ESMO Translational Research Fellowship 2014 –2015

Phsophorylated-STAT3 and Trastuzumab resistance

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Final Report

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Project title: Phsophorylated-STAT3 and Trastuzumab resistance

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Brief summary of the proposal

The main objective of this study was to determine the role of Phosphorylated (Tyr705)-STAT3 status as a predictor of benefit from trastuzumab therapy in breast cancer. Thus, our first aim was to evaluate in vitro and in-silico the transcriptome- milieu of pSTAT3 positive tumours, and to develop a pSTAT3-gene expression signature that will be correlated with other validated signatures (immune, stromal etc.) both on the TCGA data and the Fin-Her randomized prospective trial gene expression data. Our second aim was to perform immunohistochemical and RNA analysis of pSTAT3 (or pSTAT3 signature) on tumour archives of patients with breast cancer who received versus not received trastuzumab at the adjuvant setting, and correlate the results with survival data.

Project results

pSTAT3 HER2-positive breast cancers are associated with a distinct gene expression profile

Reverse phase protein analysis (RPPA) of pSTAT3 (tyrosine 705) was performed on 51 primary HER2-positive breast cancers using the Responsify retrospective dataset. No significant correlation was found between pSTAT3 status and any classic clinico-pathological features.

To determine whether pSTAT3 signalling pathway activation was associated with specific transcriptional changes, we compared gene expression profiles obtained from pSTAT3-positive (Upper quartile) and pSTAT3-negative (Lower quartile) samples using a two-sample t test). 123 genes were significantly (fdr≤0.05) and differentially expressed between pSTAT3-positive and pSTAT3-negative tumors, suggesting a distinct gene expression pattern associated with pSTAT3 signaling pathway activation (Dataset Figure 1).

To assess whether these genes could predict the phosphorylation status of STAT3, we developed a pSTAT3 gene expression signature (pSTAT3-GS) by computing the scalar product of the coefficient of the genes with their respective gene expression values. As expected, we found a positive significant correlation between the pSTAT3 RPPA and the pSTAT3-GS expression levels in the whole Responsify dataset, from which the signature was developed (r=0.62, P=0.19e-6).

To independently validate the ability of the pSTAT3-GS to determine pSTAT3 proteomic status in HER2-positive breast cancer, we used the TCGA cohort of patients with HER2-positive breast cancer in which gene expression and RRPA data are available (16). As shown in Figure 1c, the ability of the pSTAT3-GS to classify tumors based on their pSTAT3 proteomic status was significant, validating its predictive performance (area under the curve [AUC] of the receiver operating curve, 0.78; p= 0.01).

A Gene Set Enrichment Analysis (GSEA) showed enrichment with Jak-STAT-regulated genes (p=0.006; fdr=0.14), as well as with genes associated with cell surface signal transduction and protein kinase activity. This suggests that the pSTAT3-GS captures pSTAT3 signaling pathway activation.

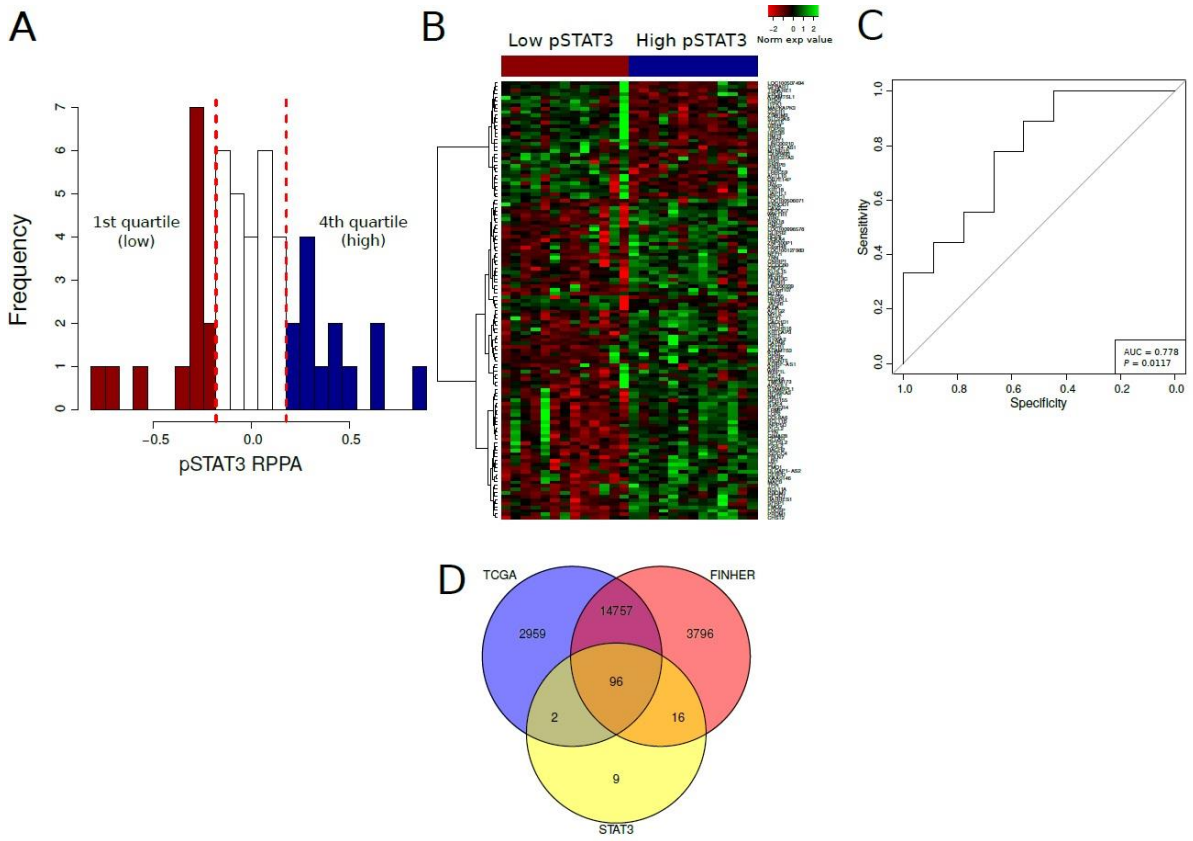


Figure 1. Schematic description of pSTAT3 gene signature building and assessment. (A) For 51 HER2-positive samples in the Responsify dataset we considered two sample groups with clear pSTAT3 "up" and "down" expression. (B) We compared gene expression profiles obtained from pSTAT3 positive (4th quartile) and pSTAT3 negative (1st quartile) samples using a two-sample t test. 123 genes were significantly (fdr≤0.05) and differentially expressed between pSTAT3 positive and pSTAT3 negative tumors. (C) ROC curve demonstrating predictive ability of the pSTAT3-GS as a continuous variable to predict pSTAT3 RPPA proteomic data in HER2-positive tumors in the TCGA repository. (D) Analysis of gene expression datasets comparing the genes (probes) of the STAT3 RPPA signatures from Responsify (blue) and genes whose expression were measured in the TCGA set (yellow) and the FinHer set (green).

Association of the pSTAT3-GS with clinical outcome in patients with HER2-positive breast cancer treated with trastuzumab

Since pSTAT3 has been reported to play a role in the pathogenesis of breast cancer through its positive effects on invasion, its modulation of the microenvironment, and its role as a negative regulator of immune cell–mediated antitumor responses, we hypothesized that pSTAT3 expression might influence response to trastuzumab therapy. To address this, we first correlated

the phosphorylation status of pSTAT3 with clinical outcome using the Responsify dataset, in which all patients received trastuzumab in the adjuvant setting and for which we had RRPA available data (N=51). No association was found between pSTAT3 protein levels and clinical outcome (Figure 2A-C).

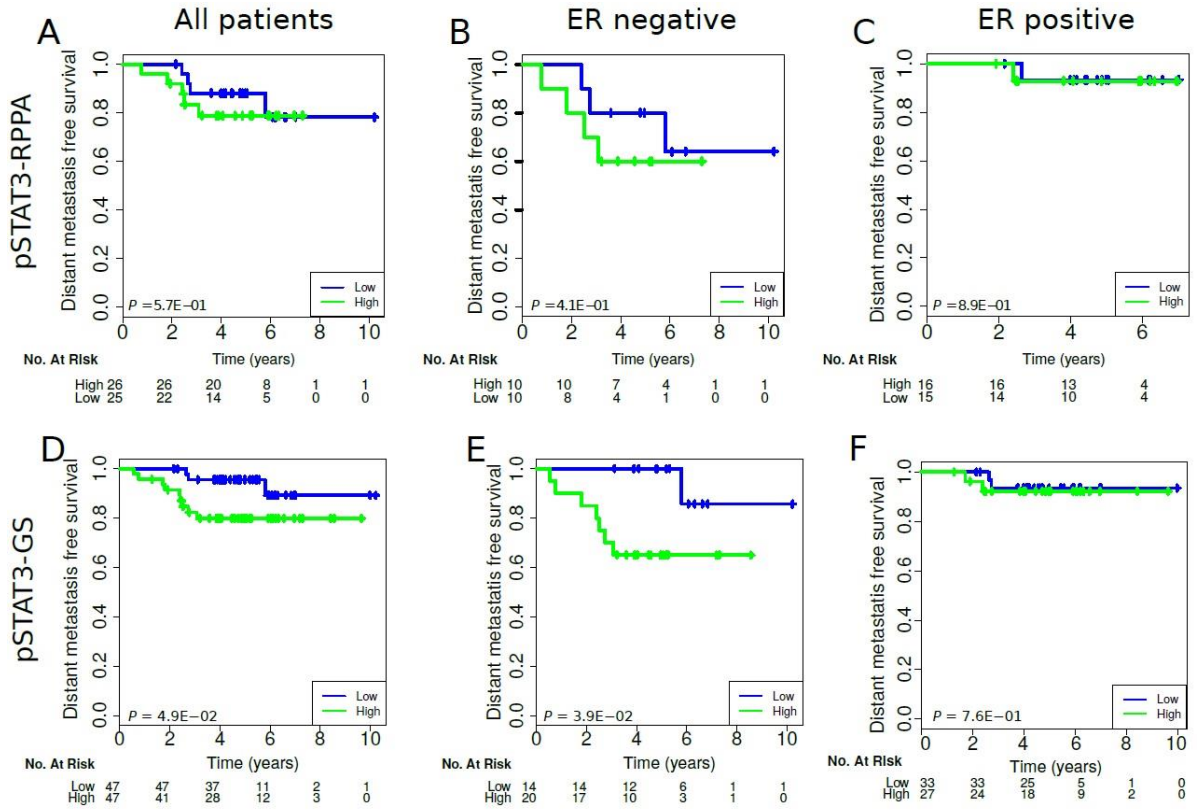
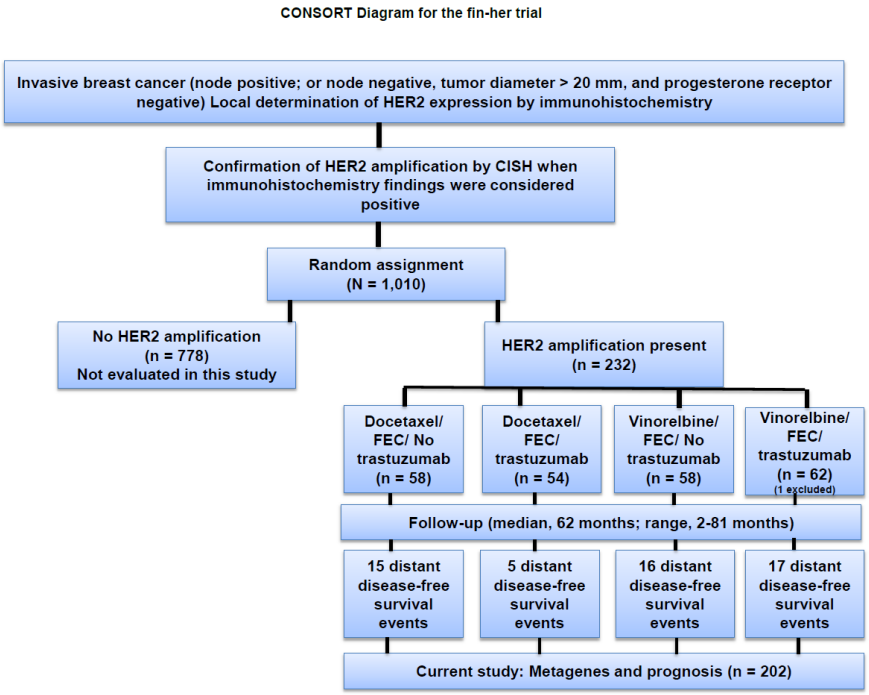


Figure 2. pSTAT3 RPPA and P-STAT3-GS survival analysis. (A-C) Kaplan-Meier curves according to pSTAT3 RPPA status in all patients (A), ER-negative (B) or ER- positive patients (C) for whom RPPA data was available in the Responsify dataset. (D-F) Kaplan-Meier curves according to pSTAT3-GS status in all patients (D), ER-negative (E) or ER-positive patients (F) for whom the gene expression data was available in the Responsify dataset.

Since the numbers were small, we sought to interrogate whether the pSTAT3-GS, which mirrors STAT3 pathway activation, could predict clinical outcome on the same dataset involving a larger number of patients with available gene expression data (N=94). Of note, the clinical outcome was not used to develop the pSTAT3-GS, and hence all the survival analyses were unbiased in their estimate of its performance. Interestingly, high pSTAT3-GS (dichotomized at the median) was significantly associated with poor outcome. This observation was mainly driven by the ER-negative subgroup (disease free survival [DFS], log rank p=0.039 for ER-negative) (Figure 2D-F).

These observations were further validated in an independent dataset of patients treated in the prospective FinHer trial, in which patients were randomized to trastuzumab in the adjuvant setting (CONSORT diagram and Table 1). In this validation series, high pSTAT3-GS was associated with lack of benefit from trastuzumab in the ER-negative subgroup when compared to low pSTAT3-GS (DDFS, p=0.003). Cox univariate analysis and multivariable analysis of the pSTAT3-GS treated as a continuous variable for each 20% increment in the FinHer study confirmed – with a significant interaction test of p=0.02, that the pSTAT3-GS could provide independent predictive information for patients with ER-negative breast cancer who had been treated with trastuzumab. Overall, our data suggest that pSTAT3 pathway activation is predictive for trastuzumab resistance in HER2-positive/ER-negative breast cancer.



	DDFS prognostic value of pSTAT3-GS (No trastuzumab)						DDFS prognostic value of pSTAT3-GS (trastuzumab)						
	Univariate			Multivariate			Univariate			Multivariate			*P* interaction
	HR	CI 95%	*P*	HR	CI 95%	*P*	HR	CI 95%	*P*	HR	CI 95%	*P*	
All	0.77	0.53-1.13	0.18	0.77	0.52-1.13	0.19	0.95	0.58-1.55	0.84	0.99	0.6-1.67	0.99	0.41
ER-	0.67	0.39-1.15	0.14	0.66	0.39-1.12	0.12	2.24	1.2-4.16	0.01	1.73	0.87-3.45	0.12	0.02
ER+	0.81	0.46-1.41	0.45	0.73	0.35-1.53	0.41	0.34	0.14-0.77	0.01	0.36	0.14-0.98	0.04	0.24

Table 1. Cox univariate and multivariable analysis of P-STAT3-GS treated as a continuous variable, in the FinHer study. For multivariate analysis, we considered the following variables: age, tumor size, grade, nodal status, and ER status. Interaction test- for the multivariate analysis.

pSTAT3 is associated with PTEN loss and stromal reactivation

Considering studies that have suggested that STAT3 could participate in oncogenesis through the up-regulation of genes encoding cell-cycle regulators (cyclins D1, c-Myc), and a recent report of in vitro data suggesting that PTEN signaling may be associated with trastuzumab resistance, we sought to investigate whether there was any relationship at the protein level between pSTAT3, PTEN and other proteins regulated by STAT3 in our clinical samples. In the Responsify dataset, we found that pSTAT3 was negatively correlated with PTEN (r=-0.4, fdr=0.025) and positively correlated with stathmin (r=0.66, fdr=0.03), a known marker of PTEN loss (Figure 3A). Other significant positive correlations with STAT3 included c-Myc (r=0.39, fdr=0.04), c-Kit (r=0.66, fdr=1.1e-5), and pEGFR (r=0.52, fdr=0.001). pcMET and CyclinD1 were also positively correlated, but did not pass the fdr≤0.05 threshold. These data confirm that in primary HER2-positive breast cancer, STAT3 participates in oncogenesis through the up-regulation of genes encoding cell-cycle regulators, and that PTEN loss may be associated with STAT3 activation.

Similar in silico analysis was performed at the gene expression level with the pSTAT3-GS and signatures involving different signalling pathways and biological processes. As seen in Figure 3B, the pSTAT3-GS was mainly correlated with several stromal signatures, suggesting a potential link between STAT3 expression and stromal activation. Of note, the pSTAT3-GS was positively correlated with IL6 (r=0.4, p=4.72e−05), which is the principal cytokine pathway through which STAT3 is activated in breast cancer and a surrogate of stromal reactivation.

Supportive findings have been reported for the TCGA dataset, in which HER2-positive breast cancers (based on the PAM50 classification) were analyzed according to their proteomic pSTAT3 status (Z±0.2 the interquartile range) using the C-bio portal. pSTAT3-positive tumors were associated with stromal reactivation genes, including high expression of POSTN, SRPX2, ADAM12, DACT1, and ADAMT6S (Fold>1, p<0.001) (figure 3C).

Overall these data suggest that there is a potential link between IL6-pSTAT3-PTEN loss, stroma reactivation, and primary trastuzumab resistance in HER2-positive primary breast cancers.

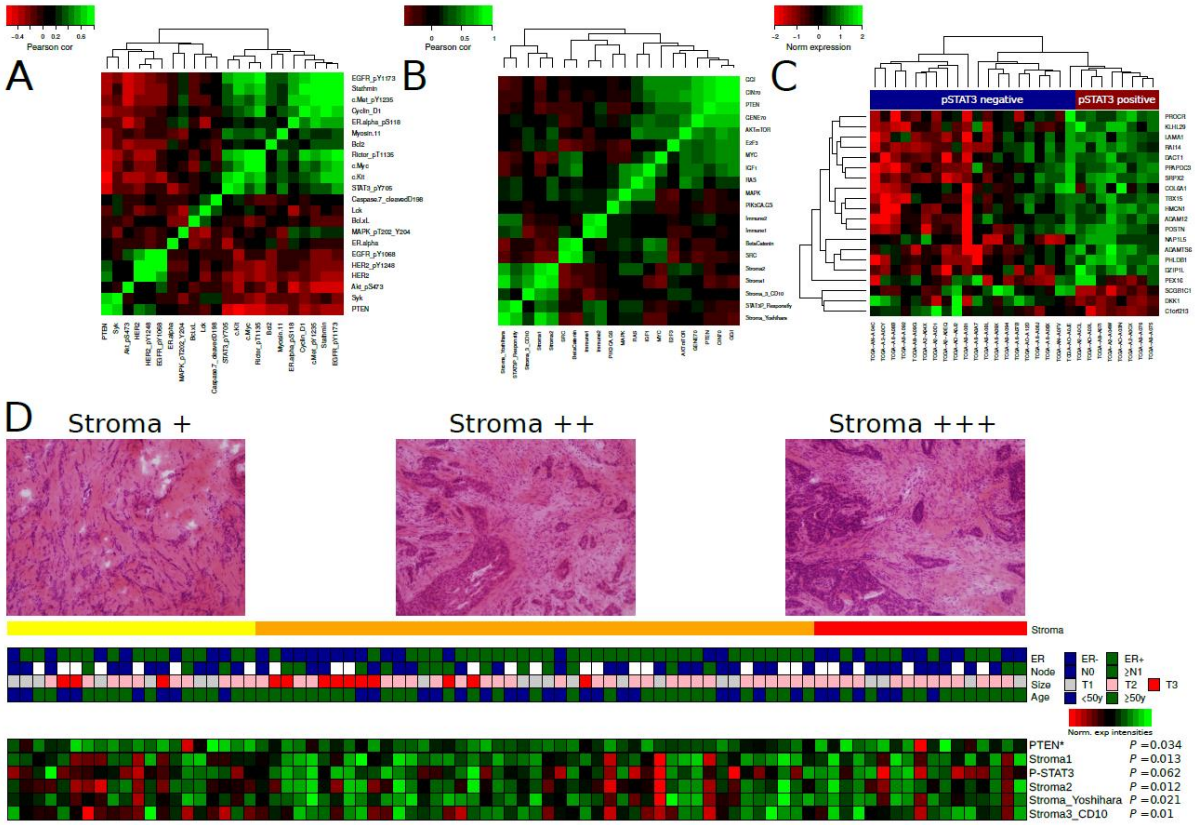
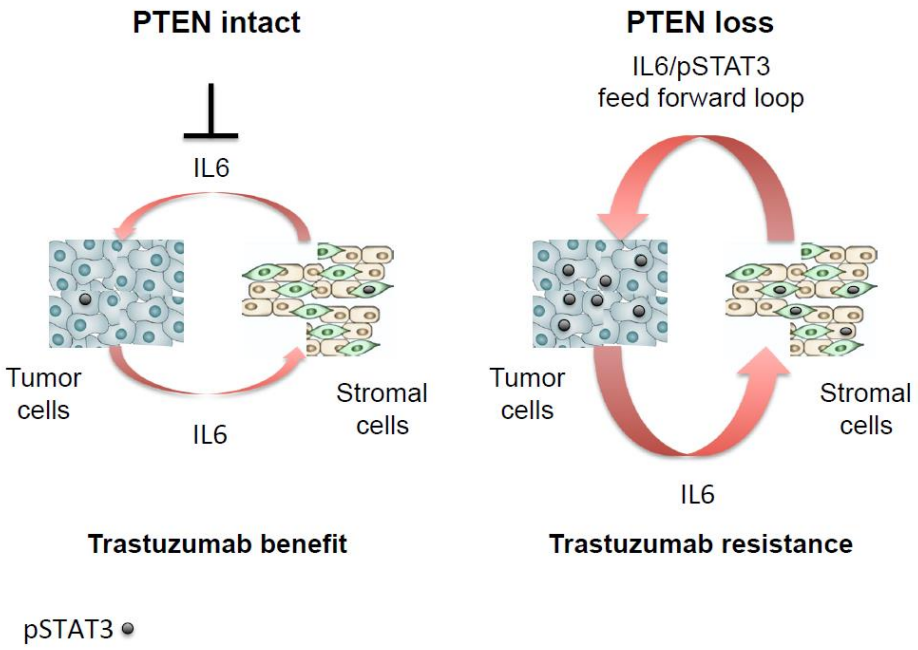


Figure 3

Figure 3. pSTAT3 and pSTAT3-GS are associated with PTEN loss and stromal reactivation. (A) pSTAT3 is associated with PTEN loss. Heatmap representation of the correlations between the RPPA values in the Responsify dataset. Cells are colored according to Pearson correlation coefficient values, with green indicating positive correlation and red negative correlations. (B) pSTAT3-GS is associated with stromal reactivation. The heat map reflects the hierarchic clustering of pairwise correlations between different gene signatures in the Responsify dataset. Cells are colored according to Pearson correlation coefficient values, with green indicating positive correlation and red negative correlations. (C) The heatmap of the top significantly enriched genes in PAM50-identified patients with HER2 positive breast cancer annotated in the TCGA ($p<0.001$,fold>1), selected according to the high or low RPPA expression level of pSTAT3. Cells are colored according to the gene expression values, with green indicating positive correlation and red negative correlation. (D) pSTAT3-GS correlates with histological stromal reactivation. Histological sections showing breast tumors containing low (+), intermediate (++) and high (+++) reactive stroma. Heatmap shows correlation of reactive stromal content with clinical pathological parameters (not significant) and correlation with different gene signatures including stromal signature and pSTAT3-GS. * Negative correlation, p values were assessed using Mann-Whitney test.

Conclusions

Overall, we propose that the STAT3-stromal feed-forward loop, which can be enhanced by PTEN loss, is predictive of primary trastuzumab resistance (model; figure 4). Inhibiting the IL6-STAT3 pathway may be a valuable addition to trastzumab treatment of primary HER2-positive breast cancer, especially those that are PTEN deficient.



Future directions

1. To determine the pSTAT3 status as a predictor of benefit from anti-HER2 therapy in breast cancer patients treated in the ALTTO trial.

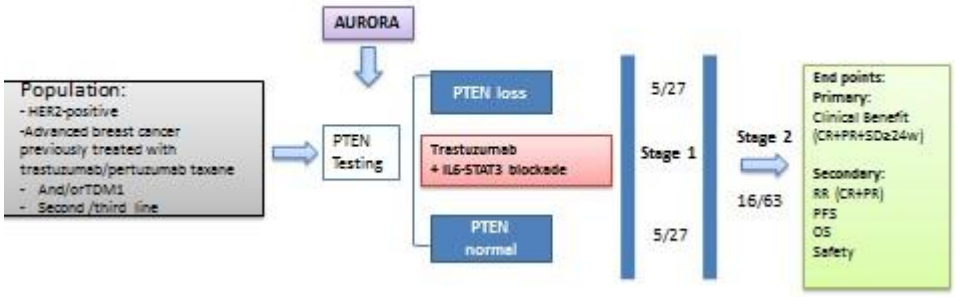
Data suggests that lapatinib is able to inhibit pSTAT3 suggesting that lapatanib may reverse trastuzumab resistance. Therefore, our goal will be to determine the role of pSTAT3 as a prognostic and predictive marker in response to trastuzumab/lapatinib based therapies in HER2 positive breast cancer patients.

Archived, formalin fixed, paraffin-embedded (FFPE) breast carcinoma TMAs from patients treated in ALTTO, which is an adjuvant randomized, phase III trial in HER2 positive patients comparing Trastuzumab and/or Lapatinib combinations, are available for translational research (300 TMA slides presenting specimens from 8,000 women in duplicates). The use of

2 TMAs slides from these specimens and research data were approved by the Translational Steering Committee of the ALTTO and BIG for our project. Upon recipient of the specimens immunodetection of pSTAT3 will be carried out and will be analyzed with the assistance of an automated cellular imaging system and blinded to all clinical information.

2**. Clinical trial phase 1/2: IL6-Jak-STAT3 axis inhibition in advanced HER2 positive breast cancer patients who developed resistance to trastuzumab.**

We have shown that the IL6- STAT3 feed forward loop, which can be enhanced by PTEN loss, is predictive of trastuzumab resistance. We therefore propose that Inhibiting the IL6-STAT3 pathway may be valuable in addition to trastuzumab for treatment of HER2 positive patients that progressed on trastuzumab especially if they are PTEN deficient. We designed a clinical phase 1/2 trial which was developed and approved at the FLIMS 2015- clinical trial workshop, that will evaluate whether inhibiting the IL6-STAT3 pathway may be valuable in addition to trastuzumab for treatment of HER2 positive patients that progressed on trastuzumab especially if they are PTEN deficient.



LIST OF PRESENTED ABSTRACTS RESULTING FROM THIS GRANT:

2014: Amir Sonnenblick, Hatem A. Azim Jr, Evandro de Azambuja, Prudence Francis, Bo Nordenskjöld, John Crown, Jorge Gutiérez, Emmanuel Quinaux, Mauro G. Mastropasqua, Lieveke Ameye , Michael Anderson, Ana Lluch, Raimund Jakesz, Aron Goldhirsch, Angelo Di Leo, Agusti Barnadas, Hernan Cortes-Funes, Martine Piccart. "Ten-year safety and efficacy analyses of the BIG 2-98 phase III trial with an exploratory analysis on the role of Ki67 in predicting benefit of adjuvant docetaxel in ER positive patients". ESMO 2014 Madrid. Poster discussion.

2014: Amir Sonnenblick, Sylvain Brohée, Debora Fumagalli, Christine Desmedt, Martine Piccart ,Heikki Jensu and Christos Sotiriou. Integrative proteomic and gene expression analysis identify annexinA1 and caveolin1 as predictive biomarkers for adjuvant trastuzumab resistance. SABCC 2014 San Antonio. Abstract

2015: Amir Sonnenblick, Hatem A. Azim Jr, Evandro de Azambuja, Prudence Francis, Bo Nordenskjöld, Jorge Gutiérez, Emmanuel Quinaux, Mauro G. Mastropasqua, Lieveke Ameye , Michael Anderson, Ana Lluch, Raimund Jakesz, Aron Goldhirsch, Angelo Di Leo, Agusti Barnadas, Hernan Cortes-Funes, Martine Piccart and John Crown. "Fianl analyses of the BIG 2-98 phase III trial and a Meta analysis on the role of Ki67 in predicting benefit of adjuvant docetaxel in ER positive patients". ISCORT 2014 Eilat. Oral Presentation.

2015: A. Sonnenblick, S. Brohée, R. F. Salgado, G. Van den Eynden, P. Neven , S. Loibl , C. Denkert , H. Joensuu, M. Piccart and C. Sotiriou. " Constitutively activated STAT3 is predictive for trastuzumab resistance in primary HER2 positive breast cancer". IMPAKT 2015 Brussels. Oral presentation. Best abstracts session

LIST OF PUBLICATIONS RESULTING FROM THIS GRANT

1. **Sonnenblick A**, Fumagalli D, Sotiriou C, Piccart M."Is the differentiation into molecular subtypes of breast cancer important for staging, local and systemic therapy, and follow up?" Cancer Treat Rev. 2014 Oct 4

2. **Sonnenblick A**, Fumagalli D, Azim HA Jr, Sotiriou C, Piccart M New Strategies in Breast Cancer: The Significance of Molecular Subtypes in Systemic Adjuvant Treatment for Small T1a,bN0M0 Tumors. Clin Cancer Res. 2014 December 15.

3. **Sonnenblick A**, de Azambuja E, Azim HA Jr, Piccart M. "An update on PARP inhibitors-moving to the adjuvant setting". Nat Rev Clin Oncol. 2015 January 12

4. **Sonnenblick A**, Piccart M. " Adjuvant systemic therapy in breast cancer: quo vadis ?". Ann Oncol. 2015 February 23

5. **Amir Sonnenblick**, Prudence A. Francis, Hatem A. Azim Jr, Evandro de Azambuja, Bo Nordenskjöld, Jorge Gutiérez, Emmanuel Quinaux, Mauro G. Mastropasqua, Lieveke Ameye, Michael Anderson, Ana Lluch, Michael Gnant, Aron Goldhirsch, Angelo Di Leo, Agusti Barnadas, Hernan Cortes-Funes, Martine Piccart, John Crown. "Final 10-year results of the Breast International Group 2–98 phase III trial and the role of Ki67 in predicting benefit of adjuvant docetaxel in patients with oestrogen receptor positive breast cancer". European Journal of Cancer. 2015 June 11.

6. **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, Denkert C, Joensuu H, Loi S, Sirtaine N, Kellokumpu-Lehtinen PL, Piccart M, Sotiriou C. "Constitutive phosphorylated STAT3-associated gene signature is predictive for trastuzumab resistance in primary HER2-positive breast cancer". BMC Med. 2015 Aug 3;13(1):177.

7. **Sonnenblick A**, Brohée S, Fumagalli D, Rothé F, Vincent D, Ignatiadis M, Desmedt C, Salgado R, Sirtaine N, Loi S, Neven P, Loibl S, Denkert C, Joensuu H, Piccart M, Sotiriou C. "Integrative proteomic and gene expression analysis identify potential biomarkers for adjuvant trastuzumab resistance: analysis from the Fin-her phase III randomized trial". Oncotarget. 2015 Aug 3

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