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Efficacy of PI3K pathway inhibitors as single agents in metastatic breast cancer

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Contents

1. Scientific abstract

Background: Molecular prescreening of tumors aims at selecting populations that can benefit most from anticancer targeted-agents. Breast cancer present several molecular alterations, such as *PIK3CA* mutations, PTEN low expression, or FGFR amplification or mutation.

Purpose: Assess the activity of single agent PI3K/AKT/mTOR pathway inhibitors (PI3Kpi) in metastatic breast cancer (MBC) patients, and explore PI3K pathway alterations as a predictor of clinical benefit to these agents.

Patients and Methods: From September 2009 to November 2012, MBC patients were assessed for PI3K pathway alterations. Some were treated with single agent pan-isoform PI3K, alpha-specific PI3K, dual PI3K/mTORC1/2, mTORC1/2, or AKT inhibitors. Their outcomes were analyzed.

Results: Of 445 patients tested, 38 received single agent PI3Kpi. Prevalence of molecular alterations: *PIK3CA* mutation 59.4%, *AKT1* mutation 13.3%, PTEN low 29.7%, PI3K/AKT/mTOR pathway dysregulation (*PIK3CA* mutation and/or *AKT1* mutation and/or PTEN low) 85.7%. Subtype distribution: HR+/HER2- 76.3%, HER2+ 13.2%, TN 10.5%. Median number of prior MBC lines: 5 (1-11). Median time to progression (TTP) was 2.6 months (95%CI 2.0-4.8), being numerically longer in *PIK3CA* WT patients (4.1 vs. 2.3 months, p=NS). Among *PIK3CA*-mutated patients, those receiving a PI3K-alpha inhibitor did better than those treated with pan-isoform PI3K inhibitor (*P*=0.025).

Conclusion: Single agent PI3K pathway inhibitors showed interesting activity in heavily pre-treated MBC patients. In general, there was no correlation between PIK3 pathway alterations and efficacy of these agents, but *PIK3CA* mutated patients did better with PI3K alpha-specific inhibitors. These results emphasize the need of an adequate molecular selection prior to targeted therapy in MBC.

2. Background

Breast cancer is the most frequently diagnosed cancer worldwide and the leading cause of cancer death among females, accounting for 23% of the total cancer cases and 14% of total cancer deaths (1). A large proportion of breast cancer cases, especially in developed countries, are now diagnosed in early stages, but 25-40% of patients will eventually develop recurrence or metastases (2). Additionally, 5-6% of patients will have overt metastatic disease at presentation (3). Metastatic breast cancer (MBC) is generally an incurable condition, with a median survival that ranges from 2 to 3 years after diagnosis (4). A better understanding of the molecular pathways involved in the pathogenesis of breast cancer has prompted the development and use of novel agents in the treatment of this disease. The paradigmatic example is the development of extremely effective anti-HER2 therapies in the last 15 years, which has dramatically changed the natural history of HER2-positive breast cancer (5-7). However, for triple negative MBC no specific targeted agents have been approved, and for ER-positive MBC overall survival has remained stable since the early nineties (8).

Personalized cancer therapy refers to the use of molecular characteristics of the tumor and microenvironment, as well as characteristics of the patient, in order to tailor therapies delivered to the patient, and treat cancer more effectively and with less toxicity (9). Routine molecular screening of primary and/or metastatic tumors is now used as part of prescreening programs to enrich phase I or early phase II trials with specific populations (10-12). This effort aims at selecting the populations that can potentially benefit most from anticancer targeted-agents. Successful histories of such matching include, among others, vemurafenib for B-RAF V600E mutant melanoma (13), crizotinib for non-small cell lung tumors with EML4-ALK translocation (14), and imatinib for KIT-mutated gastrointestinal stromal tumors (15).

The phosphatydilinositol-3-kinase/AKT/mammalian target of rapamicin (PI3K/AKT/mTOR) intracellular signaling pathway is frequently dysregulated in human cancer (16). In breast cancer, the most common alterations of this pathway

are mutations of *PIK3CA* (the gene encoding for the catalytic p110α subunit of PI3K, 10-40% depending on breast cancer subtype) and PTEN low expression (~50%) (17). Additionally, amplification of FGFR1 occurs in approximately 10% of breast cancers (18-19), and AKT1 mutation can occur in about 3% of ER+ breast cancers (16). Of note, with the increasingly widespread use of high-throughput platforms, new potential targetable molecular alterations are expected to be identified in the future.

Several agents targeting PI3K/AKT/mTOR pathway alterations at different levels are currently in clinical development. They include PI3K inhibitors (either pan-isoform PI3K, alpha-specific PI3K inhibitors, or others), dual PI3K/mTORC1/2 inhibitors, mTORC1/2 inhibitors, or AKT inhibitors (20-23). Although the rationale for targeting these alterations in breast cancer is very appealing, no compelling evidence has been provided so far that MBC patients with tumors harboring targetable molecular aberrations definitely benefit from matched targeted agents.

A pre-screening program for detection of PI3K/AKT/mTOR pathway alterations in MBC patients receiving active treatment for advanced disease was initiated in September 2009 at the Medical Oncology Department and Breast Cancer Group of Vall d'Hebron University Hospital (24). Information on *PIK3CA* and PTEN status became available for the treating physician contemporaneously to decision-making on treatment, and some of these patients were offered treatment with a PI3K/AKT/mTOR pathway inhibitor in some point of their MBC natural history.

The aim of this study was to assess the clinical activity of several PI3K pathway inhibitors when used as single agent in MBC patients. We also wanted to explore the presence of PI3K pathway alterations, such as *PIK3CA* mutations, AKT1 mutations, and/or PTEN low expression, as a predictor of clinical benefit to these agents in the clinical setting.

3. Materials and Methods

i) Mutation analysis of *PIK3CA* and determination of PTEN

From September 2009 to February 2011, somatic mutation profiling of *PIK3CA* was performed using the PCR-based DxS Mutation Test Kit (Qiagen®). DxS PI3K Mutation Test Kit is an assay for detection of 4 specific mutations in the *PIK3CA* gene (H1047R, E542K, E545D and E545K) using real-time PCR, and was used according to the manufacturer's protocol.

From February 2011 to November 2012, the MassARRAY system (Sequenom®) and OncoCarta™ v1.0 assay panel were implemented for routine molecular testing of tumor samples at our Institution. This panel performs somatic mutation profiling of 238 mutations in 19 oncogenes (including *PIK3CA*, *KRAS*, *BRAF* or *EGFR*). The panel is based on the use of IPlex chemistry (Sequenom). In brief, DNA was extracted from 5x10µm slices of formalin fixed paraffin-embedded (FFPE) tumor samples using the RecoverAll Total Nucleic Acid Isolation Kit (Ambion). Six-hundred nanograms (ng) of DNA were used for mutation profiling using OncoCarta v1.0. After quantification (nanodrop) and dilution of DNA to a 10ng/µl concentration, multiplexed PCR was performed in order to amplify the genomic regions that contain the loci to be genotyped. Each mutation was then analyzed as the single-base extension product of a probe that anneals immediately contiguous to the mutation position. Gen II SpectroCHIPs were loaded into a matrix-assisted laser desorption/ionization–time of flight (MALDI-TOF) mass spectrometer (Mass ARRAY, Sequenom) and spectra were obtained for each of the extension products. Data analysis and mutation reports were generated using the Typer Analyzer 4.0 software (Sequenom). Manual assessing of spectra was performed on each of the *PIK3CA* assays included in the panel for all samples as well as on all reported mutations by the Sequenom software. A mutation call was done in the presence of >10% mutate alleles in a given sample.

PTEN was determined by immunohistochemistry. The antibody anti-PTEN from Cell Signaling Technologies® (Clone 138G6, cat# 9559, batch 7-12) was used in a 1:100 diluted incubated at room temperature for 60 minutes. An H-score was

calculated based on the intensity of staining (0-3) and percentage of staining-positive cells (0-100). PTEN low expression was considered when H-score was ≤ 50 (0-300). PTEN null was assumed in case of absence of immunostaining (as compared with the internal control).

ii) Patients

MBC patients treated from September 2009 to November 2012 with single agent pan-isoform PI3K inhibitors, PI3K-alpha specific inhibitors, dual PI3K/mTORC1/2 inhibitors, mTORC1/2 inhibitors or AKT inhibitors in early drug development trials at the Vall d'Hebron University Hospital in Barcelona were identified. Patients were included in the analysis if tumor tissue was available for molecular testing. They provided signed informed consent to the molecular determinations as part of the routine molecular testing at our Institution.

If *PIK3CA* mutation, and/or *AKT1* mutation, and/or PTEN low were present, patients were classified as having a PI3K pathway dysregulation. If neither of the alterations was present, they were classified as having no pathway dysregulation. Patients with wild type (WT) *PIK3CA* / *AKT1* and PTEN not determined, or patients with PTEN normal and *PIK3CA* / *AKT1* not assessed, were considered as having a non-informative PI3K pathway.

Registration of the patients in the database, pathology assessments, molecular determinations and treatment were all performed at Vall d'Hebron University Hospital. Medical charts of all patients were reviewed to confirm the accuracy of the variables recorded in the database. Patients' demographics, tumor characteristics, characteristics of the metastatic disease at the time of beginning of treatment with PI3K pathway inhibitor, outcome of the treatment and vital status, including date of death or last follow-up, were collected and recorded for each patient.

iii) Treatment and evaluation

Data on type of inhibitor – pan-isoform PI3K, PI3K-alpha specific, dual PI3K/mTORC1/2, mTORC1/2 or AKT – and dose of PI3K pathway inhibitor – lower or higher than 2/3 of the declared maximum tolerated dose (MTD) – were collected for each individual patient.

All efficacy and safety assessments were done according to each protocol. Generally, this included physical examination, evaluation of adverse events and laboratory work-up at baseline and at least prior to the beginning of each cycle of treatment. Tumor assessments were done for all patients at baseline and every other cycle later on (between 6 and 8 weeks depending on the length of cycles at each different clinical trial). Response was assessed according to the Response Evaluation Criteria in Solid Tumors (RECIST) v.1.0 (23) and reported as the best response. Complete response (CR) was defined as disappearance of all lesions; partial response (PR) as a reduction of at least 30% respect to the baseline sum of target lesions without evidence of appearance of new lesions; progressive disease (PD) as at least a 20% increase in the sum of largest diameters of target lesions, taking as reference the smallest sum of largest diameters recorded since the treatment started, or appearance of unequivocal new lesions; stable disease (SD) as neither sufficient decrease in sum of largest diameters to qualify for PR nor sufficient increase in sum of largest diameters to qualify for PD.

Response rate (RR) was defined as the proportion of patients with CR or PR, and clinical benefit rate (CBR) as the proportion of patients with a CR, PR or SD lasting ≥6 months.

Treatment was continued until progressive disease or presence of unacceptable toxicity, and the reason for discontinuation was collected and recorded.

iv) Statistical analysis

This is a retrospective analysis using data from several prospective clinical trials.

Time to progression (TTP) was defined as the time (in months) from the date of beginning PI3K pathway inhibitor until the date of recording progressive disease. Patients who interrupted treatment for toxicity and did not progress thereafter or patients that were on treatment at the time of data cut-off were censored for the purpose of the TTP analysis.

Overall survival (OS) after beginning of PI3K pathway inhibitor (OSPI3Kpi) was calculated from the date of beginning of experimental treatment until the date of death or last follow-up. OS after MBC diagnosis (OSMBC) was defined from the date of first distant recurrence after completion of loco-regional therapy to the date of death or last follow-up.

All the statistical analysis was performed with the R x64 3.0.0 platform. Survival package of the R-library was used for the survival analysis (TTP and OS). Survival times were compared by the log-rank test.

Univariate analysis was performed to compare clinical characteristics according to distinct molecular alterations. Logistic regression model was used to estimate the effect (OR) and AUC to measure the predictive ability of the variable.

Comparison of times within the same patient was performed using the Wilcoxon non-parametric test.

4. Results

i) Patients

From September 2009 to November 2012, 523 MBC patients were screened for the presence of *PIK3CA* mutations and PTEN alterations at our Hospital (Figure 1). From these, 78 could not be assessed because of insufficient material, leaving a total of 445 patients with at least one molecular determination available.

Of these 445 patients, 115 (25.8%) had *PIK3CA* mutation and 115 (25.8%) had PTEN low.

Thirty-eight out of the 445 patients (8.5%) were treated with single agent PI3K pathway inhibitor, meeting the inclusion criteria for this study.

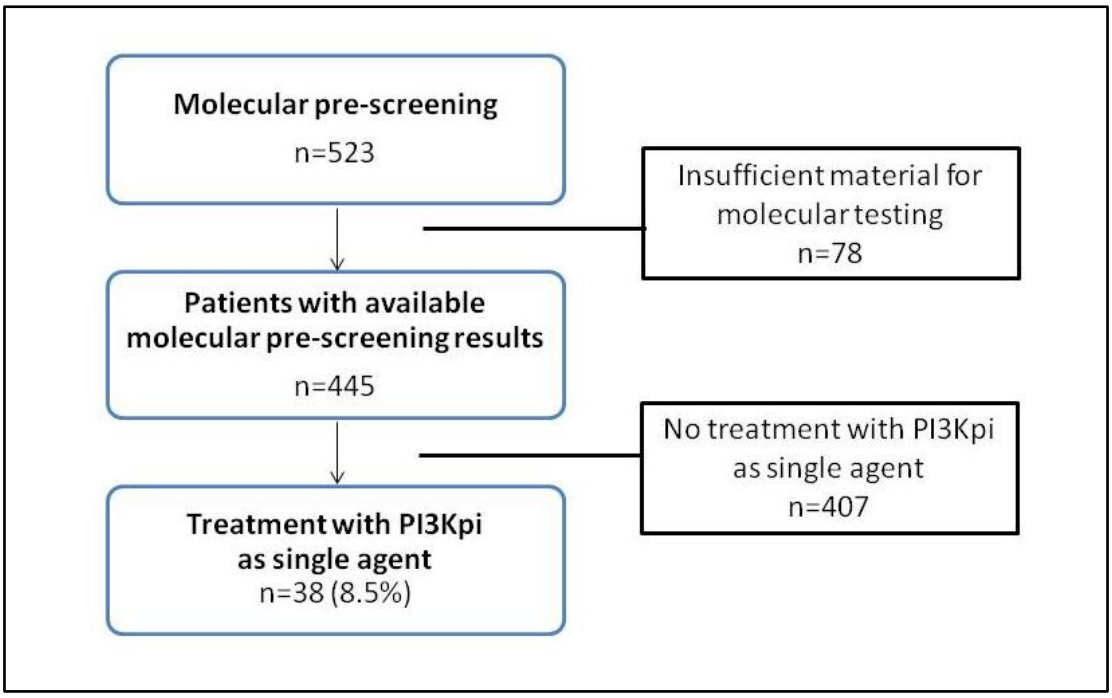

Figure 1: Disposition of patients included in the analysis. PI3Kpi, PI3K pathway inhibitor.

Patients' characteristics are shown in Table 1. Of note, the majority had HR+/HER2- tumors, visceral disease and were heavily pre-treated, with a median number of 5 lines of treatment for metastatic disease (range 1-11).

Table 1. Patients' characteristics.

Demographical or Clinical Characteristic	N (%)
Age, years	
Mean (SD)	55 (10.8)
Median (Range)	54 (36-78)
Tumor subtype	
HER2+	5 (13.2%)
HR+/HER2-	29 (76.3%)
Triple Negative	4 (10.5%)
Number of metastatic sites	
Median (Range)	2 (1-6)
1-2	20 (52.6%)
≥3	18 (47.4%)
Sites of metastasis	
Bone only	1 (2.6%)
Visceral	37 (97.4%)
Prior lines for MBC treatment	
Median (Range)	5 (1-11)
Prior Chemotherapy for MBC	33 (86.8%)
Dose of PI3K pathway inhibitor	
<2/3 MTD	4 (10.5%)
≥2/3 MTD	34 (89.5%)

HR, hormonal receptor; MBC, metastatic breast cancer; MTD, maximum tolerated dose; SD, standard deviation.

Table 2 details the molecular features of this population.

Determinations were performed mainly in primary tumor tissue (n=21, 55.3%), but metastatic tumor tissue was used to assess PI3K pathway alterations in 13 patients (34.2%).

PIK3CA status could be determined in 32 out of the 38 patients. Of these, 19 had a *PIK3CA* mutation (59.4%), mainly in exon 20 (n=10).

PTEN expression could be determined in 37 out of the 38 patients, and 11 had PTEN low expression (29.7%).

Table 2. Molecular determinations.

Site of molecular determination	**N (%)**
Primary tumor	21 (55.3%)
Metastasis	13 (34.2%)
Unknown	4 (10.5%)
Analysis with Sequenom technology	15 (39.5%)
***PIK3CA* status (N=32)**	
WT	13 (40.6%)
Mutated	19 (59.4%)
Exon 1	
E110K	1 (5.3%)
Exon 9	
E545K	6 (31.6%)
Q546K	2 (10.5%)
Exon 20	
H1047R	10 (52.6%)
PTEN (N=37)	
Normal	26 (70.3%)
Low	11 (29.7%)
Other PI3K pathway alterations (N=15)	
AKT1 E17K mutation	2 (13.3%)
PI3K pathway dysregulation (N=38)	
No	5 (13.2%)
Yes	30 (78.9%)
Non-informative	3 (7.9%)

WT, wild type.

Sequenom technology was performed in 15 patients (39.5%), allowing for screening of other mutations. From these, 2 (13.3%) had an AKT1 mutation (both E17K).

PI3K pathway status was non-informative in 3 patients (PTEN normal, *PIK3CA* not determined). Among the remaining 35 patients, 30 (78.9%) had a dysregulation in the PI3K pathway (2 patients had both *PIK3CA* mutation and PTEN low expression, and 2 patients had AKT1 mutation).

Distribution of molecular alterations (*PIK3CA* mutation, PTEN low expression, and PI3K pathway dysregulation) did not associate with age, breast cancer subtype, or number of metastatic sites (AUC<0.72 for all comparisons, univariate analysis NS).

Table 3 shows the distribution of type of treatment according to *PIK3CA* status. One third of the patients were treated with pan-isoform PI3K inhibitors, 25.7% with AKT inhibitors, 18.4% with PI3K-alpha specific inhibitor, 18.4% with dual PI3K/mTORC1/2, and 7.9% with AKT inhibitors. Of note, the majority of *PIK3CA* mutated patients received a PI3K-alpha specific inhibitor.

Table 3. Type of inhibitor received, according to *PIK3CA* status.

Type of Inhibitor	***PIK3CA* WT N=13**	***PIK3CA* MUT N=19**	***PIK3CA* ND N=6**	**Total N=38**
Pan-isoform PI3K	4 (30.8%)	5 (26.3%)	3 (50%)	**12 (31.6%)**
PI3K-alpha specific	-	7 (36.8%)	-	**7 (18.4%)**
Dual PI3K/mTORC1/2	2 (15.4%)	4 (21.1%)	1 (16.7%)	**7 (18.4%)**
mTORC1/2	3 (23.0%)	-	-	**3 (7.9%)**
AKT	4 (30.8%)	3 (15.8%)	2 (33.3%)	**9 (25.7%)**
Total	**13 (100%)**	**19 (100%)**	**6 (100%)**	**38 (100%)**

MUT, mutated; ND, not done; WT, wild type.

ii) Time to progression

Figure 2 depicts TTP for the individual patients, as well as the type of breast tumor and the distribution of molecular alterations.

Interestingly, seven patients received treatment with no evidence of progression for more than 6 months.

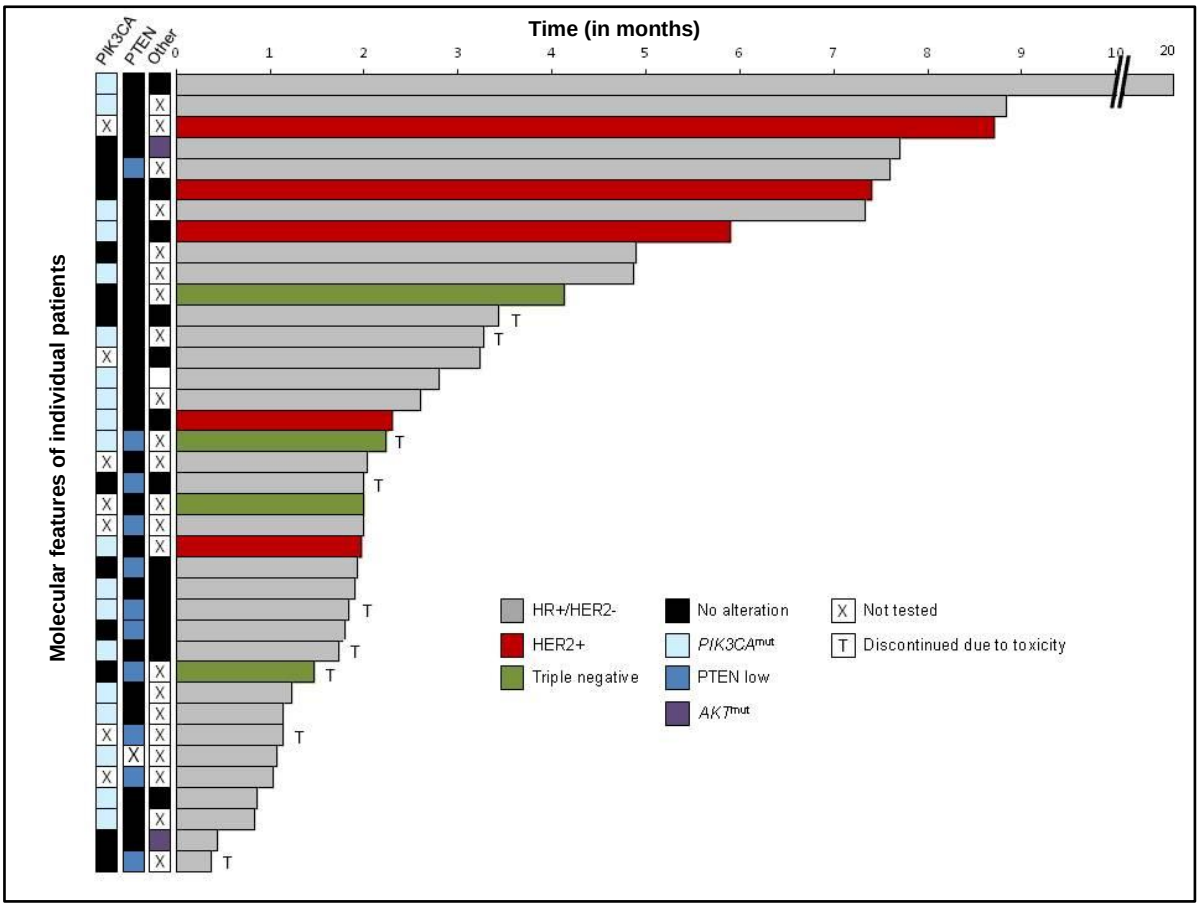

Figure 2. TTP for each individual patient.

Median TTP with the treatment with a PI3K pathway inhibitor as single agent was 2.6 months (95%CI 2.0-4.8 months).

No differences in TTP were seen according to age, breast tumor subtype or previous number of MBC lines (Table 4).

Table 4. Median TTP according to clinical characteristics.

TTP (95%CI)		***P***
Overall	2.6 (2.0-4.8)	
Age		
<50	2.5 (1.8-4.8)	*0.225*
>=50	3.8 (2.0-NR)	
Breast tumor subtype		
HR+/HER2-	2.1 (1.9-4.8)	
HER2+	5.9 (2.3-NR)	*0.745*
TN	3.6 (2.0-NR)	
Previous number of MBC lines		
0-1	1.9 (1.2-NR)	0.685
≥2	2.6 (1.0-4.9)	

CI, Confidence Interval; HR, Hormonal Receptor; NR, Not Reached; TN, Triple negative.

TTP to PI3K pathway inhibitor was numerically (but not statistically significantly) higher in *PIK3CA* WT patients (4.1 vs. 2.3 months, *P*=0.500), and in patients with no PI3K pathway dysregulation (4.9 vs. 2.3 months, *P*=0.374) (Table 5 and Figure 3). Interestingly, no differences were seen according to PTEN.

Table 5. Time to progression to PI3K pathway inhibitor and to prior line of treatment.

Time in months	**Prior line of treatment**		**PI3K pathway inhibitor**	
	TTP (95%CI)	***P***	**TTP (95%CI)**	***P***
Overall	5.0 (3.6 - 9.9)		2.6 (2.0 - 4.8)	
PIK3CA				
WT	5.1 (3.4 - NR)	*0.829*	4.1 (2.4 - NR)	*0.500*
Mutated	5.7 (3.1 - 13.1)		2.3 (1.9 - 7.3)	
PTEN				
Low	4.1 (3.3 - NR)	*0.507*	2.4 (1.9 - NR)	*0.480*
Normal	5.3 (3.9 - 11.7)		2.9 (2.0 - 7.3)	
PI3K pathway dysregulation				
No	12.3 (3.6 - NR)	*0.411*	4.9 (4.1 - NR)	*0.374*
Yes	4.1 (3.3 - 7.2)		2.3 (1.9 - 5.9)	

CI, Confidence Interval; NR, Not Reached; TTP, Time to Progression; WT, Wild Type.

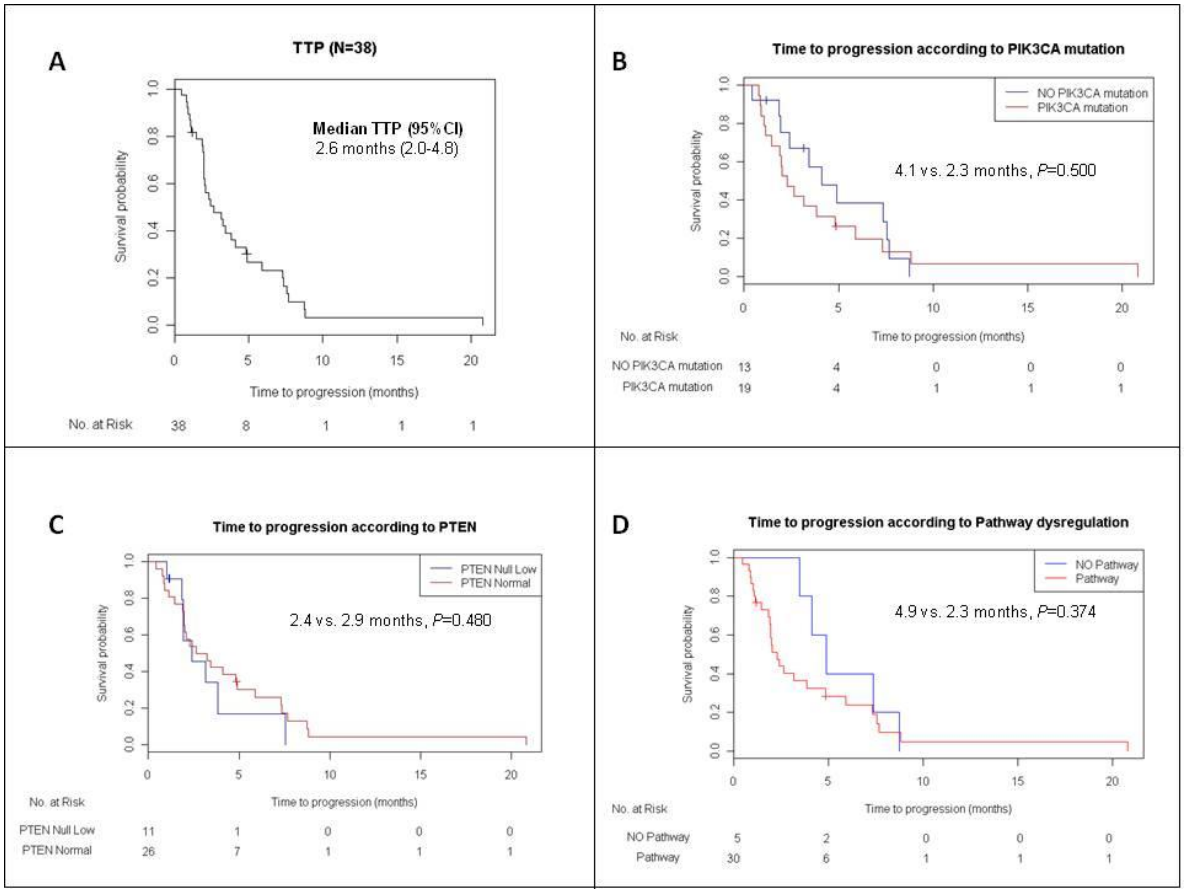

Figure 3. Time to Progression to PI3K pathway inhibitor. (A) Overall population. **(B)** According to *PIK3CA* mutation. **(C)** According to PTEN expression. **(D)** According to PI3K pathway dysregulation. TTP, Time to progression.

When we look at how the PI3K pathway inhibitor as single agent compared to the prior treatment these patients had received (Table 5), we see that in all subgroups prior treatment led to significantly longer TTP than PI3K inhibitor as single agent ($p<0.001$ for all comparisons).

Finally, we repeated all the analysis excluding those patients that received a PI3K inhibitor in first or second line of treatment for MBC, in order to see how PI3K inhibitor as single agent behaved in a population that currently has no approved standard treatment for MBC. This has left us with 33 patients, whose clinical characteristics were similar to the general population.

Molecular characteristics of these patients are shown in Table 6. Determinations were performed mainly in primary tumor tissue (n=18, 54.6%), and metastatic tumor tissue was used in 11 patients (33.3%).

Table 6. Molecular characteristics of patients treated in third or posterior lines for metastatic breast cancer.

Site of molecular determination (N=33)	
Primary tumor	18 (54.6%)
Metastasis	11 (33.3%)
Unknown	4 (12.1%)
Analysis with Sequenom technology (N=33)	13 (39.4%)
***PIK3CA* status (N=28)**	
WT	11 (39.3%)
Mutated	17 (60.7%)
Exon 1	
E110K	1 (5.8%)
Exon 9	
E545K	6 (35.3%)
Q546K	2 (11.8%)
Exon 20	
H1047R	8 (47.1%)
PTEN (N=32)	
Normal	23 (71.9%)
Low	9 (28.1%)
Other PI3K pathway alterations (N=13)	
AKT1 E17K mutation	2 (15.4%)
PI3K pathway dysregulation (N=30)	
No	4 (13.3%)
Yes	26 (86.7%)

WT, wild type.

PIK3CA status could be determined in 28 out of the 33 patients. Of these, 17 had a *PIK3CA* mutation (60.7%), mainly in exon 20 (n=8). PTEN expression could be determined in 32 out of the 33 patients, and 9 had PTEN low expression (28.1%). Sequenom technology was performed in 13 of the 33 patients (39.4%), allowing for screening of other mutations. From these, 2 (15.4%) had an AKT1 mutation (both E17K).

PI3K pathway status was non-informative in 3 patients. Among the remaining 30 patients, 26 (86.7%) had a dysregulation in the PI3K pathway.

In this selected population of patients, TTP was 2.6 months overall (95%CI 2.0-4.9), and again it was numerically (but not statistically significantly) higher in

PIK3CA WT patients (4.9 vs. 2.3 months, *P*=0.202), and in patients with no PI3K pathway dysregulation (6.1 vs. 2.4 months, *P*=0.276) (Table 7 and Figure 4).

Table 7: Time to progression to PI3K pathway inhibitor and to prior line of treatment in patients receiving PI3K pathway inhibitor in 3rd or posterior line.

Time in months	Prior line of treatment		PI3K pathway inhibitor	
	TTP (95%CI)	***p***	**TTP (95%CI)**	***p***
Overall	5.0 (3.6-11.7)		2.6 (2.0-4.9)	
PIK3CA				
WT	4.0 (3.4-NR)	*0.743*	4.9 (2.4-NR)	*0.202*
Mutated	5.5 (3.1-13.1)		2.3 (1.9-7.3)	
PTEN				
Low	4.1 (3.28-NR)	*0.374*	3.2 (1.9-NR)	*0.918*
Normal	5.1 (3.6-12.5)		2.6 (2.0-7.3)	
PI3K pathway dysregulation				
No	12.3 (3.6-NR)	*0.474*	6.1 (3.4-NR)	*0.276*
Yes	4.1 (3.1-9.9)		2.4 (1.9-7.3)	

NR, Not Reached; WT, Wild Type.

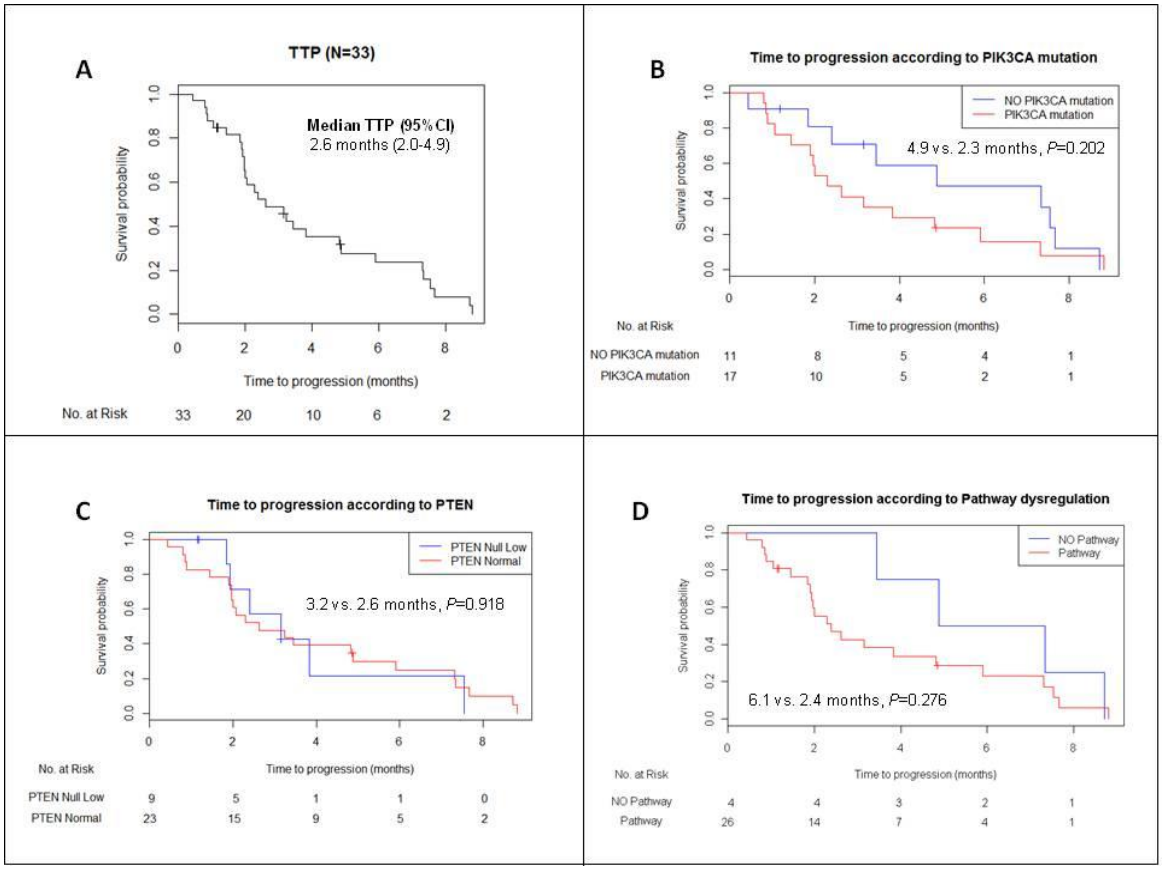

Figure 4. Time to Progression to PI3K pathway inhibitor in patients receiving PI3K pathway inhibitor in 3rd or posterior line. (A) Overall population. **(B)** According to *PIK3CA* mutation. **(C)** According to PTEN expression. **(D)** According to PI3K pathway dysregulation.

Prior treatment led to significantly longer TTP than PI3K inhibitor as single agent in all subgroups (p<0.001 for all comparisons) (Table 7).

It has been suggested that *PIK3CA* mutation predicts response to PI3K alpha-specific inhibitors, but probably not to pan-isoform PI3K, dual PI3K/mTOR, mTOR, or AKT inhibitors. To test this hypothesis, we had a look at the outcomes of the 19 patients with a *PIK3CA* mutation in our population. Seven out of these 19 patients (36.8%) received an alpha-specific inhibitor (Table 8).

Table 8. Distribution of patients with *PIK3CA* mutations according to treatment received.

Type of Treatment	***N***
Alpha-specific PI3K inhibitor Pan-isoform PI3K inhibitor	7 5
Alpha-specific PI3K inhibitor Pan-isoform PI3K, dual PI3K/mTOR, mTOR, AKT inhibitor	7 12

Notably, patients with *PIK3CA* mutation that receive a PI3K-alpha inhibitor did better than patients with *PIK3CA* mutation that receive a pan-isoform PI3K inhibitor (*P*=0.025) (Figure 5). A numerical difference was also seen when comparing treatment with alpha-specific inhibitor with other treatments (3.2 vs. 1.7 months), although it did not reach statistical significance (*P*=0.520).

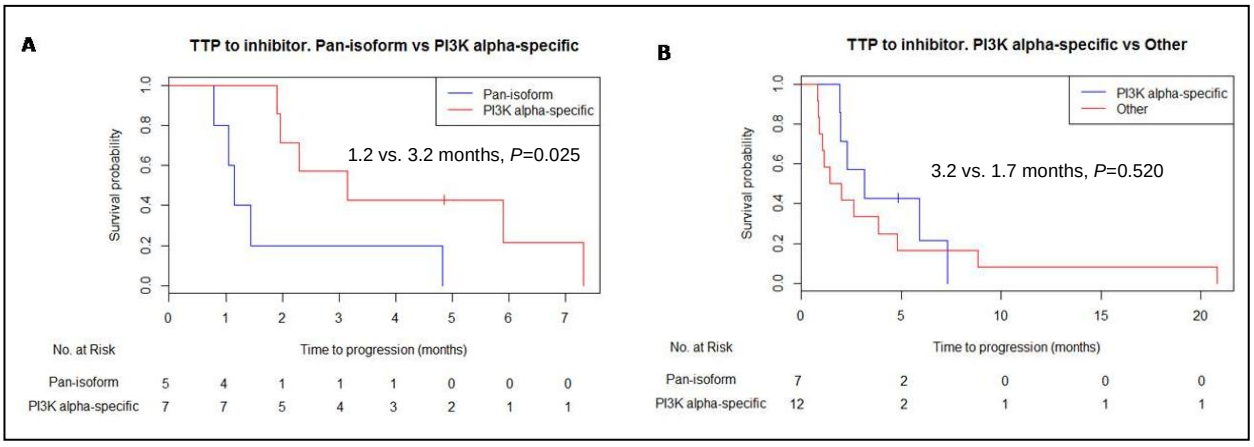

Figure 5. TTP in *PIK3CA* mutated patients. (A) Treatment with pan-isoform PI3K inhibitor versus treatment with PI3K alpha-specific inhibitor. **(B)** Treatment with PI3K alpha-specific inhibitor versus treatment with other inhibitors (pan-isoform PI3K, dual PI3-mTOR, mTORC1/2, and AKT inhibitors).

iii) Response to treatment

Table 9 shows the overall response rate of these patients.

Table 9. Response rate in the overall population.

Best response	N (%)
CR	-
PR	1 (2.6%)
SD	22 (57.9%)
PD	15 (39.5%)
SD > 6 months	7 (18.4%)
CBR	8 (21.1%)

CBR, clinical benefit rate; CR, complete response; PD, progressive disease; PR, partial response; SD, stable disease.

Twenty-nine of the 38 patients (76.3%) had measurable disease according to RECIST 1.0. Figure 6 shows the waterfall plot for these patients. One PR was recorded (RR 2.6%), in an ER+/HER2- patient with a *PIK3CA* mutation in exon 9, and 19 patients had SD as best response.

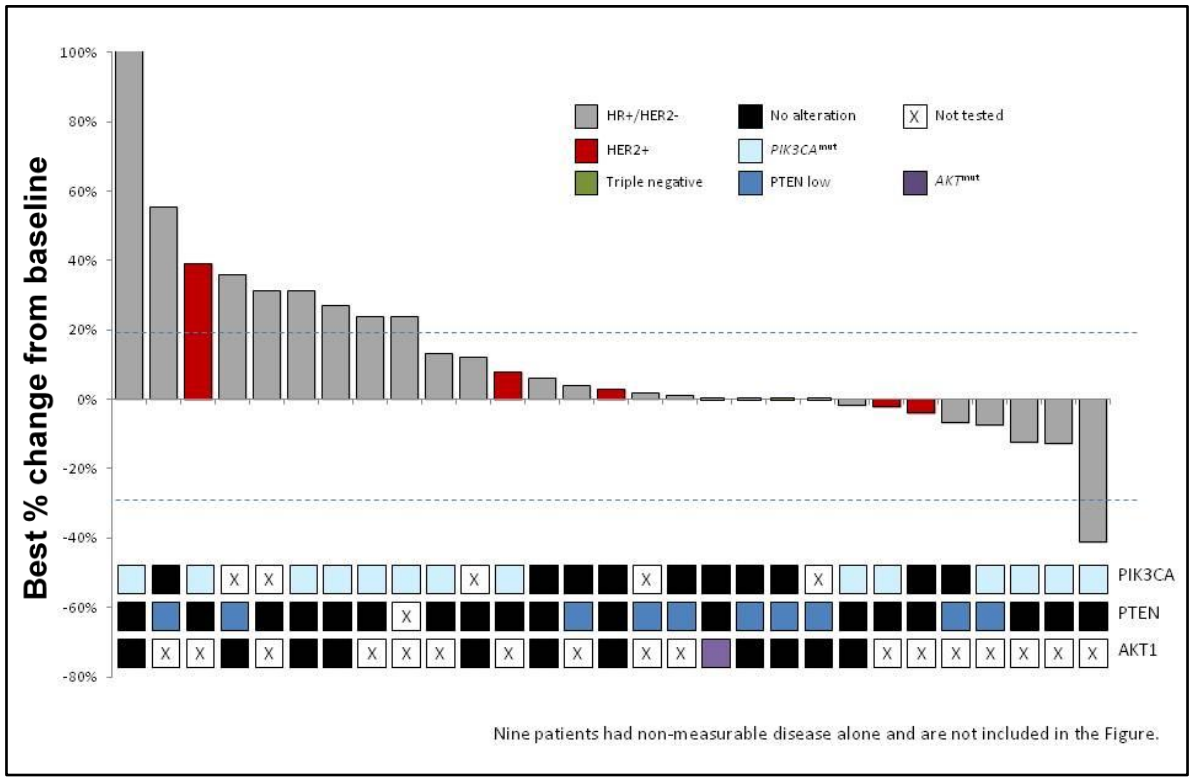

Figure 6. Waterfall plot of best response to treatment.

Of the nine patients who had non-measurable disease only, three achieved stable disease as best response (one of them >6 months) and the remaining had progressive disease at the time of first tumor assessment.

No clear association between response and *PIK3CA* status, PTEN expression or presence of a PI3K pathway dysregulation could be established.

iv) Exploratory analysis of survival

In the whole population, median OS after receiving a PI3K pathway inhibitor was 20.1 months (95%CI 8.5-NR). No statistically different results were observed according to the presence of *PIK3CA* mutation, or expression of PTEN, although patients with PI3K pathway dysregulation lived less than patients with no PI3K pathway dysregulation after having received the PI3K pathway inhibitor (Figure 7).

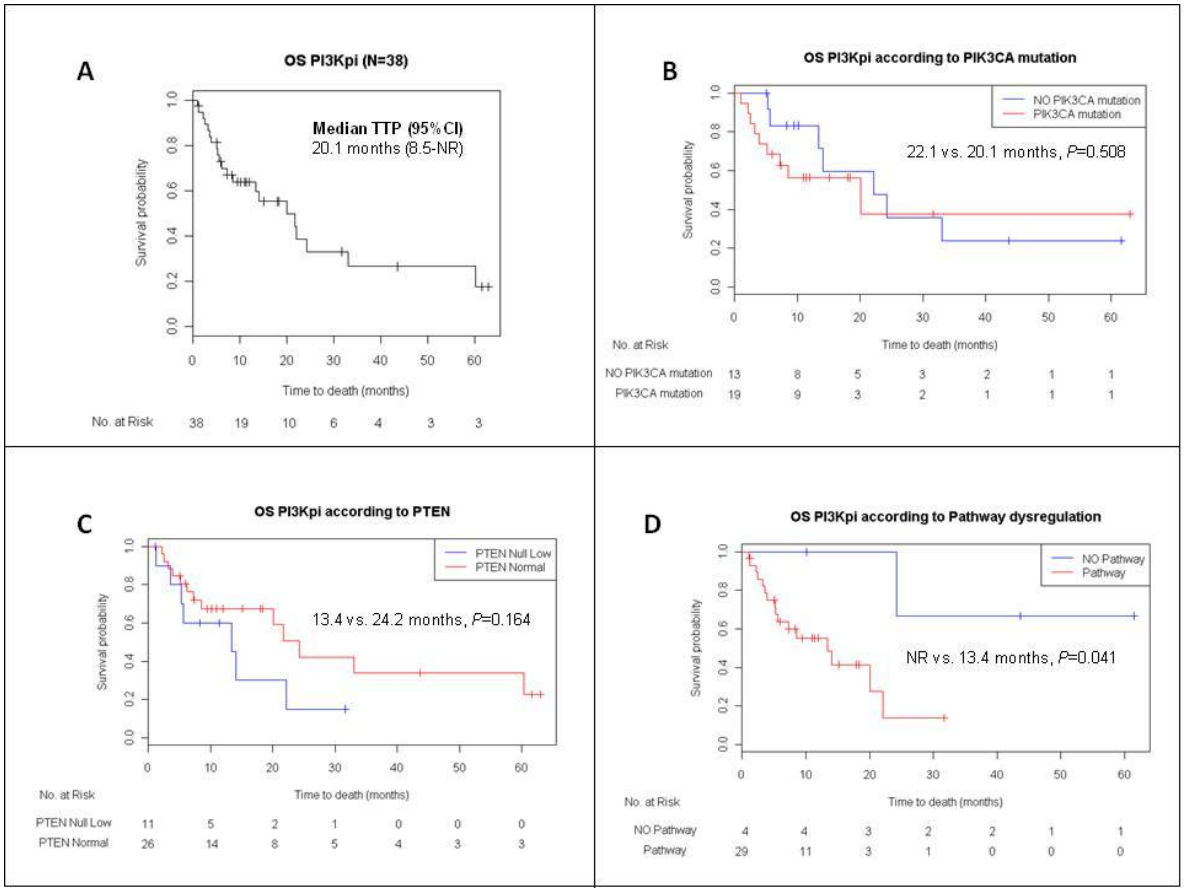

Figure 7. Overall Survival after treatment with PI3K pathway inhibitor. (A) Overall population. **(B)** According to *PIK3CA* mutation. **(C)** According to PTEN expression. **(D)** According to PI3K pathway dysregulation. NR, Not Reached.

Median OS after diagnosis of MBC was 58.1 months (Figure 8). Although patients with *PIK3CA* mutations had a shorter OS after diagnosis of MBC, this did not reach statistical significance (p=0.704).

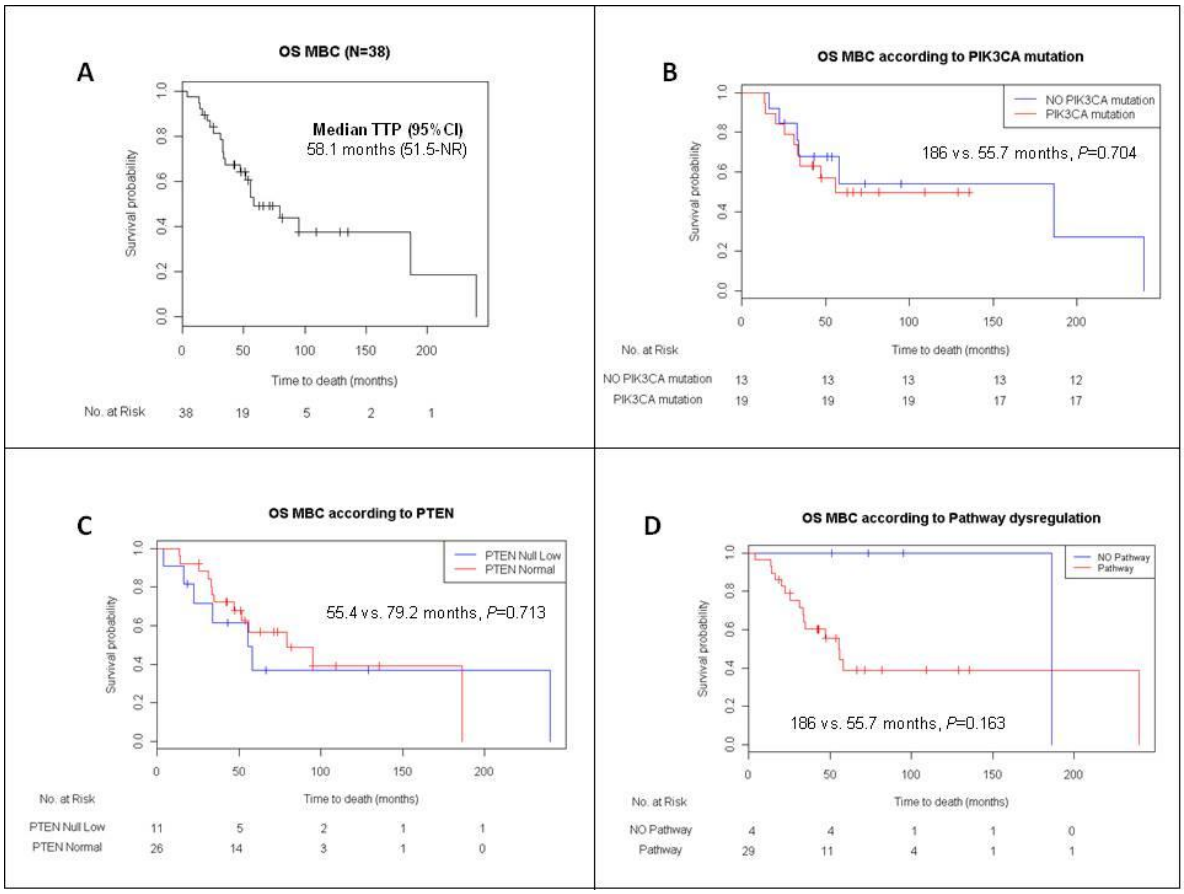

Figure 8. Overall Survival after diagnosis of metastatic breast cancer. (A) Overall population. **(B)** According to *PIK3CA* mutation. **(C)** According to PTEN expression. **(D)** According to PI3K pathway dysregulation. NR, Not Reached.

5. Discussion

The prevalence of *PIK3CA* mutations and PTEN low expression in our cohort was 59.4% and 20.7%, respectively, for a total of 78.9% of patients with a dysregulation in the PI3K pathway. These numbers are slightly higher than the usually reported in breast cancer (16, 17). This may be due to a twofold selection bias: the one introduced by treating physicians that offered a pathway-modulating agent to patients with PI3K pathway dysregulation (especially to patients harboring *a PI3KCA* mutation); or the bias of clinical trials that specifically required patients with molecular alterations as inclusion criteria.

This is, to the best of our knowledge, the largest cohort of breast cancer patients treated with single agent PI3K pathway inhibitors that has been reported to date.

In our cohort, the TTP to single agent PI3K pathway inhibitor was 2.6 months, considering both patients treated in any line and only heavily pre-treated MBC patients (i.e. patients who received the PI3K pathway inhibitor in ≥3rd line). While the majority of our patients had a non-clinically significant benefit from this treatment (52.6% had TTP of ≤2 months), it is remarkable that 8 achieved a PR or SD for more than 6 months (CBR of 21%). This is an interesting result for a single-agent targeted therapy in a refractory setting population. However, median TTP to single agent PI3K pathway inhibitor in the whole cohort was consistently inferior that median TTP to the prior line of treatment these patients had received ($p<0.01$ for all comparisons).

It has been speculated that PI3K pathway inhibitors may have limited clinical activity if used as single agents, because of the complex network of negative feedback loops involving receptor tyrosine kinases (RTK) and PI3K/AKT/mTOR and MAPK pathways (26). Examples of this feedback loops are the rebound phosphorylation of AKT after inhibition of TORC1 and S6K with everolimus (27, 28), the enhanced transcription and phosphorylation of multiple RTKs (including HER3, IGFR-1 and insulin receptor) after AKT inhibition (29), and the compensatory activation of the ERK signaling pathway as a result of activation of HER family receptors upon inhibition of p110 (30). For those patients who did not

benefit from single agent treatment, we can hypothesize that some of these feedback loops were being activated, leading to the lack of response. Analyzing tumor samples after tumor progression to these agents would clearly help us understand if these mechanisms of resistance might be involved. Combinatorial approaches involving PI3K/AKT/mTOR inhibitors, MAPK pathway inhibitors and/or other standard treatments such as anti-hormonal therapy, chemotherapy or anti-HER2 therapy have been or are currently being explored as a way of overcoming such mechanisms of resistance (31-33).

PIK3CA mutations and/or PTEN low expression have been proposed as biomarkers of response to PI3K pathway inhibitors (34-38). In our cohort, no clear association between TTP and *PIK3CA* or PTEN status could be demonstrated. However, among *PIK3CA* mutated patients, those who were treated with PI3K alpha-specific inhibitors had longer TTP than those who received pan-isoform PI3K inhibitors. Although very preliminary, this is the first time such an observation is made in the clinical setting. Given this possibility, the identification of a *PIK3CA* mutation may prompt the treating physician to offer these patients an alpha-specific inhibitor. Recently, a trial testing single agent BYL719, a selective inhibitor of PI3Kα, reported prolonged disease stabilization in patients with ER+/*PIK3CA* mutated breast cancer and one out of the 11 breast cancer patients achieved a partial response (38). Results from this and other trials with alpha isoform-specific inhibitors will be important to determine their activity in *PIK3CA* mutated MBC patients.

The suggestion that patients harboring a *PIK3CA* mutation may have worse outcomes when treated with single agent PI3K pathway inhibitors is provocative. However, it should be interpreted cautiously, as the retrospective design of the study and the small numbers of patients preclude adequate powered comparisons between groups. Of note, our results contrast to another study reporting a response rate of 30% in gynecological and breast cancer patients with *PIK3CA* mutations, compared with 10% in those without a mutation enrolled in similar trials (p=0.04) (39). It should be stressed that in this study all responses were seen in patients receiving combination therapies (44% vs. 0% for single agents, p=0.06),

while in our study all patients received PI3K pathway inhibitors as single agents. Additionally, in the study by Janku *et al.* most of the responding patients received a combination of an mTOR inhibitor with liposomal doxorubicin (an effective chemotherapy in breast and gynecological tumors) and among the 7 patients achieving PR, only one had breast cancer.

Establishing a valid predictive biomarker of response to PI3K/AKT/mTOR inhibitors in the clinical setting can be challenging for a number of reasons. First, the molecular alterations we are able to detect may be "passenger", and not true drivers of tumor proliferation. Second, the presence of a single mutation may not be a sufficient biomarker of pathway activation, and hence of potential benefit of a drug that targets the pathway. Loi et al. for instance, have described that *PIK3CA* mutations in ER+/HER2- are associated with relatively low mTORC1 signaling output, which correlated with rapamycin resistance (40). This kind of signatures may traduce more accurately the activation of the PI3K/AKT/mTOR pathway and hence may help to discriminate for treatment sensitivity. Finally, the presence of the biomarker may not be representative of the current disease. In our study, all the molecular determinations were performed in archival specimens, and the majority (55.3%) was tissue from the primary tumor. This may lead to two major limitations when looking for a predictive biomarker. The first is due to tumor heterogeneity, as a single biopsy in a single time point may not traduce the molecular complexity of the tumor (41, 42). The second is the possibility of clonal evolution between archival tumor (primary) specimens and the contemporary metastatic disease. Of note, it is estimated that about 25% of breast cancers change their *PIK3CA* status along the natural history of the disease (43, 44). Ideally, fresh tumor biopsies from target lesions prior to treatment beginning should be obtained, but this must balance against several safety and ethical issues (45).

This work presents other limitations that must be acknowledged. First, we have included outcomes from compounds acting in the PI3K signaling pathway at different sites, and we cannot exclude that their activity is neither equivalent nor comparable. Then, accuracy and sensitivity of *PIK3CA* somatic mutation determinations vary between techniques, and we cannot rule out that other

important predictive mutations were not detected by the technology that was used for most of the current analysis. It is also uncertain which the best technique to determine PTEN activity is. We have used an H-score ≤50 to consider the presence of a deficient PTEN, as it was the required cut-off for the inclusion in several of the considered clinical trials, but this is probably an approximate way of measuring PTEN function, and a threshold decided without a sound biological rationale.

6. Conclusion

In conclusion, treatment with single agent PI3K pathway inhibitors showed an interesting activity in a population of heavily pre-treated MBC patients. Correlation between PIK3 pathway alterations and efficacy of these agents in the general population could not be demonstrated, but patients harboring a *PIK3CA* mutation did better if treated with a PI3K alpha-specific inhibitor. These results emphasize the need of an adequate molecular selection prior to beginning of a targeted therapy in MBC.

Expanding the knowledge on the activity of alpha isoform-specific PI3K inhibitors in MBC patients, discovery of innovative biomarkers of response and development of rational combinations of PI3K pathway inhibitors with standard treatments are warranted to improve outcome of MBC patients, to overcome treatment resistances and to optimize the use of these targeted drugs.

7. References

1. Jemal A, Bray F, Center MM, et al. Global Statistics. CA Cancer J Clin 2011;61(2):69-90.
2. Guarneri V, Conte P. Metastatic breast cancer: therapeutic options according to molecular subtypes and prior adjuvant therapy. Oncologist 2009 Jul;14(7):645-56. Epub 2009 Jul 16.
3. O'Shaughnessy J. Extending Survival with Chemotherapy in Metastatic Breast Cancer. The Oncologist. 2005;10 (suppl 3): 20 -29.
4. Cardoso F, Costa A, Norton L, et al. 1st International consensus guidelines for advanced breast cancer (ABC 1). Breast. 2012 Jun;21(3):242-52. Epub 2012 Mar 16.
5. Slamon DJ, Leyland-Jones B, Shak S, et al. Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. N Engl J Med. 2001 Mar 15;344(11):783-92.
6. Baselga J, Cortés J, Kim SB, et al. Pertuzumab plus trastuzumab plus docetaxel for metastatic breast cancer. N Engl J Med. 2012 Jan 12;366(2):109-19. Epub 2011 Dec 7.
7. Verma S, Miles D, Gianni L, et al. Trastuzumab emtansine for HER2-positive advanced breast cancer. N Engl J Med. 2012 Nov 8;367(19):1783-91. Epub 2012 Oct 1.
8. Andre F, Slimane K, Bachelot T, et al. Breast cancer with Synchronous metastases: trends in survival during a 14-Year Period. J Clin Oncol 2004;22:3302-8.
9. Meric-Bernstam F & Mills GB. Overcoming implementation challenges of personalized cancer therapy. Nat Rev Clin Oncol 2012;9:542-548. E-pub Jul 31.
10. Kim ES, Herbst RS, Wistuba II, et al.: The BATTLE Trial: Personalizing Therapy for Lung Cancer. Cancer Discovery 1:43-53, 2011
11. Von Hoff DD, Stephenson JJ, Jr., Rosen P, et al.: Pilot study using molecular profiling of patients' tumors to find potential targets and select treatments for their refractory cancers. J Clin Oncol 28:4877-4883, 2010

12. Rodon J, Saura C, Dienstmann R, et al.: Molecular prescreening to select patient population in early clinical trials. Nat Rev Clin Oncol 9:359-366, 2012
13. Flaherty KT, Puzanov I, Kim KB, et al.: Inhibition of mutated, activated BRAF in metastatic melanoma. N Engl J Med 2010;363:809-819.
14. Kwak EL, Bang YJ, Camidge DR, et al.: Anaplastic lymphoma kinase inhibition in non-small-cell lung cancer. N Engl J Med 2010;363:1693-1703.
15. Demetri GD, von MM, Blanke CD, et al.: Efficacy and safety of imatinib mesylate in advanced gastrointestinal stromal tumors. N Engl J Med 2002;347:472-480.
16. The Cancer Genome Atlas Network. Comprehensive molecular portraits of human breast tumours. Nature. 2012 Oct 4;490(7418):61-70. Epub 2012 Sep 23.
17. Hernandez-Aya LF, Gonzalez-Angulo AM: Targeting the phosphatidylinositol 3-kinase signaling pathway in breast cancer. Oncologist 2011;16:404-414.
18. Elbauomy Elsheikh S, Green AR, Lambros MB, et al. FGFR1 amplification in breast carcinomas: a chromogenic in situ hybridisation analysis. Breast Cancer Res. 2007;9(2):R23.
19. Turner N, Pearson A, Sharpe R, et al. FGFR1 amplification drives endocrine therapy resistance and is a therapeutic target in breast cancer. Cancer Res. 2010 Mar 1;70(5):2085-94. Epub 2010 Feb 23.
20. Bendell JC, Rodon J, Burris HA, et al.: Phase I, dose-escalation study of BKM120, an oral pan-Class I PI3K inhibitor, in patients with advanced solid tumors. J Clin Oncol 30:282-290, 2012
21. Burris H, Rodón-Ahnert J, Sharma S, et al.: First-in-human phase I study of the oral PI3K inhibitor BEZ235 in patients (pts) with advanced solid tumors. J Clin Oncol 28 (suppl; abst 3005), 2010
22. Tabernero J, Saura C, Roda-Pérez R, et al.: First-in-human phase I study evaluating the safety, pharmacokinetics (PK), and intratumor pharmacodynamics (PD) of the novel, oral, ATP-competitive Akt inhibitor GDC-0068. J Clin Oncol 29 (suppl;abstr 3022), 2011
23. Tabernero J, Cervantes A, Gordon MS, et al.: A phase I, open label, dose escalation study of oral mammalian target of rapamycin inhibitor INK128

administered by intermittent dosing regimens in patients with advanced malignancies. Cancer Res 72;8(Suppl 1; abstr CT-02), 2012

24. Rodon J, Saura C, Dienstmann R, et al.: Molecular prescreening to select patient population in early clinical trials. Nat Rev Clin Oncol 9:359-366, 2012.
25. Therasse P, Arbuck SG, Eisenhauer EA, et al.: New guidelines to evaluate the response to treatment in solid tumors. European Organization for Research and Treatment of Cancer, National Cancer Institute of the United States, National Cancer Institute of Canada. J Natl Cancer Inst 92:205-216, 2000.
26. Garrett JT, Chakrabarty A, Arteaga CL: Will PI3K pathway inhibitors be effective as single agents in patients with cancer? Oncotarget 2:1314-1321, 2011.
27. Tabernero J, Rojo F, Calvo E, et al.: Dose- and schedule-dependent inhibition of the mammalian target of rapamycin pathway with everolimus: a phase I tumor pharmacodynamic study in patients with advanced solid tumors. J Clin Oncol 26:1603-1610, 2008.
28. O'Reilly KE, Rojo F, She QB, et al.: mTOR inhibition induces upstream receptor tyrosine kinase signaling and activates Akt. Cancer Res 66:1500-1508, 2006.
29. Chandarlapaty S, Sawai A, Scaltriti M, et al.: AKT inhibition relieves feedback suppression of receptor tyrosine kinase expression and activity. Cancer Cell 19:58-71, 2011.
30. Serra V, Scaltriti M, Prudkin L, et al.: PI3K inhibition results in enhanced HER signaling and acquired ERK dependency in HER2-overexpressing breast cancer. Oncogene 30:2547-2557, 2011.
31. Baselga J, Campone M, Piccart M, et al.: Everolimus in postmenopausal hormone-receptor-positive advanced breast cancer. N Engl J Med 366:520-529, 2012.
32. Krop IE, Saura C, Rodón-Ahnert J, et al.: A phase I/IB dose-escalation study of BEZ235 in combination with trastuzumab in patients with PI3-kinase or PTEN altered HER2+ metastatic breast cancer. J Clin Oncol 30, 2012 (abstr).

33. Saura C, Jones S, Mateo J, et al.: A phase Ib study of the Akt inhibitor GDC-0068 with docetaxel (D) or mFOLFOX-6 (F) in patients (pts) with advanced solid tumors. J Clin Oncol 30, 2012 (abstr).
34. Brachmann SM, Hofmann I, Schnell C, et al.: Specific apoptosis induction by the dual PI3K/mTor inhibitor NVP-BEZ235 in HER2 amplified and *PIK3CA* mutant breast cancer cells. Proc Natl Acad Sci U S A 106:22299-22304, 2009.
35. O'Brien C, Wallin JJ, Sampath D, et al.: Predictive biomarkers of sensitivity to the phosphatidylinositol 3' kinase inhibitor GDC-0941 in breast cancer preclinical models. Clin Cancer Res 16:3670-3683, 2010.
36. Roper J, Richardson MP, Wang WV, et al.: The dual PI3K/mTOR inhibitor NVP-BEZ235 induces tumor regression in a genetically engineered mouse model of *PIK3CA* wild-type colorectal cancer. PLoS One 6:e25132, 2011.
37. Serra V, Markman B, Scaltriti M, et al.: NVP-BEZ235, a dual PI3K/mTOR inhibitor, prevents PI3K signaling and inhibits the growth of cancer cells with activating PI3K mutations. Cancer Res 68:8022-8030, 2008.
38. Juric D, Rodon J, Gonzalez-Angulo AM, et al.: BYL719, a next generation PI3K-specific inhibitor: Preliminary safety, pharmacokinetics, and efficacy results from the first-in-human study. AACR 2012 Annual Meeting, 2012 (abstr).
39. Janku F, Wheler JJ, Westin SN, et al.: PI3K/AKT/mTOR Inhibitors in Patients With Breast and Gynecologic Malignancies Harboring *PIK3CA* Mutations. J Clin Oncol, 2012.
40. Loi S, Haibe-Kains B, Majjaj S, et al.: *PIK3CA* mutations associated with gene signature of low mTORC1 signaling and better outcomes in estrogen receptor-positive breast cancer. Proc Natl Acad Sci U S A 107:10208-10213, 2010.
41. Gerlinger M, Rowan AJ, Horswell S, et al.: Intratumor heterogeneity and branched evolution revealed by multiregion sequencing. N Engl J Med 366:883-892, 2012.
42. Shah SP, Roth A, Goya R, et al.: The clonal and mutational evolution spectrum of primary triple-negative breast cancers. Nature 486:395-399, 2012.

43. Gonzalez-Angulo AM, Ferrer-Lozano J, Stemke-Hale K, et al.: PI3K pathway mutations and PTEN levels in primary and metastatic breast cancer. Mol Cancer Ther 10:1093-1101, 2011.
44. Dupont JJ, Laenkholm AV, Knoop A, et al.: *PIK3CA* mutations may be discordant between primary and corresponding metastatic disease in breast cancer. Clin Cancer Res 17:667-677, 2011.
45. Olson EM, Lin NU, Krop IE, et al.: The ethical use of mandatory research biopsies. Nat Rev Clin Oncol 8:620-625, 2011.