 staining did not affect outcomes in the patients with hormone receptor-negative tumors (HR, 0.91 95% CI 0.76-1.09; *P* = .311) (Table 2). An analysis based on a mean pSTAT3 Allred score ≥2 demonstrated the same pattern of improved DFS in hormone receptor-positive breast cancer patients (Supplementary Table 2). OS was not affected by the pSTAT3 status in any subgroup (Table 2). Finally, based on the pSTAT3 status having been found to have an important role in the tumor microenvironment by strongly suppressing the antitumor immune response, a correlation was sought between stromal pSTAT3 staining with outcome, and the results failed to show any (Supplementary Table 3).

4 | DISCUSSION

The results of the present study in the setting of large adjuvant Phase III trial demonstrated that pSTAT3 is a marker of a better outcome in HR-positive/HER2-positive patients. Since STAT3 leads to activation of signaling pathways, it is suggested that tumors with active IL6-STAT3 pathway signaling would be associated with dismal outcome. Indeed, numerous in-vitro studies demonstrated that STAT3 inhibition or loss leads to tumor regression,[10,16,18,19,33-35] however, data from clinical samples showed conflicting results, with few trials having shown pSTAT3 to be associated with better

prognosis.[20,21,23,30] This was also demonstrated in other types of cancer as well.[36,37]

Studies on HER2-positive breast cancers have suggested that the activation of an STAT3 inflammatory loop leads to trastuzumab resistance by expanding stemness and EMT.[27,38] These observations were validated by clinical samples suggesting primary resistance to trastuzumab in the adjuvant setting.[25] However, in the HER2 positive/HR-positive subgroup, an opposite pattern was suggested.[25] The results of the present study support the role of STAT3 activation as a marker of favorable outcome in HR-positive/HER2-positive breast cancer patients, and did not demonstrate worse outcome or indication of trastuzumab resistance in any subset. A possible explanation is that different signal transduction pathways are involved in STAT3 activation in the HR-positive group in contrast to the HR negative group. For example, previous in-silico analysis showed that low proliferating luminal breast cancers were much more likely to possess a high pSTAT3 phenotype.[39] In addition, an analysis of TMAs from breast cancer patients revealed that pSTAT3 was associated with better prognosis, specifically, in luminal cancers.[30]

The finding that pSTAT3 is associated with better outcome suggests that tumors that activate this pathway are less aggressive than tumors that progress in the absence of STAT3 activation in some circumstances. Indeed STAT3 upregulates tissue inhibitor of metalloproteinase-1 (TIMP1) expression, which decreases invasiveness and serves as a tumor suppressor protein with a role in breast tissue cellular differentiation [40,41].

The strength of the current study was that it used prospective data from the very large cohort of the ALTTO trial. Its main limitation is that the analytical validity of pSTAT3 that employs TMAs is narrow, although the authors do not believe that these limitations detract from the significance of the findings.

While various STAT3 inhibitors are currently in the process of clinical evaluation at different stages,[14,15] their practical clinical utility and efficacy are limited. The observation that pSTAT3 status is a marker of favorable outcome in HR-positive/HER2 breast cancer raises several issues regarding patient management in this setting, and indicates that it is imperative to identify the precise context in which STAT3 inhibitors might be useful in breast cancer therapy.

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Amir Sonnenblick reports consulting or advisory role from: Eli Lilly, Pfizer, Novartis. Travel, accommodations expenses: Neopharm, Celgene, Speakers bureau: Teva, Roche, Pfizer. EdA Honoraria and/or advisory board from Roche/GNE, Novartis, SeaGen and Zodiac, Libbs; Travel grants from Roche, Novartis; Research grant to institution from Roche/GNE, Astra-Zeneca, GSK/Novartis and Servier. Miguel Izquierdo Novartis employee and own Novartis stocks. Minetta Liu Research grant to institution: EISKI, Genetech, GRAIL, MENAKINI, MERCK, Novartis, SeaGen, Teskro. Nadia Harbeck honoraria for consulting and/or lectures from Novartis. Martine Piccart Board Member (Scientific Board): Oncolytics, Consultant (honoraria): AstraZeneca, Camel-IDS, Crescendo Biologics, Debiopharm, Genentech, Immunomedics, Lilly, Menarini, MSD, Novartis, Odonate, Pfizer, Radius, Roche, Seattle Genetics. Research grants to institute: AstraZeneca, Lilly, MSD, Novartis, Pfizer, Radius, Roche-Genentech, Servier, Synthon. Alvaro Moreno-Aspita institutional research funding from Genentech, GSK, Daiichi, Novartis, Lilly, Sermonix.

DATA AVAILABILITY STATEMENT

The data will be made available upon reasonable request.

ETHICS STATEMENT

The study was approved by the ethics committees of all participating sites, and this substudy was approved by the ALTTO executive and translational committees. Clinical trial information: NCT00490139. Informed consent was obtained from all patients at study entry.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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