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**A phase II randomized study of preoperative trastuzumab alone or combined to everolimus in early HER2-positive breast cancer patients and predictive biomarkers (RADHER trial).**

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**Running title :** preoperative trastuzumab plus everolimus in breast cancer

**Keywords:** biomarker, everolimus, HER2+ breast cancer, PI3K/AKT/mTOR, trastuzumab

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## **Conflicts of interest statements**

MC received research fundings and served as speaker / advisor (compensated to the hospital) for Astra Zeneca, Novartis, Abbvie, Sanofi, Lilly, Pfizer, Sandoz, Accord, G1 Therapeutic, Pierre Fabre Oncology, Servier and Roche. TB served as speaker / advisor (compensated) for Roche and Novartis. BP received research fundings personal fees and non-financial support from Novartis. MA received research fundings and served as speaker / advisor (compensated to the hospital) for Novartis, Astra Zeneca, Seattle Genetics, Abvie and Pfizer. VD served as speaker / advisor (compensated) for Pfizer, Novartis, Daiichi Sankyo, and Lilly.

FA received research fundings and served as speaker / advisor (compensated to the hospital) for Roche, AstraZeneca, Daiichi Sankyo, Pfizer, Novartis, Lilly. JLM is a speaker for Roche and Novartis and a translational research board member for Novartis. Unicancer as the study sponsor received research funding (investigator sponsored study) by Roche and Novartis. All other authors declare no potential conflicts of interest.

## **Highlights**

- Multicentric phase II randomized study, recruiting 84 HER2 breast cancer
- Combining pre-operatively trastuzumab and everolimus in HER2 breast cancer
- No improvement of clinical or pathological response rate in the combination arm.
- Only increase of low grade toxicity (WHO 1-2) in the combination arm.
- Changes in MAPK pathway effectors in the combination arm

Abstract : 250 words

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## ABSTRACT

**Introduction:** Resistance to trastuzumab in breast cancer is an ongoing challenge. Clinical and biologic effects of co-targeting HER2 and mTOR in HER2-positive early operable breast cancer patients via the addition of everolimus to preoperative trastuzumab was evaluated in a phase II randomized study.

**Methods:** patients were randomized 1:1 to receive trastuzumab (4 mg/kg initial dose then 2 mg/kg weekly for 5 weeks) alone or combined with everolimus (10 mg/day for 6 weeks), then underwent surgery. Tumors were assessed by clinical examination and echography at baseline, and on treatment. The primary endpoint was the clinical response rate at 6 weeks. Pathologic response and safety were also evaluated. Baseline and surgery tumor samples were assessed by immunohistochemistry and multiplex immunoanalysis for predictive downstream effectors of the PI3K/AKT/mTOR and MAPK pathways.

**Results:** 82 patients were enrolled, 41 per arm. The clinical response rates were 34.1% and 43.9% with trastuzumab alone and combined with everolimus respectively. Pathologic response rates were 43.6% and, 47.5% respectively. Addition of everolimus increased toxicity, notably mucositis (82.5% versus 5.0%) and rash (57.5% versus 10.0%), but grade 3/4 events were rare. No correlation between response to treatments and baseline candidate biomarkers were identified, except for *PIK3CA* mutations which were found to predict trastuzumab resistance. Significant changes were seen in several MAPK pathway effectors after combination therapy.

**Conclusions:** The addition of everolimus did not improve the efficacy, but induced MAPK signaling. Combination therapy to overcome pathway cross-talk should be considered in order to maximize the effectiveness of trastuzumab in this setting.

ClinicalTrial.gov Identifier NCT00674414

## INTRODUCTION

Trastuzumab has considerably improved the outcome in HER2-positive breast cancer in both adjuvant and metastatic settings, with a clear survival benefit even more pronounced when combined with chemotherapy<sup>1-3</sup>. Nonetheless, 10% of operated patients treated with trastuzumab plus adjuvant chemotherapy develop resistance within 4 years, while almost all patients with metastatic disease ultimately progress under trastuzumab<sup>2</sup>.

Targeting the extracellular domain of HER2, trastuzumab, initially reported to trigger HER2 internalization and degradation, blocks downstream signaling pathways, mainly PI3K/AKT/mTOR and MAP kinase (MAPK) pathways which regulate cell proliferation and survival<sup>4</sup>. Trastuzumab was later proved to induce antibody-dependent cell cytotoxicity (ADCC)<sup>5</sup>. Resistance to trastuzumab can derive from dysregulation of multiple factors including increased signaling of upstream growth factor receptors, alterations of the HER2 receptor, mutation-induced repression of PTEN and/or activation of PI3K<sup>6</sup>. mTOR inhibitors targeting the PI3K/AKT/mTOR signaling pathway, address several of these mechanisms and have been proposed to overcome resistance to trastuzumab. Everolimus is an oral mTOR inhibitor approved for use in advanced hormone receptor-positive HER2-negative breast cancer. In preclinical studies, everolimus overcame PI3K/AKT/mTOR signaling-mediated trastuzumab resistance in PTEN-deficient breast cancer cells<sup>7</sup>, and acted synergistically with trastuzumab<sup>8</sup>, restoring inhibition of cell proliferation and induction of apoptosis. Some clinical trials reported that these preclinical benefits translate to the clinical setting; indeed, treatment with everolimus alone or with chemotherapy in trastuzumab-resistant HER2-positive metastatic breast cancer patients restored sensitivity to trastuzumab<sup>9</sup>.

A pilot study in 11 patients reported 1 pathologic complete response (pCR) and 4 clinical partial responses, suggesting the value of trastuzumab as monotherapy in the preoperative setting<sup>5</sup>. Further, the results of this study suggest that trastuzumab acts through ADCC more than to down-modulation of HER2 pathways. We confirmed these findings in HER2-positive breast cancer cell lines and in tumor specimens of patients receiving trastuzumab as pre-operative monotherapy<sup>10</sup>.

Subsequent studies of trastuzumab in combination with chemotherapy in neoadjuvant setting, presented pCR rates from 43% to 65%<sup>11-13</sup>.

The current study evaluated the addition of everolimus to preoperative trastuzumab in HER2-positive primary breast cancer in terms of clinical response at 6 weeks. Furthermore, this “window-of-opportunity” study allowed for multiple translational research on both pre and post-treatment samples. This could help understanding the pharmacodynamic activity of both

treatments and identifying potential molecular predictive biomarkers for efficacy of trastuzumab, alone or combined with everolimus.

## **PATIENTS AND METHODS**

### **Study Design**

This phase II, randomized, open-label study was performed in 6 French comprehensive cancer centers. One hundred and twenty patients were planned to be centrally randomized (1:1) to receive trastuzumab (weekly; 4 mg/kg intravenous loading dose then 2 mg/kg for 5 weeks), alone (Arm T) or combined with everolimus (10 mg/day for 6 weeks; last dose <24 hours prior to surgery) (Arm T+E), with stratification on primary tumor, regional lymph nodes, and estrogen receptor status. Two everolimus dose reductions for toxicity were permitted after recovery to grade 1. All patients underwent breast-conserving surgery after 6 weeks. Adjuvant chemotherapy, radiotherapy, and hormone therapy were left to the physician's decision according to local guidelines. The study was approved by a national ethics committee and the French Competent Health Authority, and was conducted in accordance with the Declaration of Helsinki.

### **Patients**

Patients had to be 18 years or older, have histologically-confirmed non-metastatic breast cancer with no prior treatment, HER2-positive (3+ immunohistochemistry (IHC) score or 2+ with gene amplification using *in situ* hybridization), and be candidate for breast-conserving surgery (T0-3, N0-2,  $\geq 15$  mm measured with ultrasonography). Patients had an Eastern Cooperative Oncology Group performance status of 0 or 1, and adequate hematologic, renal, hepatic, cardiac and pulmonary function. An amendment allowed the inclusion of patients with bilateral disease and with multifocal disease for whom a breast conserving surgery was not possible. Patients with inflammatory breast cancer, cardiac disease or other serious illness were not eligible. All patients gave written informed consent prior to enrolment.

### **Tumor assessments**

Tumor response was assessed by clinical examination and echography at baseline, then after 3 and 6 weeks (immediately before surgery). Three core needle biopsies were collected at baseline and 2 punch samplings were done on the surgery specimen for biomarker analyses. At each time point, one specimen was formalin-fixed paraffin-embedded (FFPE) for IHC and the others were snap-frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until analyzed.

Initially included as mandatory in the study protocol, the biopsy during pre-operative treatment *e.g.* 3 weeks after treatment initiation was amended as optional since it was a source of refusal to participate to the study by many patients.

### **Translational studies**

We selected candidate biomarkers to be tested before and after exposition to treatment for their potential interest in assessing efficacy and predicting sensitivity to trastuzumab and/or everolimus + trastuzumab. We hypothesized that trastuzumab and everolimus + trastuzumab combination could induce an arrest of proliferation, induction of apoptosis, inhibition of PI3K/AKT/mTOR and MAPK signaling pathways. Finally, anti-tumor immune reaction was assessed before and after treatment through evaluation of lymphocyte and macrophage infiltration. The pharmacokinetics and the metabolomic analyses associated to this study were already published<sup>14-15</sup>.

### ***PIK3CA alterations analysis***

As routinely used at the time of this study, *PIK3CA* gene (NM\_006218.2) exon 9 and 20 hot-spot pathogenic variants were analyzed by targeted next generation sequencing of DNA isolated from 63 FFPE tumor specimens (58/63) and frozen specimens (5/63), upon standard protocol with Ion Torrent PGM (ThermoFisher Scientific) following the manufacturer's instruction with an entire coverage of the two exons and an average depth of more than 1000X. The NestGENe software (SoftGenetics) was used for sequence alignment and variation calling.

### ***Immunohistochemistry***

All biomarkers but PTEN were assessed on FFPE specimens from baseline core needle biopsies and surgical residues. PTEN IHC was performed either on FFPE specimen from baseline biopsies or from FFPE surgical residues. FFPE blocks were cut at 4 µm thickness. Deparaffinization was performed on Discovery XT automate (Roche). After heat induced antigen retrieval in CC1 buffer (pH8.4) and blockage of the endogenous peroxidases in H<sub>2</sub>O<sub>2</sub>, the slides were incubated with anti-phospho-4EBP1 (p4EBP1) (Ser65), anti-phospho-S6 Ribosomal Protein (pS6RP) (Ser235/236) and anti-PTEN (138G6) (Cell Signaling), anti-LKB1 and anti-phospho-AKT1 (pAKT1) (Ser473) (Abcam), or with anti-Ki67 (SP6) (Thermo Scientific). Antibody binding was revealed using OmniMap anti-rabbit HRP kit (Roche) for LKB1, p4EBP1, pS6RP, Ki67, and PTEN antibodies or UltraMap anti-rabbit HRP kit (Roche)

for pAKT1 antibody followed by ChromoMap DAB kit (Roche). Sections were then counterstained with hematoxylin and bluing (Roche).

For eIF4E and Caspase 3, deparaffinization and antigen retrieval were performed on a PTLINK (Dako) using the target retrieval solution High pH (Dako). The slides were then incubated in Autostainer (Dako) for blockade of the endogenous peroxidases in H<sub>2</sub>O<sub>2</sub> followed by anti-eIF4E (Cell Signaling) or anti-caspase 3 (BD Biosciences). Antibody binding was revealed using Flex Envision kit (Dako). Sections were counterstained with hematoxylin (Dako).

Blinded to the clinical data, the slides were analyzed for both the percentage and the intensity (1+ =low, 2+ =intermediate, 3+ =high) of cytoplasmic staining in the infiltrative carcinomatous cells for pS6RP, caspase3 and eIF4E antibodies or nuclear staining for p4EBP1, PTEN and Ki67 antibodies and both cytoplasmic and nuclear staining for LKB1 and pAKT1 antibodies. A single score (from 0 to 6) was derived from intensity and percentage parameters of pAKT1, pS6RB, p4EBP1, eIF4E, LKB1 and PTEN staining, according to Allred et al.<sup>16</sup>. Allred scores at baseline and at surgery were compared. PTEN repression was considered when less than 50% of the cells were stained.

### ***Multiplex Bead Immunoassay***

The expression of pMEK1 and pERK1/2 was quantitatively analyzed using the Bio-Plex<sup>®</sup> Multiplex Immunoassay (Bio-Rad), as described by Chergui et al.<sup>17</sup>. Protein extracts from baseline and surgical frozen tumor samples (15-20 mg with at least 50% of tumor cells) were incubated overnight at 37°C with fluorescent capturing beads bearing antibodies against the target phosphoproteins (Bio-Rad), then exposed to streptavidin-phycoerythrin and detected with fluorescein-conjugated biotinylated antibodies. Results were recorded as mean fluorescence intensity of triplicated analyses.

### ***Immunological study***

Tumor slides from the baseline and surgical FFPE tumor samples were assessed for the infiltration by CD8<sup>+</sup> lymphocytes (Spring Bioscience, Roche) and CD68<sup>+</sup> macrophages (Dako) performed on a BenchMark XT immunostainer (Ventana). Antigen retrieval was performed by incubating slides on CC1 buffer for 36 minutes. Then the antibody was incubated for 1 hour at 37°C and revealed by ultraView Universal DAB Detection Kit (Roche). Scoring was performed as previously described<sup>18</sup>.

### **Endpoints**



The primary endpoint was the clinical response rate at 6 weeks (WHO criteria), analyzed in all randomized patients (intent to treat [ITT]). Secondary endpoints included the pCR rate at 6 weeks, safety graded by NCI-CTCAE v3.0, identification of candidate biomarkers predictive of response, pharmacodynamic and pharmacokinetic parameters of trastuzumab and everolimus plus trastuzumab combination. Using Sataloff classification<sup>19</sup>, pathologic response was centrally reviewed on the complete set of slides from the surgical specimen, and defined as pCR if total or near total therapeutic effect was established, or partial pathological response if a decrease in tumor cellularity of at least 50% was observed, excluding nodes.

### Statistical Analyses

With  $P_0=10\%$  (clinical response at 6 weeks with trastuzumab alone <sup>20</sup>) and using  $\alpha=0.05$  and  $\beta=0.05$ , 60 patients per arm were required to demonstrate a true improvement in clinical response to 40% ( $P_1$ ) with combined trastuzumab and everolimus. For missing clinical response data at 6 weeks, data from 3 weeks treatment were used. Clinical and pathologic responses were compared between treatment arms using Chi<sup>2</sup> tests. Non-parametric Wilcoxon tests were used for associations between baseline biomarkers and response. Cochran–Mantel–Haenszel tests were used to compare trastuzumab alone arm (arm T) and trastuzumab plus everolimus arm (arm T+E) parameters. Analyses were performed with SAS software (version 9.3) and R (version 3.3.2), using a 5% significance threshold.

## RESULTS

Due to slow recruitment rate, an independent data monitoring committee recommended stopping the study after randomization of 82 patients (41 per arm, **Figure 1**) between July 2008 and April 2012. Patient and tumor characteristics were well balanced between the two arms (**Table 1**).

### Antitumor Activity

The overall clinical rates were 34.1% and 43.9% and the pathological response rates were 43.6% and 47.5%, for arm T and arm T+E, respectively. No statistically significant difference was observed between both arms (**Table 2**). No significant difference in clinical ( $P=0.82$ ) and pathological response rate ( $P=0.36$ ) was observed between hormone receptor positive (HR+) and negative (HR-) tumors in both treatment arm (**Table S1**)

## Safety

Broadly speaking, the incidence of the adverse events (AEs) associated with trastuzumab treatment increased with the addition of everolimus (**Table S2**). Nonetheless, toxicity was largely mild to moderate, grade 3-4 AEs were only reported in arm T+E: Seven patients (17.5%) experienced mucositis, 2 (5.0%) hyperglycemia, 1 (2.5%) neutropenia, and 1 (2.5%) transaminitis. The occurrence rate of grade 3-4 AEs was significantly higher in patients in arm T+E with 22.5% of the patients having at least one grade 3-4 AE *versus* none in arm T ( $P=0.002$ , Fisher test). In arm T+E, 2 patients stopped both agents early, 1 due to grade 4 mucositis and the other one due to nausea/vomiting, transaminase elevations, and headache (all grade 2), and 3 patients stopped everolimus only due to grade 2-3 mucositis. In addition, 7 patients had everolimus dose reduction exclusively for grade 1 to 3 mucositis.

## Translational Research results

### Proliferation and apoptosis

Ki67 and caspase-3 were assessed on 55 matched baseline and surgery tumor samples. As a surrogate marker for proliferation, Ki67 significantly decreased between baseline and surgery in both arms ( $P<0.001$  in each arm). No difference was observed between the two arms (**Table 3**). While no difference was observed in arm T between clinical complete responders and non-complete responders, a significant difference was observed in arm T+E ( $P=0.036$ ) (**Figure 2**).

No difference in apoptosis induction, analyzed through activated caspase-3 expression, was observed at baseline and at surgery between arm T and arm T+E. Additionally, no treatment-induced variation was observed in arm T and in arm T+E. Furthermore, no relationship was observed with clinical response in both arms.

### HER2 downstream signaling biomarkers

#### *PTEN repression and PIK3CA alteration*

From 59 interpretable NGS, 14 were positive for a *PIK3CA* hot-spot pathogenic variant (23.7%). PTEN was obtained from 21 baseline biopsies or 38 surgery samples. Considering the entire patients population, no significant variation in *PIK3CA* mutation ( $P=0.991$ ) and in PTEN repression rates was observed between baseline and surgery ( $P=0.300$ ) (**Table S3**). There was no inter-arm significant difference in the variation in *PIK3CA* mutation and PTEN

expression rates, considered either alone or combined (PTEN loss or *PIK3CA* mutation). Moreover, *PIK3CA* mutational status was found to be related to clinical response ( $P=0.029$ ) with a higher proportion of responders (complete and partial responses) observed in patients with wild-type (92%) versus mutated *PIK3CA* tumors (8%) (**Table 4**). No variation in Ki67 labelled nuclei rate was observed between *PIK3CA* mutated and wild type tumors ( $P=0.95$ )

#### ***PI3-kinase – AKT – mTOR pathway***

pS6RP was assessed by IHC on 55 matched baseline and surgery tumor samples, pAKT1, p4EBP1, eIF4E on 54, and LKB1 on 53. No difference was observed at baseline and at surgery in PI3K/AKT/mTOR signaling biomarker expression between arm T and arm T+E. A significant decrease in pAKT1 (cytoplasmic and nuclear), pS6RP and nuclear LKB1 was observed between baseline and surgery (**Table 3**) while no variation was observed for cytoplasmic LKB1, eIF4E, and p4EBP1 in both arms. When PTEN repression was observed, no consequent decrease in pAKT expression was observed. Furthermore, no relationship was observed between any biomarker and clinical complete response in both treatment arms (**Figure S2**).

#### ***MAP kinase signaling pathway***

pMEK1 and pERK1/2 expression were obtained from 37 matched baseline and surgery tumor samples. Within arm T no variation in pMEK1 and pERK1/2 was observed between baseline and surgery, while within arm T+E, significant inductions of pMEK1 ( $P=0.005$ ) and pERK1/2 ( $P=0.030$ ) were observed, leading to a significant difference between the two treatment arms ( $P=0.014$  and  $0.017$  respectively) (**Table 3**).

#### **Lymphocyte and macrophage infiltration**

Lymphocyte and macrophage infiltration was assessed in 47 matched baseline and surgery tumor samples. On the 3 points scale, CD8 score increased significantly in both arms between baseline and surgery (mean increase 0.63 (SD=0.76),  $P=.007$  and 0.37 (SD=0.76),  $P=0.018$  for arm A and B, respectively) with no difference in the mean increase rate between arm A and B ( $P=0.32$ ). (**Table 3**). Furthermore, no difference was observed between the two arms (**Figure S1**). No difference was observed in CD68 tumor infiltrating macrophages rate between baseline and surgery in arm T and T+E, and between the two arms (**Table 3**). No relationship was found with complete clinical response (**Figure S1**).

#### **Relationship with pathological response**

For all biomarkers, no relationship was observed with complete clinical or overall pathological response (data not shown).

## DISCUSSION

PI3K/AKT/mTOR signaling represents a potential resistance mechanism to trastuzumab. We thus evaluated the clinical and biologic effects of a first line pre-operative mTOR and HER2 co-targeting therapy in HER2-positive early operable breast cancer patients. Our results confirm the clinical efficacy of trastuzumab given alone for a short period. The addition of everolimus proved a trend but not a significant increase in clinical response rate. In addition, pathologic response rates were similar for patients treated with or without everolimus. Although the premature termination of this study weakened the statistical power, the 95% level aimed in the hypothesis was ambitious. The 82 patients included still represents an acceptable 83% power, but our results are inconclusive on that point.

In this preoperative setting, none of the candidate biomarkers tested but *PIK3CA* mutational status was predictive of trastuzumab efficacy. Patients with wild type *PIK3CA* tumor had a higher clinical response rate than patients with *PIK3CA* mutated tumor in the overall population, potentially predicting trastuzumab efficacy. Andre *et al.*<sup>21</sup> showed that in HER2-positive advanced breast cancer, *PIK3CA* mutations may be a predictive biomarker for everolimus efficacy. However, sub-population analysis showed that only patients with hormone receptor-negative tumors had statistically significant progression free survival benefit with everolimus treatment. In the present study, only 17 out of the 41 patients randomized in the everolimus arm had hormone receptor-negative tumors. Given the low number of samples in this subgroup of patients, it was futile to draw conclusion for the predictive value of *PIK3CA* mutations for everolimus efficacy in this setting.

In the present trial, patients with HER2-positive breast cancers received trastuzumab alone or combined with everolimus in pre-operative setting. This drastically differs from other trials considering the impact of alteration of PTEN/PI3-kinase/AKT pathway of the clinical efficacy of drugs targeting this pathway, but mostly performed in HER-negative advanced breast cancers resistant to mTOR inhibitors<sup>22</sup>, in HER2-negative metastatic breast cancers resistant to aromatase inhibitors<sup>23,24</sup>, in metastatic triple negative breast cancers<sup>25,26</sup>.

In hormone receptor-positive, HER2-negative advanced breast cancers, data from BOLERO-2 trial<sup>27</sup>, showed that the PFS benefit with everolimus was maintained regardless of the hyperactivation status of PI3-kinase/mTOR pathway *i.e.* in tumor bearing at least one mutation in *PIK3CA*, *PTEN*, *AKT1*, or *PIK3R1* genes or low PTEN expression.

Further, even if the clinical benefit of PI3-kinase inhibitors<sup>22,28</sup> and AKT inhibitors<sup>24-26</sup>, compared to placebo, was observed in both PTEN/PI3-kinase/AKT altered and unaltered tumor bearing patients, no direct statistical comparison of the two groups was assessed to

ascertain the higher benefit in the PTEN/PI3-kinase/AKT altered subgroup, making any extrapolation very hazardous.

In PrE0102 trial<sup>23</sup>, as previously in BOLERO-2 and TAMRAD trials<sup>29,30</sup>, the improved PFS achieved by adding everolimus to fulvestrant in aromatase inhibitors-resistant tumors could be related to the development of aromatase inhibitors resistance that may select tumors being highly dependent on PI3K/AKT/mTOR pathway and more sensitive to mTOR inhibition. In BELLE-3 trial<sup>22</sup>, it was suggested that the higher efficacy of buparlisib in *PIK3CA* mutated tumors could be due to pretreatment of all patients with an mTOR inhibitor which may upregulate the PI3-kinase pathway via feedback activation. In LOTUS trial<sup>25</sup>, the differential effect of ipatasertib and paclitaxel in *PIK3CA/AKT1/PTEN* altered tumors was not statistically demonstrated with median PFS values of 9.0 (95% CI 4.6-NA) and 5.3 (95% CI 3.6-7.3) months, respectively in altered and non-altered tumors. In PAKT trial<sup>26</sup>, it was hypothesized that the benefit of capivasertib and paclitaxel might be limited to the *PIK3CA/AKT1/PTEN* altered tumors subgroup although an overall survival benefit cannot be excluded in patient with nonaltered tumors. In SOLAR-1 trial<sup>28</sup>, clinically relevant PFS benefit was initially reported in patients with hormone-refractory, HER2-negative advanced breast cancers with a *PIK3CA* mutation, but, more recently, updated data<sup>31</sup> reported no significant difference in OS with 39.3 months (95% CI, 34.1-44.9) in the alpelisib plus fulvestrant arm *versus* 31.4 months (95% CI, 26.8-41.3) in the placebo plus fulvestrant arm (HR = 0.86; 95% CI, 0.64-1.15; *P* = 0.15).

In FAKTION phase 2 trial<sup>24</sup>, the significant improvement of PFS observed with capivasertib and fulvestrant *versus* placebo observed in the overall population was preserved in the PI3-kinase/PTEN nonaltered group (HR 0.56, 0.33-0.96, *P*=0.035) but not in the altered group (HR 0.59, 0.34-1.03, *P*=0.064).

As compared to everolimus, using PI3-kinase inhibitors are associated with higher incidence of adverse events and higher cost. Therefore, the combination of everolimus to endocrine therapy could remain an effective and reasonably well-tolerated option in HR+ HER2-negative breast cancers progressing after endocrine therapy, independently of *PIK3CA* mutational status<sup>21,32</sup>

We reported here a rapid decrease of the PI3K/AKT/mTOR activity and tumor cell proliferation in response to trastuzumab treatment. Such pharmacodynamics of downstream pathways of HER2 were expected following trastuzumab therapy<sup>33</sup>. The addition of everolimus to trastuzumab did not synergized the PI3K/AKT/mTOR inhibition, but resulted in the activation of the MAPK pathway. PI3K/AKT/mTOR and MAPK pathways are interconnected with multiple crosstalk and feedback loops. Indeed, tumor biopsies obtained

from patient with melanoma, breast, and colon cancer treated in the neoadjuvant setting with everolimus displayed ERK activation<sup>34,35</sup>. The negative feedback loop suppression by mTOR inhibitor can explain the activation of MEK-ERK pathway in the combination arm suggesting the potential interest of combining mTOR with MEK inhibitors to allow full blockade of HER2 downstream signaling.

On the other hand, we detected rapid decrease of PTEN protein expression. PTEN, a negative regulator of the PI3K signaling, is mutated or lost in various cancers and loss of PTEN causes resistance to trastuzumab<sup>36</sup>. In our study, no variation in *PIK3CA* gene mutation and PTEN repression rates was detected between baseline and surgery and did not suggest any early induction of resistance to HER2 inhibition through the alteration of PI3-kinase signaling pathway.

There is increased evidence that ADCC is an important mechanism of action of trastuzumab. Furthermore, preclinical and clinical studies demonstrated that tumor-infiltrating lymphocytes can predict outcome of trastuzumab-containing treatment<sup>37,38</sup>. Our analyses showed an increase of tumor-associated lymphocytes infiltrates following only 6 weeks of trastuzumab confirming its efficacy to induce immune reactions, independently of everolimus treatment. This provide strong rationale for combining trastuzumab with enhancer of immunity such as checkpoint inhibitors. No relationship was observed between *PIK3CA* mutations and lymphocyte infiltration either at diagnosis ( $P=.099$ ) or at surgery ( $P=.043$ ). Our findings contrast from those achieved in post-operative setting in the ShortHER trial<sup>39</sup> with a better DFS within the HER2-enriched subtype, in *PIK3CA* mutated tumors, hypothetically in relation with an upregulation of immune-related genes. However our study differs from ShortHER trial regarding the duration of treatment by trastuzumab (6 weeks in our study vs 9 weeks or 1 year in ShortHER) and the combination of trastuzumab with chemotherapy in ShortHER trial.

In conclusion, combining everolimus with trastuzumab, altered the regulation of both PI3K and MAPK pathways, with evidence of early cell adaptation to treatment. Moreover, the inability to identify predictive biomarkers of activity suggests that response to trastuzumab alone or combined with everolimus may be driven by additional mechanisms.

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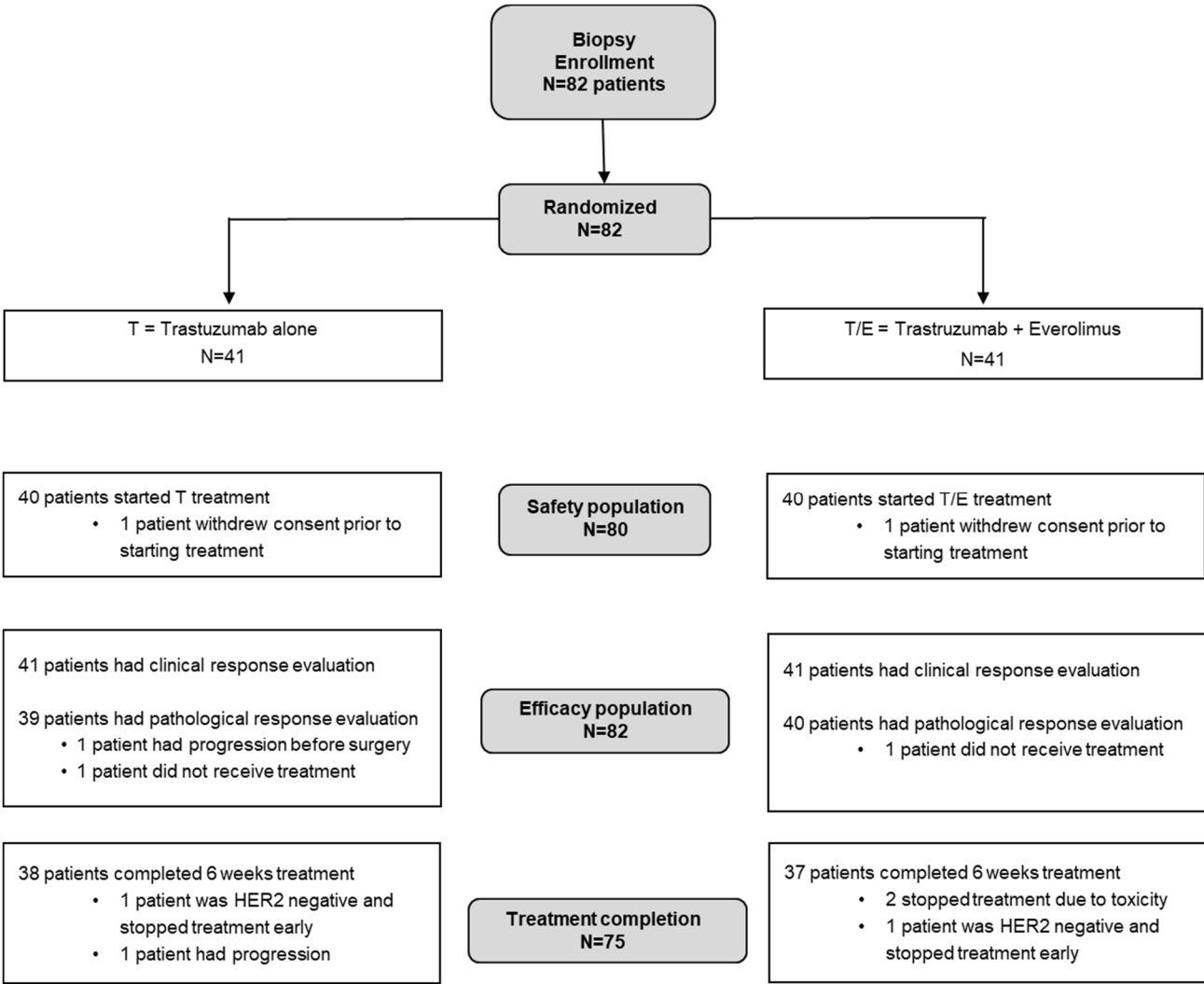


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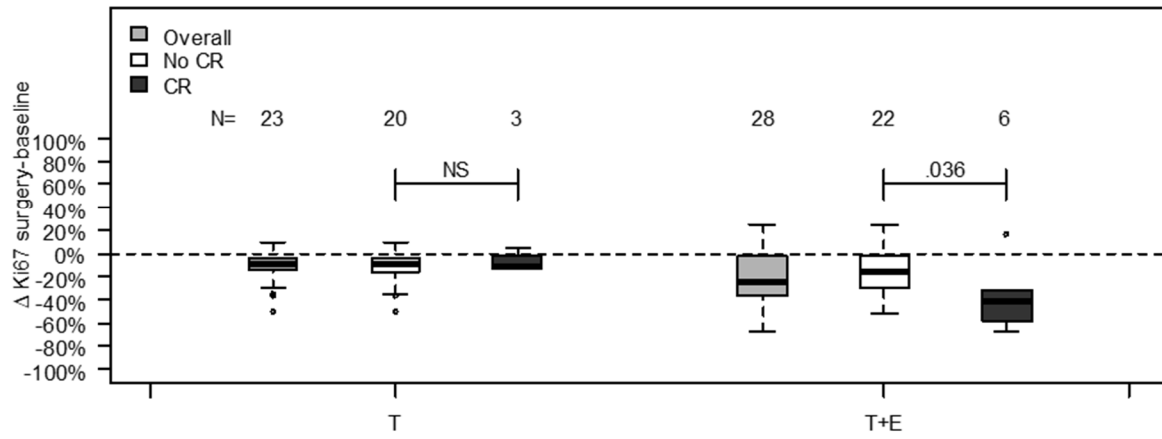
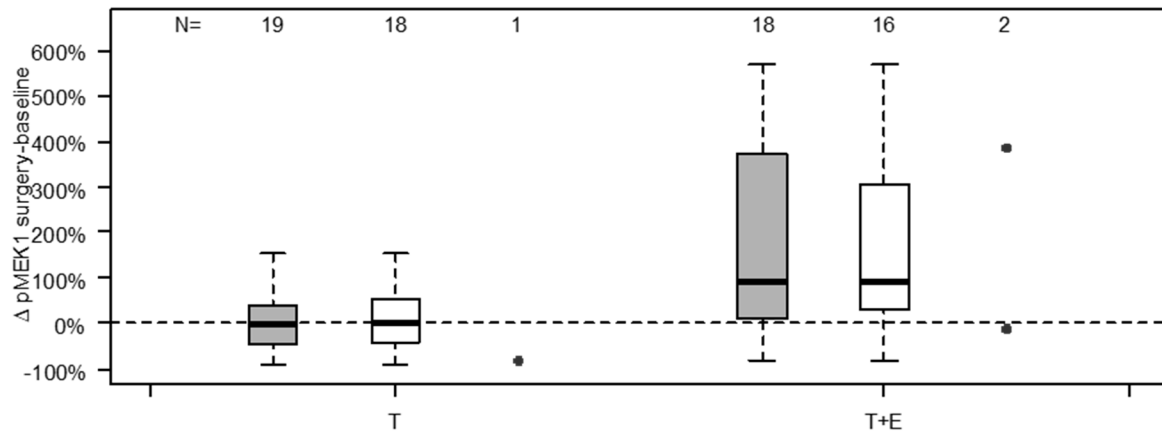
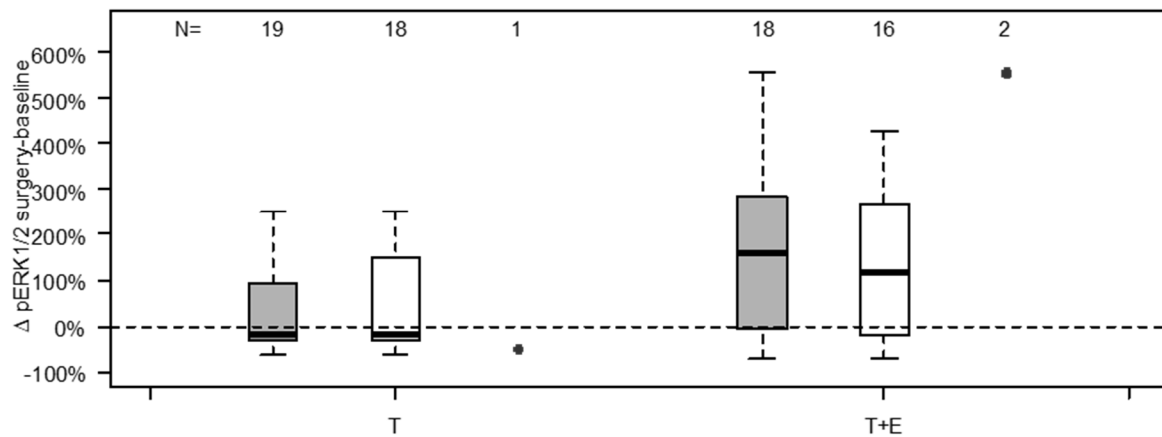
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**Figure 1: Flow-Chart of the study**



**Figure 2 : Relative Ki67 (A), pMEK1 (B) and pERK1/2 (C) variation calculated between initial biopsy and surgery following pre-operative treatment by trastuzumab alone (arm T) and trastuzumab + everolimus (arm T+E).**

*Light grey boxes are calculated from all patients' data, dark grey boxes from complete clinical responders and white boxes from non-complete responders.*

**A****B****C**

## TABLES

**Table 1. Patient, tumor and treatment characteristics, ITT population**

	<b>Arm T Trastuzumab alone N=41</b>	<b>Arm T+E Trastuzumab + Everolimus N=41</b>
Median age in years (range)	51 (25-78)	53 (28-75)
Performance status, <i>N (%)</i> <sup>a</sup>		
0	34 (85.0%)	38 (92.7%)
1	6 (15.0%)	3 (7.3%)
Tumor size		
Median in mm (range)	25 (0-90)	25 (15-80)
<15 mm, <i>N (%)</i>	9 (23.7%)	2 (5.3%)
Clinical T stage, <i>N (%)</i>		
T0	3 (7.3%)	1 (2.3%)
T1-T2	36 (87.8%)	42 (97.7%)
T3	2 (4.9%)	0
Regional lymph nodes, <i>N (%)</i> <sup>b</sup>		
N0	32 (78.1%)	33 (76.7%)
N1-N2	9 (21.9%)	10 (23.3%)
Histology grade, <i>N (%)</i> <sup>b</sup>		
I	1 (2.4%)	2 (4.7%)
II-III	39 (95.1%)	40 (95.3%)
HER2-positive, <i>N (%)</i> <sup>b,c</sup>	39 (95.1%)	41 (95.3%)
ER and/or PR positive, <i>N (%)</i> <sup>b</sup>	24 (58.5%)	24 (55.8%)
Adjuvant therapy, <i>N (%)</i>		
Chemotherapy	31 (75.6%)	28 (68.3%)
Hormone therapy	15 (65.2%)	11 (55.0%)
Radiotherapy	21 (80.8%)	22 (88.0%)

ER, estrogen receptor; PR, progesterone receptor

a. Missing data for 1 trastuzumab patient

b. N=43 in the combination arm due to two patients with bilateral disease

c. HER2 3+ or HER2 2+ with FISH/CISH/SISH +; Note that 3 treated patients (2 in the trastuzumab arm, 1 in the combination arm) were retrospectively identified as having HER2-negative tumors following systematic expert pathologist review. One patient with bilateral disease (in the combination arm) had an undetermined HER2 status on the contralateral tumor.

**Table 2. Summary of efficacy outcomes**

	<b>Arm T Trastuzumab alone</b>	<b>Arm T+E Trastuzumab + Everolimus</b>	<b>P-value</b>
<b>Clinical response (WHO)<sup>a,b</sup></b>	<i>N</i> =41	<i>N</i> =41	
Overall response rate, <i>N</i> (%)	14 (34.1%)	18 (43.9%)	0.365
Complete response, <i>N</i> (%)	7 (17.1%)	10 (24.4%)	
Partial response, <i>N</i> (%)	7 (17.1%)	8 (19.5%)	
<b>Pathologic response (Sataloff)<sup>b,c</sup></b>	<i>N</i> =39	<i>N</i> =40	
Overall response rate, <i>N</i> (%)	17 (43.6%)	19 (47.5%)	0.727
Complete response, <i>N</i> (%)	6 (15.3%)	3 (7.5%)	
Partial response, <i>N</i> (%)	11 (28.2%)	16 (40.0%)	

WHO, World Health Organization

- a. Complete response + partial response at 6 weeks or after 3 weeks in the absence of 6-week data, in the ITT population.
- b. Note that for the 2 bilateral patients, one breast was identified before initiating the treatment for the response assessment (one patient was HER2-positive on both sides and presented a stable disease and a complete pathologic response on the selected breast, while the contralateral tumor did not respond; the other patient was HER2-positive on one side, selected for response assessment, and had an undetermined HER2 status on contralateral tumor)
- c. Three patients were not evaluable; two not treated (one per arm), one patient progressed and was treated with chemotherapy before surgery (trastuzumab arm).



**Table 3 : Ki67 and signaling biomarkers analysis.**

	$\Delta$ arm T <sup>d,f</sup>	<i>P</i> value	$\Delta$ arm T+E <sup>d,f</sup>	<i>P</i> value	T vs T+E <sup>e</sup>
Ki67 <sup>a</sup>	-12.4 (14.5), <i>N</i> =23	<0.001	-20.8 (23.2), <i>N</i> =32	<0.001	NS
n_pAKT1 <sup>b</sup>	-2.09 (1.90), <i>N</i> =23	<0.001	-2.26 (1.98), <i>N</i> =31	<0.001	NS
c_pAKT1 <sup>b</sup>	-1.35 (2.25), <i>N</i> =23	0.009	-2.26 (2.41), <i>N</i> =31	<0.001	NS
pS6RP <sup>b</sup>	-0.78 (1.65), <i>N</i> =23	0.033	-1.50 (2.05), <i>N</i> =32	<0.001	NS
n_LKB1 <sup>b</sup>	-1.26 (2.28), <i>N</i> =23	0.015	-1.97 (2.50), <i>N</i> =30	<0.001	NS
c_LKB1 <sup>b</sup>	0.39 (1.50), <i>N</i> =23	NS	-0.40 (1.89), <i>N</i> =30	NS	NS
eIF4E <sup>b</sup>	0.04 (1.72), <i>N</i> =23	NS	0.42 (2.51), <i>N</i> =31	NS	NS
p4EBP1 <sup>b</sup>	0.00 (0.67), <i>N</i> =23	NS	0.03 (0.55), <i>N</i> =31	NS	NS
pMEK1 <sup>c</sup>	-1.3 (52.6), <i>N</i> =19	NS	115.2 (195.1), <i>N</i> =18	0.005	0.014
pERK1/2 <sup>c</sup>	0.04 (39.8), <i>N</i> =19	NS	48.3 (103.8), <i>N</i> =18	0.030	0.017
CD8	0.63 (0.76), <i>N</i> =19	0.007	0.37 (0.76), <i>N</i> =30	0.018	NS
CD68	0.33 (0.69), <i>N</i> =18	NS	0.10 (0.77), <i>N</i> =29	NS	NS

n\_pAKT1: nuclear phosphorylated AKT1, c\_pAKT1 : cytoplasmic phosphorylated AKT1, pS6RP : phosphorylated S6 ribosomal protein, n\_LKB1 nuclear LKB1, c\_LKB1: cytoplasmic LKB1

<sup>a</sup> % stained nuclei from IHC. <sup>b</sup> Allred scoring from IHC. <sup>c</sup> normalized fluorescence intensities from multiplex immunoassays. Relative variations ( $\Delta$ ) were calculated from baseline and surgery specimens and compared <sup>d</sup> within arm T and arm T+E and <sup>e</sup> between arms. Data are presented as <sup>f</sup> mean variation (SD, %).

**Table 3 : Ki67 and signaling biomarkers analysis.**

	$\Delta$ arm T <sup>d,f</sup>	<i>P</i> value	$\Delta$ arm T+E <sup>d,f</sup>	<i>P</i> value	T vs T+E <sup>e</sup>
Ki67 <sup>a</sup>	-12.4 (14.5), <i>N</i> =23	<0.001	-20.8 (23.2), <i>N</i> =32	<0.001	0.201
n_pAKT1 <sup>b</sup>	-2.09 (1.90), <i>N</i> =23	<0.001	-2.26 (1.98), <i>N</i> =31	<0.001	0.742
c_pAKT1 <sup>b</sup>	-1.35 (2.25), <i>N</i> =23	0.009	-2.26 (2.41), <i>N</i> =31	<0.001	0.269
pS6RP <sup>b</sup>	-0.78 (1.65), <i>N</i> =23	0.033	-1.50 (2.05), <i>N</i> =32	<0.001	0.211
n_LKB1 <sup>b</sup>	-1.26 (2.28), <i>N</i> =23	0.015	-1.97 (2.50), <i>N</i> =30	<0.001	0.293
c_LKB1 <sup>b</sup>	0.39 (1.50), <i>N</i> =23	0.278	-0.40 (1.89), <i>N</i> =30	0.304	0.228
eIF4E <sup>b</sup>	0.04 (1.72), <i>N</i> =23	0.539	0.42 (2.51), <i>N</i> =31	0.265	0.654
p4EBP1 <sup>b</sup>	0.00 (0.67), <i>N</i> =23	0.999	0.03 (0.55), <i>N</i> =31	0.790	0.663
pMEK1 <sup>c</sup>	-1.3 (52.6), <i>N</i> =19	0.953	115.2 (195.1), <i>N</i> =18	0.005	0.014
pERK1/2 <sup>c</sup>	0.04 (39.8), <i>N</i> =19	0.709	48.3 (103.8), <i>N</i> =18	0.030	0.017
CD8	0.63 (0.76), <i>N</i> =19	0.007	0.37 (0.76), <i>N</i> =30	0.018	0.317
CD68	0.33 (0.69), <i>N</i> =18	0.066	0.10 (0.77), <i>N</i> =29	0.488	0.248

n\_pAKT1: nuclear phosphorylated AKT1, c\_pAKT1 : cytoplasmic phosphorylated AKT1, pS6RP : phosphorylated S6 ribosomal protein, n\_LKB1 nuclear LKB1, c\_LKB1: cytoplasmic LKB1

<sup>a</sup> % stained nuclei from IHC. <sup>b</sup> Allred scoring from IHC. <sup>c</sup> normalized fluorescence intensities from multiplex immunoassays. Relative variations ( $\Delta$ ) were calculated from baseline and surgery specimens and compared <sup>d</sup> within arm T and arm T+E and <sup>e</sup> between arms. Data are presented as <sup>f</sup> mean variation (SD, %).

**Table 4 : *PIK3CA* mutation and PTEN expression loss rates, considered either alone or combined in trastuzumab alone (arm T) and trastuzumab + everolimus (arm T+E) and relationship with clinical response rate.**

		Arm T	Arm T+E	<i>P</i> value (T vs T+E)	Entire population	Clinical response rate (CR+PR) <sup>c</sup>	<i>P</i> value (No vs Yes)	Complete clinical response rate (CR) <sup>c</sup>	<i>P</i> value (No vs Yes)
<i>PIK3CA</i> mutation <sup>b</sup> N=59	No	23 (85.2%)	22 (68.8%)	0.242	45 (76.3%)	22 (91.7%)	0.029	10 (90.9%)	0.269
	Yes	4 (14.8%)	10 (31.2%)		14 (23.7%)	2 (8.3%)		1 (9.1%)	
PTEN loss <sup>a</sup> N=59	No	7 (29.2%)	14 (40%)	0.393	21 (35.6%)	10 (38.4%)	0.683	5 (41.7%)	0.623
	Yes	17 (70.8%)	21 (60%)		38 (64.4%)	16 (61.5%)		7 (58.3%)	
PTEN loss or <i>PIK3CA</i> mutation N=55	No	5 (21.7%)	7 (21.9%)	0.990	12 (21.8%)	7 (30.4%)	0.190	3 (30%)	0.673
	Yes	18 (78.3%)	25 (78.1%)		43 (78.2%)	16 (69.6%)		7 (70%)	

<sup>a</sup> PTEN expression was analysed using IHC, <sup>b</sup> *PIK3CA* mutations using NGS.

<sup>c</sup> calculated from data collected from the entire patient population (arm T and arm T+E)