



HAL
open science

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

Mario Campone, Thomas Bachelot, Isabelle Treilleux, Barbara Pistilli, Julia Salleron, Valérie Seegers, Monica Arnedos, Delphine Loussouarn, Qing Wang, Laurence Vanlemmens, et al.

► To cite this version:

Mario Campone, Thomas Bachelot, Isabelle Treilleux, Barbara Pistilli, Julia Salleron, et al.. A phase II randomised study of preoperative trastuzumab alone or combined with everolimus in patients with early HER2-positive breast cancer and predictive biomarkers (RADHER trial). *European Journal of Cancer*, 2021, 158, pp.169-180. 10.1016/j.ejca.2021.09.017 . hal-03469622

HAL Id: hal-03469622

<https://hal.science/hal-03469622v1>

Submitted on 5 Jan 2024

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Distributed under a Creative Commons Attribution - NonCommercial 4.0 International License

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).

Mario Campone¹, Thomas Bachelot², Isabelle Treilleux³, Barbara Pistilli⁴, Julia Salleron⁵, Valérie Seegers⁶, Monica Arnedos⁴, Delphine Loussouarn⁷, Qing Wang⁸, Laurence Vanlemmens⁹, Marta Jimenez¹⁰, Maria Rios¹¹, Véronique Diéras¹², Agnès Leroux¹³, Gilles Paintaud¹⁴, Keyvan Rezai¹⁵, Fabrice André⁴, Maëva. Lion¹³, Jean-Louis Merlin¹³

1. Department of Medical Oncology/ Cancer Research Center UMR-INSERM U892/CNRS 6299/ Bioinformatics Unit, Institut de Cancérologie de l'Ouest, Nantes, France
2. Department of Medical Oncology, Centre Léon Bérard, INSERM U1052, Lyon, France
3. Department of Pathology and Biopathology, Centre Léon Bérard, Lyon, France
4. Department of Medicine, Gustave Roussy, Villejuif, France
5. Biostatistics Unit, Institut de Cancérologie de Lorraine, Vandoeuvre-Les-Nancy, France
6. Oncology Data Factory and Analytics, Institut de Cancérologie de l'Ouest, Nantes, France
7. Department of Pathology, Hôpital G&R Laënnec, St. Herblain, France
8. Genomic Platform-Cancer Research Center of Lyon, Centre Léon Bérard, Lyon, France,
9. Department of Medical Oncology, Centre Oscar Lambret, Lille, France
10. R&D Unicancer, UNICANCER, Paris, France
11. Department of Medical Oncology, Institut de Cancérologie de Lorraine, Vandoeuvre-les-Nancy, France
12. Department of Medical Oncology, Institut Curie, Paris, France
13. Department of Biopathology, Institut de Cancérologie de Lorraine, Université de Lorraine, CNRS UMR 7039, CRAN, Vandoeuvre-Les-Nancy, France
14. François Rabelais University, CNRS, UMR 7292, Genetics, Immunotherapy, Chemistry and Cancer, Tours, France
15. Radio-Pharmacology Department, Institut Curie-Hôpital Rene Huguenin, Saint-Cloud, France

Running title : preoperative trastuzumab plus everolimus in breast cancer

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

Corresponding author

Jean-Louis Merlin
Institut de Cancérologie de Lorraine
Avenue de Bourgogne
54519 Vandoeuvre les Nancy, France
Tel: +33 3 83 65 60 62
jl.merlin@nancy.unicancer.fr

Financial support

Jean-Louis Merlin received grant from the French Health Ministry (PHRC 2008/17-2). Unicancer as the study sponsor received support from Novartis, and Roche.

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

Highlights : 5 bullets < 85 characters incl. spaces

Text : 3549 words (excluding abstract, highlights, acknowledgements and references)

Tables and figures : 6

Supplementary tables : 3

Supplementary figure : 1

References : 39

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 chemotherapy¹⁻³. 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 trastuzumab².

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 survival⁴. Trastuzumab was later proved to induce antibody-dependent cell cytotoxicity (ADCC)⁵. 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 PI3K⁶. 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 cells⁷, and acted synergistically with trastuzumab⁸, 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 trastuzumab⁹.

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 setting⁵. 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 monotherapy¹⁰.

Subsequent studies of trastuzumab in combination with chemotherapy in neoadjuvant setting, presented pCR rates from 43% to 65%¹¹⁻¹³.

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, ≥ 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°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 published¹⁴⁻¹⁵.

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 μ m 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 H₂O₂, 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 H₂O₂ 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.¹⁶. 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[®] Multiplex Immunoassay (Bio-Rad), as described by Chergui et al.¹⁷. 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⁺ lymphocytes (Spring Bioscience, Roche) and CD68⁺ 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 described¹⁸.

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 classification¹⁹, 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 alone²⁰) 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 Chi² 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.*²¹ 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 inhibitors²², in HER2-negative metastatic breast cancers resistant to aromatase inhibitors^{23,24}, in metastatic triple negative breast cancers^{25,26}.

In hormone receptor-positive, HER2-negative advanced breast cancers, data from BOLERO-2 trial²⁷, 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^{22,28} and AKT inhibitors²⁴⁻²⁶, 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 trial²³, as previously in BOLERO-2 and TAMRAD trials^{29,30}, 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 trial²², 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 trial²⁵, 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 trial²⁶, 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 trial²⁸, 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 data³¹ 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 trial²⁴, 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^{21,32}

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 therapy³³. 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^{34,35}. 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 trastuzumab³⁶. 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^{37,38}. 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 trial³⁹ 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.

ACKNOWLEDGEMENTS

We thank the patients and their families who participated in this study, the study sites for trial management, Céline Mahier Aït-Oukhtar from Unicancer for study management, EuraxiPharma for study management support, independent data monitoring committee

members (Jean-Luc Canon, Stefan Michiels, Serge Evrard), Sarah MacKenzie (funded by Unicancer, France) and Lilian Amrein from Unicancer for medical writing and editing.

REFERENCES

1. Slamon DJ, Leyland-Jones B, Shak S, Fuchs H, Paton V, Bajamonde A, et al. Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. *N Engl J Med*. 2001;344(11):783-92.
2. Romond EH, Perez EA, Bryant J, Suman VJ, Geyer CE, Jr., Davidson NE, et al. Trastuzumab plus adjuvant chemotherapy for operable HER2-positive breast cancer. *N Engl J Med*. 2005;353(16):1673-84.
3. Piccart-Gebhart MJ, Procter M, Leyland-Jones B, Goldhirsch A, Untch M, Smith I, et al. Trastuzumab after adjuvant chemotherapy in HER2-positive breast cancer. *N Engl J Med*. 2005;353(16):1659-72.
4. Lewis GD, Figari I, Fendly B, Wong WL, Carter P, Gorman C, et al. Differential responses of human tumor cell lines to anti-p185HER2 monoclonal antibodies. *Cancer Immunol Immunother*. 1993;37(4):255-63.
5. Gennari R, Menard S, Fagnoni F, Ponchio L, Scelsi M, Tagliabue E, et al. Pilot study of the mechanism of action of preoperative trastuzumab in patients with primary operable breast tumors overexpressing HER2. *Clin Cancer Res*. 2004;10(17):5650-5.
6. Vernieri C, Milano M, Brambilla M, Mennitto A, Maggi C, Cona MS, et al. Resistance mechanisms to anti-HER2 therapies in HER2-positive breast cancer: Current knowledge, new research directions and therapeutic perspectives. *Crit Rev Oncol Hematol*. 2019;139:53-66.
7. Lu CH, Wyszomierski SL, Tseng LM, Sun MH, Lan KH, Neal CL, et al. Preclinical testing of clinically applicable strategies for overcoming trastuzumab resistance caused by PTEN deficiency. *Clin Cancer Res*. 2007;13(19):5883-8.
8. Zhu Y, Zhang X, Liu Y, Zhang S, Liu J, Ma Y, et al. Antitumor effect of the mTOR inhibitor everolimus in combination with trastuzumab on human breast cancer stem cells in vitro and in vivo. *Tumour Biol*. 2012;33(5):1349-62.
9. Andre F, O'Regan R, Ozguroglu M, Toi M, Xu B, Jerusalem G, et al. Everolimus for women with trastuzumab-resistant, HER2-positive, advanced breast cancer (BOLERO-3): a randomised, double-blind, placebo-controlled phase 3 trial. *Lancet Oncol*. 2014;15(6):580-91.
10. Lion M, Harlé A, Salleron J, Ramacci C, Campone M, Merlin JL. Trastuzumab as a preoperative monotherapy does not inhibit HER2 downstream signaling in HER2-positive breast cancer. *Oncol Lett*. 2016;12(3):2028-2032.
11. Coudert BP, Largillier R, Arnould L, Chollet P, Campone M, Coeffic D, et al. Multicenter phase II trial of neoadjuvant therapy with trastuzumab, docetaxel, and carboplatin for human epidermal growth factor receptor-2-overexpressing stage II or III breast cancer: results of the GETN(A)-1 trial. *J Clin Oncol*. 2007;25(19):2678-84.
12. Coudert BP, Arnould L, Moreau L, Chollet P, Weber B, Vanlemmens L, et al. Pre-operative systemic (neo-adjuvant) therapy with trastuzumab and docetaxel for HER2-overexpressing stage II or III breast cancer: results of a multicenter phase II trial. *Ann Oncol*. 2006;17(3):409-14.
13. Buzdar AU, Ibrahim NK, Francis D, Booser DJ, Thomas ES, Theriault RL, et al. Significantly higher pathologic complete remission rate after neoadjuvant therapy with trastuzumab, paclitaxel, and epirubicin chemotherapy: results of a randomized trial in human epidermal growth factor receptor 2-positive operable breast cancer. *J Clin Oncol*. 2005;23(16):3676-85.

14. Bernadou G, Campone M, Merlin JL, Gouilleux-Gruart V, Bachelot T, Lokiec F, et al. Influence of tumour burden on trastuzumab pharmacokinetics in HER2 positive non-metastatic breast cancer. *Br J Clin Pharmacol*. 2016;81(5):941-8.
15. Jobard E, Trédan O, Bachelot T, Vigneron AM, Mahier Aït-Oukhatar C, Arnedos M, et al. Longitudinal serum metabolomics evaluation of trastuzumab and everolimus combination as pre-operative treatment for HER-2 positive breast cancer patients. *Oncotarget*. 2017;8(48):83570-83584.
16. Allred DC, Bustamante MA, Daniel CO, Gaskill HV, Cruz AB, Jr. Immunocytochemical analysis of estrogen receptors in human breast carcinomas. Evaluation of 130 cases and review of the literature regarding concordance with biochemical assay and clinical relevance. *Arch Surg*. 1990;125(1):107-13.
17. Chergui F, Chretien AS, Bouali S, Ramacci C, Rouyer M, Bastogne T, et al. Validation of a phosphoprotein array assay for characterization of human tyrosine kinase receptor downstream signaling in breast cancer. *Clin Chem*. 2009;55(7):1327-36.
18. Ladoire S, Mignot G, Dabakuyo S, Arnould L, Apetoh L, Rebe C, et al. In situ immune response after neoadjuvant chemotherapy for breast cancer predicts survival. *J Pathol*. 2011;224(3):389-400.
19. Sataloff DM, Mason BA, Prestipino AJ, Seinige UL, Lieber CP, Baloch Z. Pathologic response to induction chemotherapy in locally advanced carcinoma of the breast: a determinant of outcome. *J Am Coll Surg*. 1995;180(3):297-306.
20. Menard S, Balsari A, Casalini P, Tagliabue E, Campiglio M, Bufalino R, et al. HER-2-positive breast carcinomas as a particular subset with peculiar clinical behaviors. *Clin Cancer Res*. 2002;8(2):520-5.
21. Andre F, Hurvitz S, Fasolo A, Tseng LM, Jerusalem G, Wilks S, et al. Molecular Alterations and Everolimus Efficacy in Human Epidermal Growth Factor Receptor 2-Overexpressing Metastatic Breast Cancers: Combined Exploratory Biomarker Analysis From BOLERO-1 and BOLERO-3. *J Clin Oncol*. 2016;34(18):2115-24.
22. Di Leo A, Johnston S, Lee KS, Ciruelos E, Lønning PE, et al. Buparlisib plus fulvestrant in postmenopausal women with hormone-receptor-positive, HER2-negative, advanced breast cancer progressing on or after mTOR inhibition (BELLE-3): a randomised, double-blind, placebo-controlled, phase 3 trial. *Lancet Oncol*. 2018 Jan;19(1):87-100.
23. Kornblum N, Zhao F, Manola J, Klein P, Ramaswamy B, Brufsky A, et al. Randomized Phase II Trial of Fulvestrant Plus Everolimus or Placebo in Postmenopausal Women With Hormone Receptor-Positive, Human Epidermal Growth Factor Receptor 2-Negative Metastatic Breast Cancer Resistant to Aromatase Inhibitor Therapy: Results of PrE0102. *J Clin Oncol*. 2018 Jun 1;36(16):1556-1563.
24. Jones RH, Casbard A, Carucci M, Cox C, Butler R, Alchami F, et al. Fulvestrant plus capivasertib versus placebo after relapse or progression on an aromatase inhibitor in metastatic, oestrogen receptor-positive breast cancer (FAKTION): a multicentre, randomised, controlled, phase 2 trial. *Lancet Oncol*. 2020 Mar;21(3):345-357.
25. Kim SB, Dent R, Im SA, Espié M, Blau S, Tan AR, et al. LOTUS investigators. Ipatasertib plus paclitaxel versus placebo plus paclitaxel as first-line therapy for metastatic triple-negative breast cancer (LOTUS): a multicentre, randomised, double-blind, placebo-controlled, phase 2 trial. *Lancet Oncol*. 2017 Oct;18(10):1360-1372.
26. Schmid P, Abraham J, Chan S, Wheatley D, Brunt AM, Nemsadze G, et al. Capivasertib Plus Paclitaxel Versus Placebo Plus Paclitaxel As First-Line Therapy for Metastatic Triple-Negative Breast Cancer: The PAKT Trial. *J Clin Oncol*. 2020 Feb 10;38(5):423-433.

27. Hortobagyi GN, Chen D, Piccart M, Rugo HS, Burris HA 3rd, Pritchard KI, et al. Correlative Analysis of Genetic Alterations and Everolimus Benefit in Hormone Receptor-Positive, Human Epidermal Growth Factor Receptor 2-Negative Advanced Breast Cancer: Results From BOLERO-2. *J Clin Oncol*. 2016 Feb 10;34(5):419-26.
28. André F, Ciruelos E, Rubovszky G, Campone M, Loibl S, Rugo HS, et al. Alpelisib for PIK3CA-Mutated, Hormone Receptor-Positive Advanced Breast Cancer. *N Engl J Med*. 2019 May 16;380(20):1929-1940.
29. Piccart M, Hortobagyi GN, Campone M, Pritchard KI, Lebrun F, Ito Y, et al. Everolimus plus exemestane for hormone-receptor-positive, human epidermal growth factor receptor-2-negative advanced breast cancer: overall survival results from BOLERO-2†. *Ann Oncol*. 2014 Dec;25(12):2357-2362.
30. Bachelot T, Bourcier C, Cropet C, Ray-Coquard I, Ferrero JM, Freyer G, et al. Randomized phase II trial of everolimus in combination with tamoxifen in patients with hormone receptor-positive, human epidermal growth factor receptor 2-negative metastatic breast cancer with prior exposure to aromatase inhibitors: a GINECO study. *J Clin Oncol*. 2012 Aug 1;30(22):2718-24.
31. André F, Ciruelos EM, Juric D, Loibl S, Campone M, Mayer IA, et al. Alpelisib plus fulvestrant for PIK3CA-mutated, hormone receptor-positive, human epidermal growth factor receptor-2-negative advanced breast cancer: final overall survival results from SOLAR-1. *Ann Oncol*. 2021 Feb;32(2):208-217.
32. Vernieri C, Corti F, Nichetti F, Ligorio F, Manglaviti S, Zattarin E, Rea CG, Capri G, Bianchi GV, de Braud F. Everolimus versus alpelisib in advanced hormone receptor-positive HER2-negative breast cancer: targeting different nodes of the PI3K/AKT/mTORC1 pathway with different clinical implications. *Breast Cancer Res*. 2020 Apr 6;22(1):33.
33. Nahta R. Pharmacological strategies to overcome HER2 cross-talk and Trastuzumab resistance. *Curr Med Chem*. 2012;19(7):1065-75.
34. Saini KS, Loi S, de Azambuja E, Metzger-Filho O, Saini ML, Ignatiadis M, et al. Targeting the PI3K/AKT/mTOR and Raf/MEK/ERK pathways in the treatment of breast cancer. *Cancer Treat Rev*. 2013;39(8):935-46.
35. Carracedo A, Ma L, Teruya-Feldstein J, Rojo F, Salmena L, Alimonti A, et al. Inhibition of mTORC1 leads to MAPK pathway activation through a PI3K-dependent feedback loop in human cancer. *J Clin Invest*. 2008;118(9):3065-74.
36. Nagata Y, Lan KH, Zhou X, Tan M, Esteva FJ, Sahin AA, et al. PTEN activation contributes to tumor inhibition by trastuzumab, and loss of PTEN predicts trastuzumab resistance in patients. *Cancer Cell*. 2004;6(2):117-27.
37. Loi S, Michiels S, Salgado R, Sirtaine N, Jose V, Fumagalli D, et al. Tumor infiltrating lymphocytes are prognostic in triple negative breast cancer and predictive for trastuzumab benefit in early breast cancer: results from the FinHER trial. *Ann Oncol*. 2014;25(8):1544-50.
38. Varadan V, Gilmore H, Miskimen KL, Tuck D, Parsai S, Awadallah A, et al. Immune Signatures Following Single Dose Trastuzumab Predict Pathologic Response to Preoperative Trastuzumab and Chemotherapy in HER2-Positive Early Breast Cancer. *Clin Cancer Res*. 2016;22(13):3249-59.
39. Guarneri V, Dieci MV, Bisagni G, Brandes AA, Frassoldati A, Cavanna L, Musolino A, Giotta F, Rimanti A, Garrone O, Bertone E, Cagossi K, Nanni O, Piacentini F, Orvieto E, Griguolo G, Curtarello M, Urso L, Paré L, Chic N, D'Amico R, Prat A, Conte P. PIK3CA Mutation in the ShortHER Randomized Adjuvant Trial for Patients with Early HER2+

Breast Cancer: Association with Prognosis and Integration with PAM50 Subtype. Clin Cancer Res. 2020 Nov 15;26(22):5843-5851.

Figure 1: Flow-Chart of the study

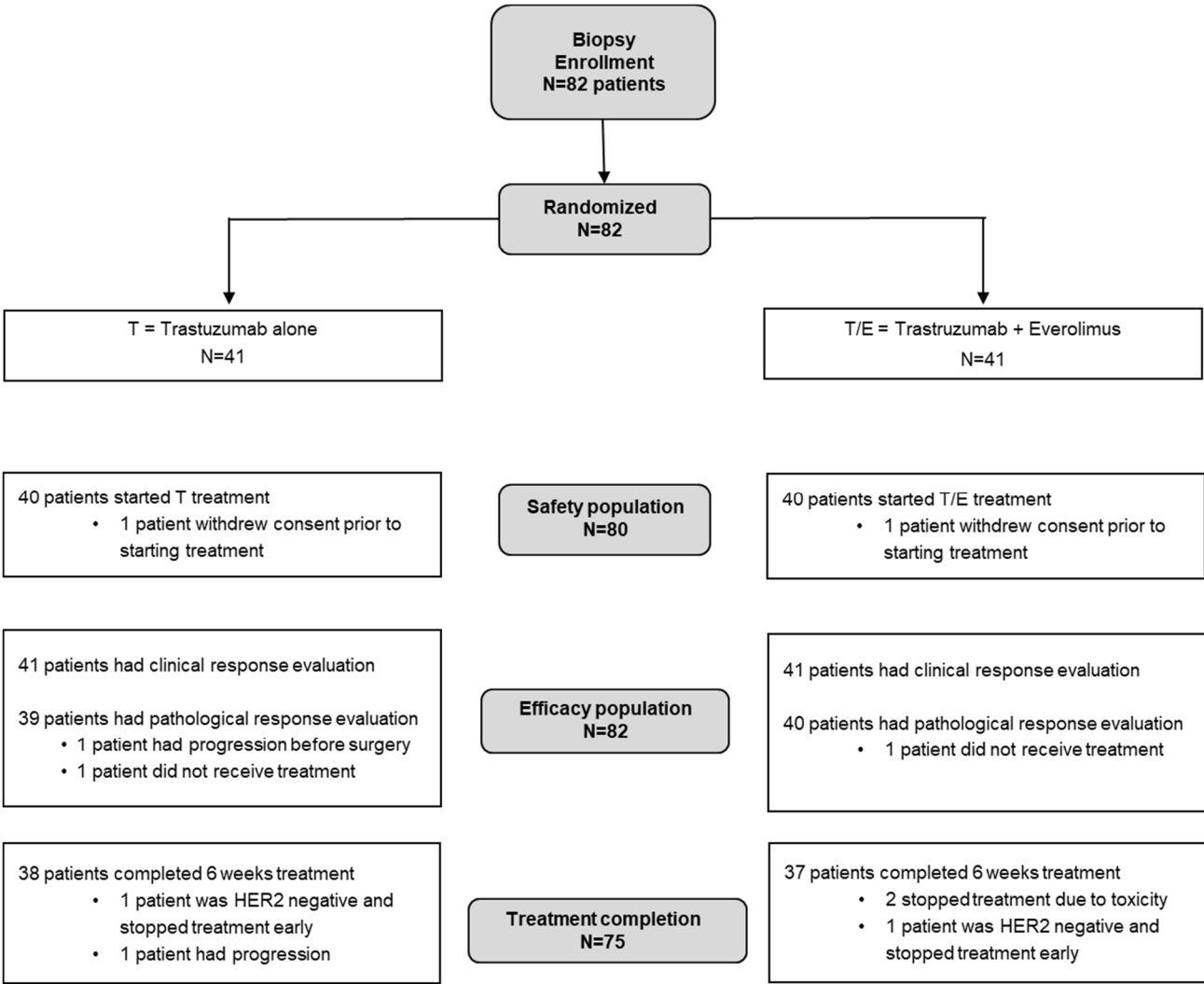
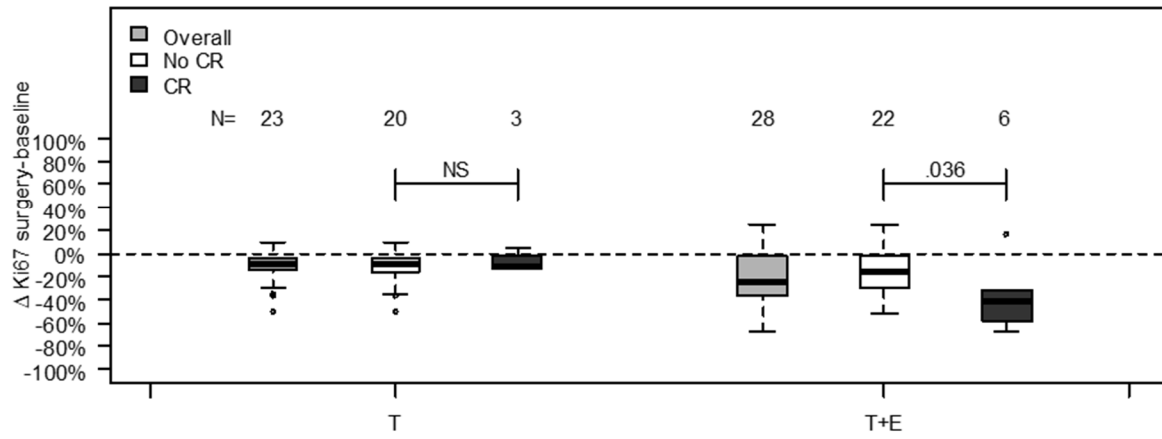
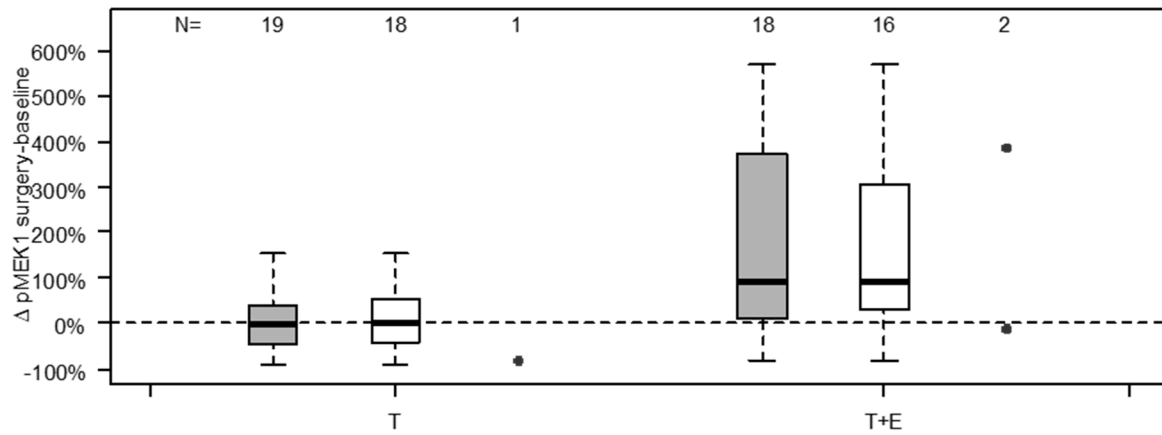
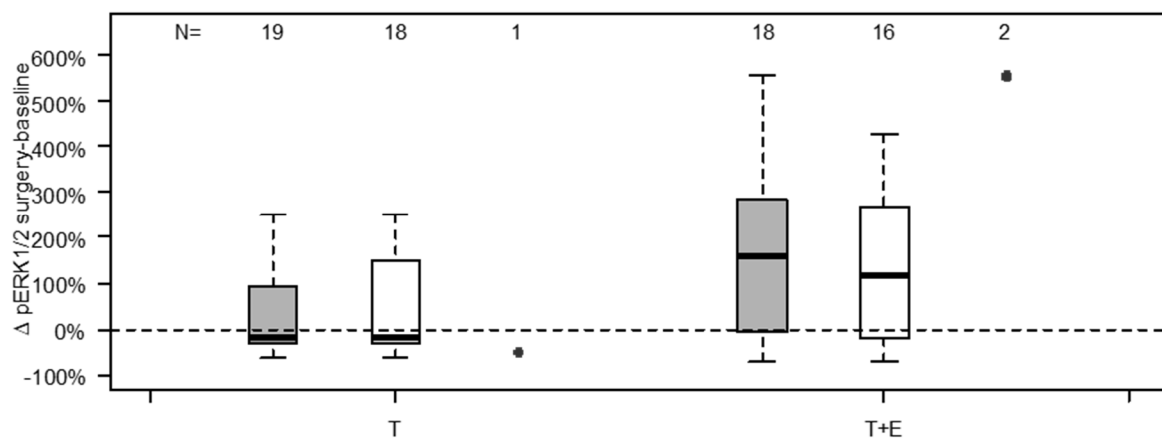


Figure 2 : Relative Ki67 (A), pMEK1 (B) and pERK1/2 (C) variation calculated between initial biopsy and surgery following pre-operative treatment by trastuzumab alone (arm T) and trastuzumab + everolimus (arm T+E).

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

	Arm T Trastuzumab alone N=41	Arm T+E Trastuzumab + Everolimus N=41
Median age in years (range)	51 (25-78)	53 (28-75)
Performance status, <i>N (%)</i> ^a		
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> ^b		
N0	32 (78.1%)	33 (76.7%)
N1-N2	9 (21.9%)	10 (23.3%)
Histology grade, <i>N (%)</i> ^b		
I	1 (2.4%)	2 (4.7%)
II-III	39 (95.1%)	40 (95.3%)
HER2-positive, <i>N (%)</i> ^{b,c}	39 (95.1%)	41 (95.3%)
ER and/or PR positive, <i>N (%)</i> ^b	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

	Arm T Trastuzumab alone	Arm T+E Trastuzumab + Everolimus	P-value
Clinical response (WHO)^{a,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%)	
Pathologic response (Sataloff)^{b,c}	<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

- Complete response + partial response at 6 weeks or after 3 weeks in the absence of 6-week data, in the ITT population.
- 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)
- 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.

	Δ arm T ^{d,f}	<i>P</i> value	Δ arm T+E ^{d,f}	<i>P</i> value	T vs T+E ^e
Ki67 ^a	-12.4 (14.5), <i>N</i> =23	<0.001	-20.8 (23.2), <i>N</i> =32	<0.001	NS
n_pAKT1 ^b	-2.09 (1.90), <i>N</i> =23	<0.001	-2.26 (1.98), <i>N</i> =31	<0.001	NS
c_pAKT1 ^b	-1.35 (2.25), <i>N</i> =23	0.009	-2.26 (2.41), <i>N</i> =31	<0.001	NS
pS6RP ^b	-0.78 (1.65), <i>N</i> =23	0.033	-1.50 (2.05), <i>N</i> =32	<0.001	NS
n_LKB1 ^b	-1.26 (2.28), <i>N</i> =23	0.015	-1.97 (2.50), <i>N</i> =30	<0.001	NS
c_LKB1 ^b	0.39 (1.50), <i>N</i> =23	NS	-0.40 (1.89), <i>N</i> =30	NS	NS
eIF4E ^b	0.04 (1.72), <i>N</i> =23	NS	0.42 (2.51), <i>N</i> =31	NS	NS
p4EBP1 ^b	0.00 (0.67), <i>N</i> =23	NS	0.03 (0.55), <i>N</i> =31	NS	NS
pMEK1 ^c	-1.3 (52.6), <i>N</i> =19	NS	115.2 (195.1), <i>N</i> =18	0.005	0.014
pERK1/2 ^c	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

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

Table 3 : Ki67 and signaling biomarkers analysis.

	Δ arm T ^{d,f}	<i>P</i> value	Δ arm T+E ^{d,f}	<i>P</i> value	T vs T+E ^e
Ki67 ^a	-12.4 (14.5), <i>N</i> =23	<0.001	-20.8 (23.2), <i>N</i> =32	<0.001	0.201
n_pAKT1 ^b	-2.09 (1.90), <i>N</i> =23	<0.001	-2.26 (1.98), <i>N</i> =31	<0.001	0.742
c_pAKT1 ^b	-1.35 (2.25), <i>N</i> =23	0.009	-2.26 (2.41), <i>N</i> =31	<0.001	0.269
pS6RP ^b	-0.78 (1.65), <i>N</i> =23	0.033	-1.50 (2.05), <i>N</i> =32	<0.001	0.211
n_LKB1 ^b	-1.26 (2.28), <i>N</i> =23	0.015	-1.97 (2.50), <i>N</i> =30	<0.001	0.293
c_LKB1 ^b	0.39 (1.50), <i>N</i> =23	0.278	-0.40 (1.89), <i>N</i> =30	0.304	0.228
eIF4E ^b	0.04 (1.72), <i>N</i> =23	0.539	0.42 (2.51), <i>N</i> =31	0.265	0.654
p4EBP1 ^b	0.00 (0.67), <i>N</i> =23	0.999	0.03 (0.55), <i>N</i> =31	0.790	0.663
pMEK1 ^c	-1.3 (52.6), <i>N</i> =19	0.953	115.2 (195.1), <i>N</i> =18	0.005	0.014
pERK1/2 ^c	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

^a % stained nuclei from IHC. ^b Allred scoring from IHC. ^c normalized fluorescence intensities from multiplex immunoassays. Relative variations (Δ) were calculated from baseline and surgery specimens and compared ^d within arm T and arm T+E and ^e between arms. Data are presented as ^f 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) ^c	<i>P</i> value (No vs Yes)	Complete clinical response rate (CR) ^c	<i>P</i> value (No vs Yes)
<i>PIK3CA</i> mutation ^b 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 ^a 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%)	

^a PTEN expression was analysed using IHC, ^b *PIK3CA* mutations using NGS.

^c calculated from data collected from the entire patient population (arm T and arm T+E)