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# PI3K/PTEN/mTOR pathway dynamic tracking and prognostic value in HR+/HER2− BC patients with residual disease after neoadjuvant chemotherapy: a cohort study

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► Additional supplemental material is published online only. To view, please visit the journal online (http://dx.doi.org/10.1136/jcp-2023-208856).

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Received 23 February 2023
Accepted 8 June 2023



**To cite:** Miglietta F, Carraro V, Amato O, *et al*. *J Clin Pathol* Epub ahead of print: [*please include* Day Month Year]. doi:10.1136/jcp-2023-208856

## ABSTRACT

**Aims** Hormone receptor-positive (HR)+/HER2− breast cancer (BC) is highly heterogeneous, with PI3K/PTEN/mTOR pathway alterations emerging as possible players within this complexity. We longitudinally tracked PI3K/PTEN/mTOR pathway dynamics from baseline biopsy to residual disease (RD)—and to metastases in case of relapse—in HR+/HER2− BC patients receiving neoadjuvant chemotherapy (NACT).

**Methods** HR+/HER2− BC patients with RD after NACT were identified. We assessed *PIK3CA* mutational, Pten-loss and phosphorylation levels of mTOR and its substrates (p70S6K and 4EBP1) on baseline biopsies and matched RD samples; in case of disease relapse, we also assessed *PIK3CA* mutational status on metastatic samples. Recurrence-free survival (RFS) was adopted as endpoint.

**Results** 92 patient were included. The conversion rate of *PIK3CA* mutational status was 12.8%; 1 patient acquired *PIK3CA* mutation at relapse; the rate of Pten conversion was 33.3%; mTOR phosphorylation levels significantly increased from baseline biopsy to RD, while its substrates significantly decreased. Baseline phosphorylated-mTOR significantly predicted poorer RFS in patients with *PIK3CA* wild-type status; baseline phosphorylated-70S6K was positively associated with RFS.

**Conclusions** We observed that PI3K/PTEN/mTOR pathway is highly dynamic under NACT exposure and the assessment of *PIK3CA* mutations may capture only a small fraction of such complexity. In this context, mTOR activation trough alternative pathways with respect to *PIK3CA* signalling may have a crucial role in shaping the molecular landscape of HR+/HER2− BC with RD after NACT. It is imperative to further elucidate the role of *PIK3CA* and mTOR-dependent pathways in shaping chemoresistance and endocrine resistance in high-risk HR+/HER2− early/locally advanced BC patients.

**WHAT IS ALREADY KNOWN ON THIS TOPIC**

⇒ PI3K/PTEN/mTOR pathway alterations are emerging as key players within the complex molecular landscape of HR+/HER2− BC, but little is known on PI3K/PTEN/mTOR pathway dynamics during disease evolution in patients undergoing neoadjuvant treatment.

**WHAT THIS STUDY ADDS**

⇒ PI3K/PTEN/mTOR pathway proved to be highly dynamic under neoadjuvant treatment exposure with PIK3CA mutational status only capturing a small fraction of such complexity.

⇒ Neoadjuvant treatment-induced mTOR activation may be driven by alternative pathways, both upstream and downstream, with mTOR relying on PI3K-independent triggers and different substrates to mediate its action.

**HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY**

⇒ It is imperative to further elucidate the role of PIK3CA and mTOR-dependent pathways in shaping chemoresistance and endocrine resistance in high-risk HR+/HER2− early/locally advanced BC patients.

## BACKGROUND

Hormone receptor-positive(HR+)/HER2-negative (HER2−) breast cancer (BC) encompasses almost two-thirds of all breast tumours.[1 2] It is now widely accepted that HR+/HER2− BC represents a highly heterogeneous disease, where the clinical complexity reflects an even greater—and only partially uncovered—complexity at a molecular and biological level. Indeed, in the past years, major efforts have been made to unravel the mechanisms underlying different clinical behaviours in terms of prognosis and treatment response for apparently clinicopathologically similar diseases,[3] with PI3K/PTEN/mTOR pathway alterations emerging as one of the possible players within this complex landscape.[4 5] Moreover, the role of this major intracellular network has gained increasingly appeal in the light of the progressive availability of treatment strategies selectively targeting and inhibiting this pathway.[6–9] Within this framework, available evidence is consistent in highlighting a crucial role of PI3K/PTEN/mTOR pathway in shaping the resistance to endocrine-based treatments, mostly in the advanced setting. In the early setting (including

locally advanced BC, LABC), accumulating evidence suggests PI3K-mediated signalling as possibly implicated in both chemoresistance[10 11] and endocrine resistance,[10–13] with conflicting data regarding the prognostic impact.[5 14] However, a comprehensive assessment of the dynamic behaviour of PIK3CA mutations and its major downstream effectors in HR+/HER2− early/LABC (EBC/LABC) is lacking.

Neoadjuvant treatment, besides the undeniable benefits from a purely clinical perspective, represents a strategical research platform,[15] offering a unique translational window of opportunity, allowing to dynamically assess tissue biomarkers immediately before and after the exposure to systemic treatment. In HR+/HER2− EBC/LABC, neoadjuvant chemotherapy (NACT) represents the standard of care in case of locally advanced/large tumours or when optimal surgery is not feasible upfront.[16]

The main aim of the present work was to longitudinally track PI3K/PTEN/mTOR pathway dynamic behaviour from baseline biopsy to residual disease (RD) and metastases in case of relapse, and assess its prognostic value in HR+/HER2− BC patients undergoing NACT.

## METHODS

### Patient cohort

Consecutive patients with stages II–III HR+/HER2− BC treated with NACT at our Institution (2000–2013) were identified from a prospectively maintained database. Those failing to achieve pathological complete response (pCR) were included in the present study.

HR-positivity was defined as positive immunohistochemistry (IHC) staining in ≥10% of tumour cells for either oestrogen receptor (ER), progesteron receptor or both. HER2-negativity was defined as IHC score 0/1+/2+ in the absence of gene amplification by in situ hybridisation.

### Histomolecular characterisation

Formalin-fixed paraffin-embedded baseline biopsies and matched surgical specimens were retrieved from the Surgical Pathology Unit of the Padua University Hospital and cases were jointly re-evaluated by two experienced pathologists. In baseline biopsies, all the available carcinoma-positive tissue samples were considered for molecular testing and were processed and microdissected to reach an adequate tumour cellularity, as described. The detailed methodology for DNA extraction, PIK3CA mutational analysis and IHC is provided as online supplemental data. In summary, PIK3CA mutational status was assessed by performing real-time analysis after DNA extraction; Pten expression (online supplemental figure 1) and ph-mTOR, ph-p70S6K and ph-4EBP1 phosphorylation status have been evaluated by IHC.

### Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics software (V.28.0).

Descriptive statistics were performed for patient clinicopathological features. Median, ranges and quartiles were computed for continuous variables. The Kolmogorov-Smirnov non-parametric test was applied to test the normality distribution of continuous variables. The Student's t-test and the Mann-Whitney U test non-parametric test were used to compare the distribution of continuous variables across subgroups. The $\chi^2$ was used to test associations between categorical variables.

mTOR, 70S6K and 4EBP1 phosphorylation levels (H-score) were considered both a continuous variables and dichotomous variables by adopting the median H-score as cut-off.

The Kaplan-Meier method was applied to estimate survival curves; the log-rank test was applied to compare variables between groups. Univariate Cox regression model was used to calculate HRs and 95% CIs.

Recurrence-free survival (RFS) was adopted as survival endpoint and was defined as the time from surgery to recurrence or death from any cause. Patients alive without RFS event and patients lost to follow up were censored at the date of last follow-up.

All reported p values are two sided, and significance level was set at $p<0.05$.

Given the descriptive purposes of the present work, no predefined sample size has been estimated.

## RESULTS

### Patient characteristics

Ninety-two patients with HR+/HER2− BC failing to achieve pCR after NACT were included. Clinicopathological features of the overall cohort are shown in table 1.

Briefly, median age at diagnosis was 51 years. Approximately half of the patients were premenopausal. The majority of patients had ductal histology, grade 3 and clinical stage III tumours. In the great majority of cases, NACT consisted of anthracycline-taxane-based treatment. In addition, almost a half of patients exhibited RCB class III aftyer NACT. Almost all patients received adjuvant endocrine therapy, consisting on tamoxifen in one-third of cases and aromatase inhibitor in two third of cases. Finally, less than 25% of patients underwent further chemotherapy in the adjuvant setting.

Almost a half of the patients experienced disease relapse, mostly consisting of distant relapse.

*PIK3CA* mutational status at baseline was available for 74 patients. A *PI3KCA* mutation was detected in 25.7% of cases (n=19/74). The spectrum of *PIK3CA* mutation type is reported in table 2.

*PIK3CA* mutational status at baseline was significantly associated with tumour grade and proliferation index, with *PIK3CA* mutated cases being enriched for G2 (p=0.017) and less proliferating (lower ki67) tumours (p=0.017).

Pten loss status at baseline was available for 57 cases. Loss of Pten was detected in 40.3% of cases (n=23). mTOR, 70SK6 and 4EBP1 phosphorylation levels at baseline were available for 49, 50 and 55 cases, respectively. mTOR dephosphorylation status was significantly associated with lobular histology (p=0.045), with a similar association of borderline statistical significance observed for dephosphorylated-4EBP1 (p=0.054).

*PIK3CA* mutational status on RD was available for 87 patients. A *PI3KCA* mutation was detected in 25.2% of cases (n=22/87). The spectrum of *PIK3CA* mutation type is reported in table 2. Two patients harboured a double *PIK3CA* mutation.

Pten loss status on RD was available for 89 cases. Loss of Pten was detected in 69.7% of cases (n=62/89).

mTOR, 70SK6 and 4EBP1 phosphorylation levels on RD were available for 88, 87 and 88 cases, respectively.

### PI3K/PTEN/mTOR pathway dynamics

*PIK3CA* mutational status evolution from baseline biopsy to RD is shown in figure 1 (n=70). In particular, 5/53 (total: 7.1%) and 4/17 (total: 5.7%) patients gained and lost *PIK3CA* mutation, respectively, with a total conversion rate of 12.8%. In addition, two patients harbouring a *PIK3CA* mutation at baseline (one patient with H1047x and one patient with E545x mutation), acquired a second mutation on RD after NACT (C420R and

**Table 1** Clinicopathological features of the overall cohort

| Clinicapathological features, n=92 | | Median (Q1–Q3) | % (n) |
|---|---|---|---|
| Age at diagnosis, years, median (Q1–Q3) | | 51 (44–62) | |
| Menopausal status, % (n) | Premenopausal | – | 46.7 (43) |
| | Postmenopausal | – | 50.0 (46) |
| | NA | – | 3.3 (3) |
| Clinical stage at diagnosis (AJCC), % (n) | II | – | 38.1 (35) |
| | III | – | 59.8 (55) |
| | NA | – | 2.1 (2) |
| Histotype, % (n) | Ductal (NST) | – | 68.5 (63) |
| | Lobular | – | 26.1 (24) |
| | Other | – | 3.3 (3) |
| | NA | – | 2.1 (2) |
| Baseline tumour grade, % (n) | 1 | – | 2.1 (2) |
| | 2 | – | 35.9 (33) |
| | 3 | – | 41.4 (38) |
| | NA | – | 20.6 (19) |
| Baseline ER expression, %, median (Q1–Q3) | | 90 (70–90) | – |
| Baseline PgR expression, %, median (Q1–Q3) | | 55 (5–80) | – |
| Baseline Ki67 expression, %, median (Q1–Q3) | | 27 (18–40) | – |
| Tumour phenotype | ER+/PgR+ | | 70.6 (65) |
| | ER+/PgR- | | 27.2 (25) |
| | ER–/PgR+ | | 0.0 (0) |
| | NA | | 2.2 (2) |
| Neoadjuvant chemotherapy, % (n) | Anthracycline-taxane | – | 93.5 (86) |
| | Anthracyline | – | 6.5 (6) |
| RD-ER expression, %, median (Q1–Q3) | | 90 (80–90) | – |
| RD-PgR expression, %, median (Q1–Q3) | | 30 (5–70) | – |
| RD-Ki67 expression, %, median (Q1–Q3) | | 12 (5–23) | – |
| Adjuvant chemotherapy, % (n) | | – | 22.8 (21) |
| Adjuvant endocrine therapy, % (n) | | – | 96.7 (89) |
| Endocrine therapy, type, % (n) | Tamoxifene | – | 32.6 (29) |
| | Aromatase inhibithor | – | 39.3 (35) |
| | Switch | – | 28.1 (25) |
| Disease recurrence, % (n) | | – | 45.7 (42) |
| Isolated local relapse | | – | 6.5 (6) |
| Distant relapse | | | 42.4 (39) |
| RCB score, median (Q1–Q3) | | 3.364 (2.311–3.944) | |
| RCB class, % (n) | 1 | – | 3.3 (3) |
| | 2 | – | 39.1 (36) |
| | 3 | – | 48.9 (45) |
| | NA | – | 8.7 (8) |

AJCC, American Joint Committee on Cancer; ER, oestrogen receptor; NA, not available; NST, no-special type; PgR, progesterone receptor; RCB, residual cancer burden; RD, residual disease.

**Table 2** Spectrum of *PIK3CA* mutation type

| | Baseline | RD |
|---|---|---|
| PIk3CA mutation | N (%) | N (%) |
| Total | 19 (25.7) | 22 (25.3) |
| CA420R | 1 (1.3) | 2 (2.3)* |
| N345x | 2 (2.7) | 2 (2.3) |
| H1047x | 9 (12.3) | 12 (13.8)* |
| E542x | 4 (5.4) | 2 (2.3) |
| E545x | 3 (4.0) | 6 (6.9)* |

*Two patients had a double PIK3CA mutation on RD (C420R+H1047× and E545x+H1074×).
RD, residual disease.

H1047x, respectively). No statistically significant association was observed between the acquisition of double *PIK3CA* mutation and clinicopathological features.

We tracked *PIK3CA* status on metastatic tissue in patients experiencing disease relapse (n=12), as shown in figure 1. In particular, 1/12 (8.3%) patient gained a *PIK3CA* mutation (C420R), while no patients experienced *PIK3CA* loss. The association between PIK3CA mutational status and the metastatic site and the distribution of PIK3CA mutations across different metastatic sites are shown in online supplemental table 1.

Regarding Pten status evolution from baseline biopsy to RD (n=57), 2/23 patients (total: 3.5%) lost Pten expression, while 17/34 patients (total: 29.8%) showing Pten-loss at baseline gained Pten expression on RD, with a total rate of Pten instability of 33.3%.

mTOR phosphorylation levels significantly increased from baseline biopsy to RD (median ph-mTOR delta=+30, p<0.001), while both its substrates significantly decreased (median ph-70S6k delta=−47, p<0.001; median ph-4EBP1 delta=−44, p<0.001).

During NACT, phosphorylation dynamics of mTOR and 4EBP1 were found to be independent from *PIK3CA* mutational status, while phosphorylation dynamics of 70S6K showed an association with *PIK3CA* mutational status, with a greater decrease of 70S6K phosphorylation levels in *PIK3CA* mutated tumours (p=0.05). We then deepened 70S6K phosphorylation level dynamics according to *PIK3CA* evolution under NACT exposure and we found that both the subgroup of patients with concordant mutated *PIK3CA* status and that of patients gaining *PIK3CA* mutation on RD showed significantly greater magnitude of 70S6K phosphorylation level reduction after NACT as compared with those losing *PIK3CA* mutation or with concordant *PIK3CA* wild-type status (p=0.001), as shown in figure 2.

No association was found between phosphorylation dynamics of mTOR, 4EBP1 and 70S6K and Pten loss.

## Correlation between PI3K/PTEN/mTOR alterations

At baseline, PI3K/PTEN/mTOR alterations were not significantly correlated with each other, with the exception of a moderate positive correlation between *PIK3CA* mutation and ph-70S6K (Spearman coefficient 0.366, p=0.05).

On RD, ph-70S6K was moderately negatively correlated with Pten-loss (Spearman coefficient −0.331, p=0.002) and weakly positively correlated with ph-4EBP1 (Spearman coefficient 0.275, p=0.10).

Finally, delta ph-70S6K and delta ph-4EBP1 were moderately positively correlated with each other (Spearman's coefficient 0.476, p<0.001).

## Prognostic role of PI3K/PTEN/mTOR pathway alterations

The median follow-up of the present patient cohort was 9.3 years.

At baseline, *PIK3CA* mutational status was not significantly associated with RFS.

At baseline, ph-mTOR showed a negative association of borderline significance with RFS (HR 2.39, 95%CI 0.94 to 6.02, p=0.064) (figure 3A). When testing ph-mTOR prognostic association according to PIK3CA mutational status, ph-mTOR

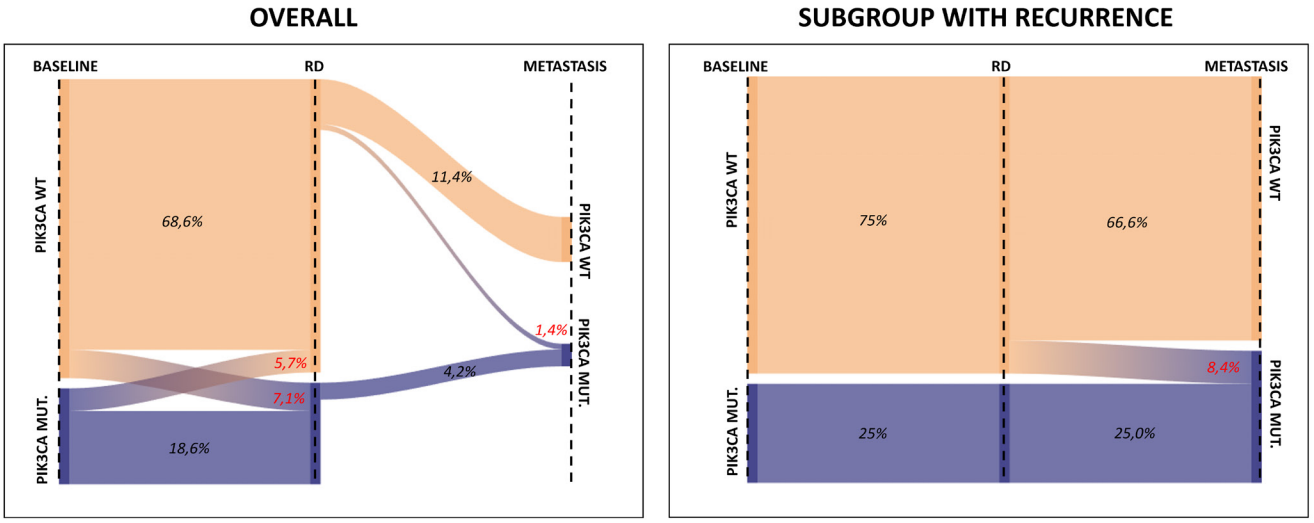


**Figure 1** PIK3CA mutational status evolution from baseline biopsy to RD to metastatic tissue (in case of disease relpase). RD, residual disease.

negative prognostic impact was statistically significant in patients with *PIK3CA* wild-type status (HR 3.78 95% CI 11.67 to 12.23, p=0021) (figure 3B), while no prognostic association was seen in patients harbouring a *PIK3CA* mutation at baseline.

At baseline, ph-70S6K was significantly and positively associated with RFS (HR 0.311, 95% CI 0.11 to 0.87, p=0.026) (figure 4).

When assessing the prognostic impact of ph-70S6K according to upstream players, the prognostic impact appeared independent from *PIK3CA* mutational status.

At baseline, 4EBP1 phosphorylation status was not significantly associated with RFS overall nor in patients stratified according to mTOR phosphorylation status.

On RD, none of the PIK3CA/PTEN/mTOR alterations was significantly associated with RFS.

## DISCUSSION

In the present study, we dynamically/longitudinally tracked PI3K/PTEN/mTOR pathway alterations on tumour tissue and assessed their prognostic role in HR+/HER2− BC patients with RD after NACT.

In our cohort, the rate of PIK3CA mutation was slightly more than 25% both at baseline and on RD, which was relatively lower than expected based on previous studies. Indeed, the rate of PIK3CA mutation in BC patients has been reported as ranging from ≈30% to 60% across studies. In addition, a negative association between the presence of *PIK3CA* mutation at baseline and pCR after NACT has been consistently reported.[10 11 17–19] For this reason, being our population entirely represented by patients with RD after NACT, we would have expected an enrichment for *PIK3CA* mutated cases. However, it should be acknowledged that data regarding *PIK3CA* prevalence in high-risk EBC/LABC are limited as compared with those gathered in the advanced setting, and mostly derived from small retrospective series, thus making cross-study comparisons of limited value.

Interestingly, we found two patients harbouring a N345K *PIK3CA* mutation, which would have been missed by applying the FDA-approved companion diagnostic Therascreen-PIK3CA-RGQ PCR test. This observation adds to the accumulating evidence, which overall highlights a suboptimal coverage of the Therascreen panel in terms of *PIK3CA* mutational status detection.[20 21] In this context, the N345x mutation represents the

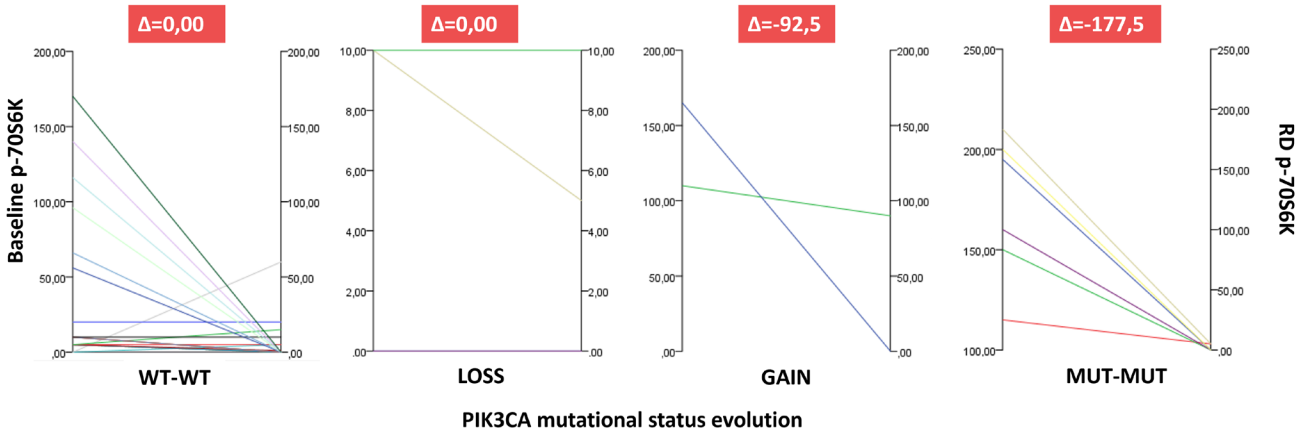


**Figure 2** 70S6K phosphorylation level dynamics according to PIK3CA mutational status evolution from baseline biopsy to RD. RD, residual disease WT, wild-type.

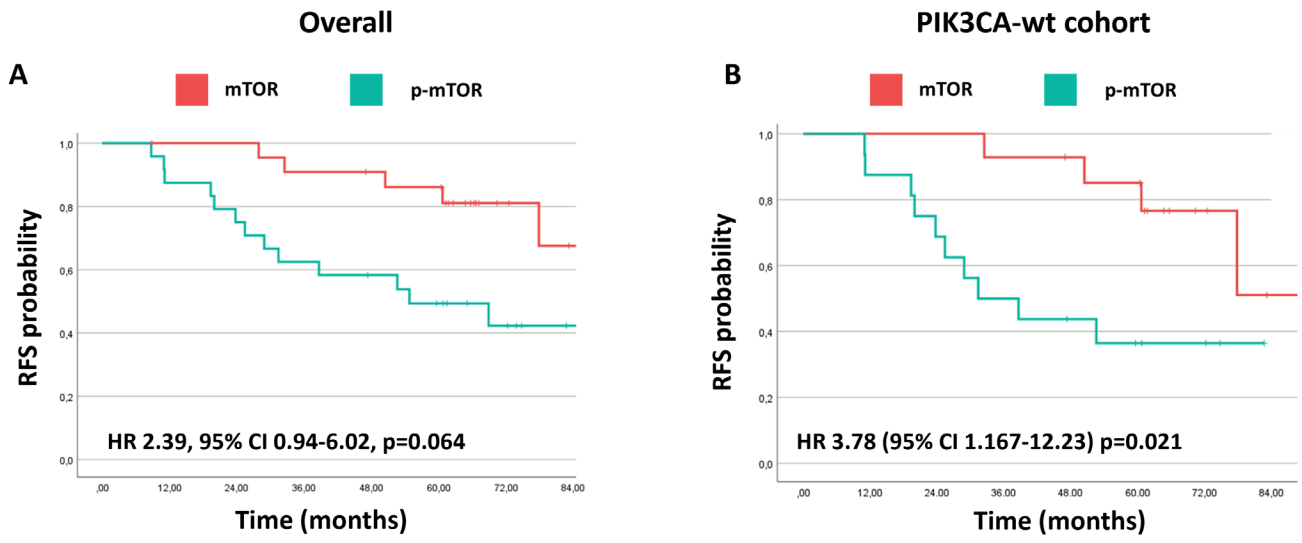


**Figure 3** Kaplan-Meier curves of RFS according to mTOR phosphorylation status. (A) Overall; (B) PIK3CA-wt cohort. RFS, recurrence-free survival.

most prevalent among the Therascreen-neglected *PIK3CA* alterations.[20 21] Within this framework, if from one hand the implementation of more comprehensive *PIK3CA* panels is warranted, on the other it is imperative to make additional research efforts aiming at unravelling the heterogeneity of *PIK3CA* mutational landscape.

Regarding Pten expression, we detected Pten loss in more than 40% of patients at baseline and almost 70% of cases on RD, thus adding to available evidence overall suggesting Pten loss as a relatively frequent phenomenon in BC patients.[22]

Regarding the clinicopathological landscape of PIK3/PTEN/mTOR pathway alterations, *PIK3CA* mutational status was significantly associated with features of restricted biological aggressiveness, consistently with previous findings.[23] Another intriguing observation was the significant association between dephosphorylation (as a surrogate of inactivated status) of both mTOR and its substrate 4EBP1, and lobular histology, which appears consistent with a previous report where lobular cancers were associated with significantly lower frequency of phosphorylated-mTOR as compared with ductal tumours.[24]

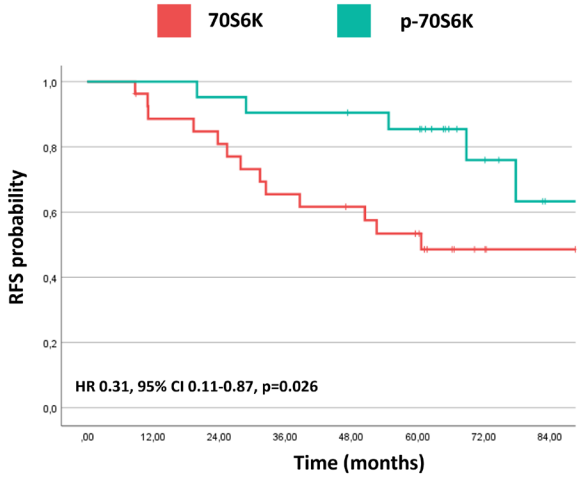


**Figure 4** Kaplan-Meier curves of RFS according to 70S6K phosphorylation status. RFS, recurrence-free survival.

The main aim of the present work was to evaluate PI3K/PTEN/mTOR pathway dynamics during NACT. Regarding *PIK3CA* mutational landscape evolution, we observed a 12.8% rate of instability from baseline biopsy to matched samples of RD after NACT, with 7% of patients with wild-type PIK3CA at baseline gaining *PIK3CA* mutation on RD and 26% of *PIK3CA*-mutated patients at baseline exhibiting *PIK3CA* loss.

Notably, we also tracked *PIK3CA* mutational landscape in the subgroup of patients experiencing disease relapse and undergoing metastatic tissue resampling, with only one patient gaining *PIK3CA* mutation, with no *PIK3CA* loss events observed. These observations raise two orders of considerations. First, the relatively low rate of *PIK3CA* mutational shift under NACT exposure (and at disease relapse) solidifies data, mostly gathered in the advanced setting, overall highlighting the propensity of *PIK3CA* mutational phenotype to be clonally dominant during BC evolution.[25] Second, we confirmed in a homogenous population of HR+/HER2− BC mostly treated with anthracycline-taxane-based NACT, that, of patients showing *PIK3CA* mutational status instability, *PIK3CA* mutation loss among *PIK3CA* mutated patients was more frequent than *PIK3CA* mutation gain among *PIK3CA* wild-type patients, as suggested in prior studies conducted in unselected BC patients, receiving heterogeneous NACT regimens.[11 26]

Notably, in our study, two patients with *PIK3CA* single mutation at baseline, acquired a second *PIK3CA* mutation on RD. Although the acquisition of double *PIK3CA* mutations has been already described in previous studies,[27 28] the actual biological and clinical value of such phenomenon is not fully understood. A multihistology study reported that double *PIK3CA* mutations may determine an enhanced activation of PI3K pathway as compared with single *PIK3CA* mutations, causing increased *PIK3CA*-driven cell proliferation and tumour growth.[27] Interestingly, the direct consequence of such phenomenon may be

the association between multiple *PIK3CA* mutations, enhanced PIK3CA oncogene addiction and subsequent greater sensitivity to PI3K inhibition in HR+/HER2− advanced BC patients receiving fulvestrant+placebo/taselisib (PI3K inhibitor) in the context of the SANDPIPER phase III trial.[27] In our study, the presence of double PIK3CA mutations did not appear as a parental phenomenon but rather an acquired one, triggered by NACT exposure. Although the small number of patients with double *PIK3CA* mutations precluded the possibility to deepen this association and to properly assess the role of chemotherapy in enhancing this phenomenon, it may be speculated that patients acquiring a second *PIK3CA* mutational event after NACT may represent ideal candidates for postneoadjuvant treatment with PI3K inhibitors+endocrine therapy given the potential enhanced sensitivity to PI3K-inhibition.

When exploring Pten landscape dynamics, we observed a distinct behaviour as compared with *PIK3CA*, both in terms of higher instability and the direction of such instability, with a substantially higher tendency to Pten loss rather than Pten restoration after NACT. It should, however, be acknowledged that Pten expression evaluation may be limited in scope by the lack of consistency and reproducibility of Pten assessment across studies, with regard to type of assay, scoring system, cutoffs and interpathologists' agreement.[22]

Interestingly, we also observed a significant increase of mTOR functional status after NACT, while that of its substrates (4EBP1 and 70S6K) significantly decreased. Interestingly, the magnitude of decrease of 70S6K phosphorylation levels from baseline to RD appeared to differ according to *PIK3CA* mutational status evolution, with a significantly greater decrease in patients either maintaining or gaining *PIK3CA* mutation during NACT as compared with those losing *PIK3CA* mutation or with concordant PIK3CA wild-type status. This observation may suggest that NACT-induced impairment of 70S6K functional status may be prerogative of tumours with intrinsically PIK3CA-mutated tumours or made them so under NACT exposure. PI3K/PETN/mTOR functional status in EBC/LABC and its evolution during NACT represents an unexplored phenomenon so far. Although our findings need to be interpreted as only descriptive, they generate the hypothesis that PI3K-related pathway is highly dynamic under NACT exposure, possibly affected by multiple cross-talking pathways, with—so far—unpredictable biological consequences, and such phenomenon may not be properly recapitulated or surrogated by only focusing on *PIK3CA* mutational status. This consideration finds a possible corroboration from the observation of lower/weaker than expected correlations between the different players of the PI3K/PTEN/mTOR pathway. In particular, at baseline, *PIK3CA* was positively and weakly associated with 70S6K phosphorylated status, thus indirectly implying the engagement of mTOR. On RD, under the effect of NACT exposure, while the two classical mTOR substrates (4EBP1 and 70S6K) were positively correlated with each other, 70S6K was no longer correlated with PIK3CA mutational status. These findings may potentially imply that, while in treatment-naïve HR+/HER2− EBC/LABC the biological hubs represented by PI3K and mTOR may work, at least in part, as a series circuit, under the exposure of NACT they seem to rather function as parallel circuits, disengaging from the mutual biological dependence. Another piece of the puzzle is the observation of an opposite dynamic behaviour of mTOR and its substrates after NACT exposure. This observation may suggest that NACT-induced mTOR activation may be driven by alternative pathways, both upstream and downstream, with mTOR relying on alternative triggers (PI3K-independent) and substrates (different from 70S6K and 4EBP1) to mediate its action. This hypothesis may be further strengthened by our exploratory survival analyses, which revealed appealing hints. In particular, we observed baseline phosphorylated-mTOR being significantly associated with poorer RFS and this negative prognostic impact seemed to be restricted to patients with *PIK3CA* wild-type status. This observation, although exploratory, may suggest that in HR+/HER2− BC patients with RD after standard NACT, the activation of mTOR may be clinically relevant in terms of prognostic impact only when it reflects PIK3CA-independent mechanisms. Conversely, we observed a positive prognostic impact of phosphorylated-70S6K, which was independent from *PIK3CA* mutational status. Although the opposite prognostic value of mTOR and its classical substrate 70S6K may appear counterintuitive, it might further underly the complexity of PI3K/PTEN/mTOR interaction and crosstalk with other pathways. Of course, it should be acknowledged that the interpretation of our survival data may be complicated by the fact that almost all patients received adjuvant endocrine therapy and no definitive and solid conclusions may be drawn in this regard.

The present work has several strengths. First, this represents one of the first attempts to uncover the role of PI3K/PTEN/mTOR dynamic behaviour under NACT exposure in HR+/HER2− BC patients with evidence of RD at surgery. In addition, the majority of patients received anthracycline-taxane-based NACT and subsequent adjuvant endocrine therapy, thus representing a homogenously treated population. In addition, the long follow-up period (>9 years) allowed us to explore the long-term prognostic value of PI3K pathway-related biomarkers, thus increasing the reliability of our exploratory survival analyses, if considering that HR+/HER2− BC recurrence pattern is typically characterised by a sustained risk even after several years from diagnosis.[29–31]

Some limitations should be acknowledged as well. In particular, the retrospective nature may have been responsible for selection bias. In addition, only patients with RD after NACT were included, thus precluding the possibility to assess the role of baseline PI3K/PTEN/mTOR pathway alterations in patients achieving pCR. Moreover, none of the patients included received adjuvant abemaciclib as treatment escalation, thus making our patient population not entirely representative of the contemporary therapeutic landscape of high-risk HR+/HER2 EBC/LABC patients. Finally, although in our study, PIK3CA mutational status tended to be stable under NACT exposure, we cannot exclude that the potential suboptimality of core biopsy-based sampling in capturing tumour heterogeneity, could have been responsible for hindering the presence of a PIK3CA mutation at baseline among the five patients exhibiting PIK3CA mutation gain on RD. However, it has been reported that the genotypic intratumoural heterogeneity of PIK3CA mutations within the primary tumour is rare,[32] thus reassuring on the reliability of core biopsy in the assessment of PIK3CA mutational status within primary BC.

In conclusion, we observed in a population of HR+/HER2− BC with RD after NACT that PI3K/PTEN/mTOR pathway is highly dynamic under NACT exposure with *PIK3CA*-dependent signalling possibly capturing only a small fraction of such complexity. In this context, mTOR activation trough alternative pathways may have a crucial role in shaping the molecular landscape of this challenging patient population. Within this framework, it is imperative to further elucidate the role of PIK3CA and mTOR-dependent pathways in shaping chemoresistance and endocrine resistance in order to improve therapeutic selection

and prognostic stratification of high-risk HR+/HER2− EBC/LABC patients.

**Handling editor** Vikram Deshpande.

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**Funding** The authors acknowledge the following fundings: Fondazione AIRC under 5 per mille 2019 - ID. 22 759 program- G.L.VG; Veneto Institute of Oncology IOV-IRCCS to MVD, and VG; DOR funding from the University of Padova to GG, MVD, VG; Italian Ministry of Health Ricerca Corrente.

**Competing interests** FM received personal fee from Roche, Novartis and Gilead, outside the submitted manuscript. GG reports personal fee from Novartis, Eli Lilly, and Gilead outside the submitted work. VG reports personal fees for advisory board membership from Eisai, Eli Lilly, Exact Sciences, Gilead, Merck Serono, MSD, Novartis, Olema Oncology and Sanofi; personal fees as an invited speaker from Amgen, Eli Lilly, GSK, Astra Zeneca, Novartis; personal fees for expert testimony from Eli Lilly; institutional funding as a local PI from AstraZeneca, BMS, Daiichi Sankyo, Eli Lilly, GlaxoSmithKline, Merck, MSD, Nerviano, Novartis, Roche and Synton Biopharmaceutical. MF has been involved in consulting/advisory roles in Astellas Pharma, Tesaro, MSD, Pierre Fabre, Astra Zeneca, Incyte, GlaxoSmithKline, Diaceutics and Roche, received research funding from Astellas Pharma, QED Therapeutics, and Macrophage Pharma and is supported by grants from the Italian Health Ministry/Veneto region research program NET-2016-02363853 and AIRC 5 per mille 2019 (ID. 22759 programme). MVD reports personal fees from Eli Lilly, MSD, Exact Sciences, Novartis, Pfizer, Seagen, outside the submitted work.

**Patient consent for publication** Not applicable.

**Ethics approval** This study involves human participants and was approved by Istituto Oncologico Veneto IOV—IRCCS: NEO_TIL 2016-10. Participants gave informed consent to participate in the study before taking part.

**Provenance and peer review** Not commissioned; externally peer reviewed.

**Data availability statement** Data are available on reasonable request. The datasets that support the findings of this study are not publicly available in order to protect patient privacy. The data will be available on reasonable request from the corresponding author: FM (federica.miglietta@unipd.it)



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## REFERENCES

1 WHO. Breast cancer. 2020 Available: https://www.who.int/news-room/fact-sheets/detail/breast-cancer#:~:text=In%202020%2C%20there%20were%202.3,the%20world's%20most%20prevalent%20cancer
2 Bray F, Ferlay J, Soerjomataram I, *et al*. Global cancer Statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin* 2018;68:394–424.
3 The Cancer Genome Atlas Network. Comprehensive molecular portraits of human breast tumours. *Nature* 2012;490:61–70.
4 Fusco N, Malapelle U, Fassan M, *et al*. Pik3Ca mutations as a molecular target for hormone receptor-positive, Her2-negative metastatic breast cancer. *Front Oncol* 2021;11:644737.
5 Fillbrunn M, Signorovitch J, André F, *et al*. Pik3Ca Mutation status, progression and survival in advanced HR + /Her2- breast cancer: a meta-analysis of published clinical trials. *BMC Cancer* 2022;22:1002.
6 André F, Ciruelos E, Rubovszky G, *et al*. Alpelisib for Pik3Ca -Mutated, hormone receptor–positive advanced breast cancer. *N Engl J Med* 2019;380:1929–40.
7 Baselga J, Campone M, Piccart M, *et al*. Everolimus in postmenopausal hormone-receptor–positive advanced breast cancer. *N Engl J Med* 2012;366:520–9.
8 Jones RH, Casbard A, Carucci M, *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. *The Lancet Oncology* 2020;21:345–57.
9 Nicholas T, Mafalda O, Sacha H, *et al*. Capivasertib and Fulvestrant for patients with Aromatase inhibitor-resistant hormone receptor-positive/human Epidermal growth factor receptor 2-negative advanced breast cancer: results from the phase III Capitello-291 trial. presented at: San Antonio breast cancer symposium. San Antonio, Texas, USA, 2022.
10 Dong C, Wu J, Chen Y, *et al*. Activation of Pi3K/AKT/mTOR pathway causes drug resistance in breast cancer. *Front Pharmacol* 2021;12.
11 Yuan H, Chen J, Liu Y, *et al*. Association of Pik3Ca Mutation status before and after Neoadjuvant chemotherapy with response to chemotherapy in women with breast cancer. *Clin Cancer Res* 2015;21:4365–72.
12 Liu C-Y, Wu C-Y, Petrossian K, *et al*. Treatment for the endocrine resistant breast cancer: Current options and future perspectives. *J Steroid Biochem Mol Biol* 2017;172:166–75.
13 Miller TW, Hennessy BT, González-Angulo AM, *et al*. Hyperactivation of Phosphatidylinositol-3 kinase promotes escape from hormone dependence in estrogen receptor-positive human breast cancer. *J Clin Invest* 2010;120:2406–13.
14 Zhang S, Hu B, Lv X, *et al*. The Prognostic role of Ribosomal protein S6 kinase 1 pathway in patients with solid tumors: A meta-analysis. *Front Oncol* 2019;9.
15 Miglietta F, Dieci MV, Griguolo G, *et al*. Neoadjuvant approach as a platform for treatment Personalization: focus on Her2-positive and triple-negative breast cancer. *Cancer Treat Rev* 2021;98:102222.
16 Cardoso F, Kyriakides S, Ohno S, *et al*. Early breast cancer: ESMO clinical practice guidelines for diagnosis, treatment and follow-up. *Ann Oncol* 2019;30:1674.
17 Guarneri V, Dieci MV, Frassoldati A, *et al*. Prospective biomarker analysis of the randomized CHER-LOB study evaluating the dual anti-Her2 treatment with Trastuzumab and Lapatinib plus chemotherapy as Neoadjuvant therapy for Her2-positive breast cancer. *Oncologist* 2015;20:1001–10.
18 Chatzipli A, Bonnefoi H, MacGrogan G, *et al*. Patterns of Genomic change in residual disease after Neoadjuvant chemotherapy for estrogen receptor-positive and Her2-negative breast cancer. *Br J Cancer* 2021;125:1356–64.
19 Loibl S, Majewski I, Guarneri V, *et al*. Corrections to "Pik3Ca mutations are associated with reduced pathological complete response rates in primary Her2-positive breast cancer: pooled analysis of 967 patients from five prospective trials investigating Lapatinib and Trastuzumab *Annals of Oncology* 2019;30:1180.
20 Martínez-Sáez O, Chic N, Pascual T, *et al*. Frequency and spectrum of Pik3Ca somatic mutations in breast cancer. *Breast Cancer Res* 2020;22:45.
21 Rugo HS, Raskina K, Schrock AB, *et al*. Biology and Targetability of the extended spectrum of Pik3Ca mutations (Pik3Cam) detected in breast carcinoma. *Clin Cancer Res* 2023;29:1056–67.
22 Carbognin L, Miglietta F, Paris I, *et al*. Prognostic and predictive implications of PTEN in breast cancer: unfulfilled promises but intriguing perspectives. *Cancers* 2019;11:1401.
23 López-Knowles E, Segal CV, Gao Q, *et al*. Relationship of Pik3Ca Mutation and pathway activity with antiproliferative response to Aromatase inhibition. *Breast Cancer Res* 2014;16:R68.
24 Walsh S, Flanagan L, Quinn C, *et al*. mTOR in breast cancer: differential expression in triple-negative and non-triple-negative tumors. *Breast* 2012;21:178–82.
25 O'Leary B, Cutts RJ, Liu Y, *et al*. The genetic landscape and Clonal evolution of breast cancer resistance to Palbociclib plus Fulvestrant in the PALOMA-3 trial. *Cancer Discov* 2018;8:1390–403.
26 Jiang Y-Z, Yu K-D, Bao J, *et al*. Favorable Prognostic impact in loss of Tp53 and Pik3Ca mutations after Neoadjuvant chemotherapy in breast cancer. *Cancer Res* 2014;74:3399–407.
27 Vasan N, Razavi P, Johnson JL, *et al*. Double Pik3Ca mutations in cis increase Oncogenicity and sensitivity to Pi3Kα inhibitors. *Science* 2019;366:714–23.
28 Migliaccio I, Paoli M, Risi E, *et al*. Pik3Ca Co-occurring mutations and copy-number gain in hormone receptor positive and Her2 negative breast cancer. *NPJ Breast Cancer* 2022;8:24.
29 Voduc KD, Cheang MCU, Tyldesley S, *et al*. Breast cancer subtypes and the risk of local and regional relapse. *J Clin Oncol* 2010;28:1684–91.
30 Ignatov A, Eggemann H, Burger E, *et al*. Patterns of breast cancer relapse in accordance to biological subtype. *J Cancer Res Clin Oncol* 2018;144:1347–55.
31 Pan H, Gray R, Braybrooke J, *et al*. 20-year risks of breast-cancer recurrence after stopping endocrine therapy at 5 years. *N Engl J Med* 2017;377:1836–46.
32 Kalinsky K, Heguy A, Bhanot UK, *et al*. Pik3Ca mutations rarely demonstrate Genotypic Intratumoral heterogeneity and are selected for in breast cancer progression. *Breast Cancer Res Treat* 2011;129:635–43.