CLINICAL TRIAL

PIK3CA mutations early persistence in cell-free tumor DNA as a negative prognostic factor in metastatic breast cancer patients treated with hormonal therapy

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Abstract

Purpose The identification of biomarkers of hormonal therapy (HT) failure would allow tailored monitoring in metastatic breast cancer (mBC) patients. *PIK3CA* gene mutation is one of the most frequent events in mBC and is associated with HT resistance. We evaluated the early prognostic value of cell-free DNA (cfDNA) *PIK3CA* detection in first-line HT-treated mBC patients.

Methods Between June 2012 and January 2014, 39 patients were prospectively included in a dedicated clinical trial (NCT01612871). Blood sampling was performed before (M0) and 4 weeks (M1), 3 months (M3) and 6 months (M6) after HT initiation, and at tumor progression. Patients were followed until progression or until the end of the study (2 years). Mutation detection was performed using droplet-based digital PCR (ddPCR). Progression-free survival (PFS) was used as primary endpoint.

Results Median age at inclusion was 63 years (range 40–86). Most patients (34/39) received an aromatase inhibitor and presented a non-measurable disease (71.8%). *PIK3CA* mutations were reported in 10 (27.8%) and 5 (14.3%) cases at M0 and M1, respectively. The persistence of a detectable circulating mutation at M1 was highly correlated with a worse progression-free survival (PFS), rate at 1 year: 40% versus 76.7%; $p = 0.0053$).

Conclusions Four-week persistence of cfDNA *PIK3CA* mutation appears highly correlated with PFS.

Trial registration NCT01612871, registered on June 6th, 2012; https://clinicaltrials.gov/ct2/show/NCT01612871.

Abbreviations

BC	Breast cancer
cfDNA	Cell-free DNA
CDK	Cyclin-dependent kinase
ER+	Estrogen-receptor-positive
HT	Hormonal therapy
mBC	Metastatic breast cancer
mTOR	Mammalian target of rapamycin
PI3K	Phosphatidylinositol 3-kinase
PI3KCA	PI3K catalytic subunit alpha
PFS	Progression-free survival
RB	Retinoblastoma
95% CI	95% confidence interval

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Background

Hormonal therapies (HT) have improved the patients' outcomes in estrogen-receptor-positive (ER+) metastatic breast cancer (mBC). However, the effectiveness is limited by de novo or acquired resistance, occurring in nearly all mBC patients. Multiple mechanisms responsible for HT resistance have been proposed, including activation of various intracellular pathways mainly the phosphatidylinositol 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR), and cyclin-dependent kinase (CDK) 4/6/retinoblastoma protein

(RB) pathways. The occurrence of mutations in the PI3K catalytic subunit alpha (*PIK3CA*), which generally occur in one of the two hotpot regions (Exon 9: E545K or E542K, and Exon 20: H1047R), is one of the most frequent events in ER + BC and has been involved in HT resistance [1–8]. However, the clinical impact of the *PIK3CA* status analyzed in tissue samples remains debated and most patients affected by estrogen-receptor-positive/HER2-negative (ER+/HER2−) tumors presented only bone metastases, a metastatic site associated with technical issues regarding pathological and biological evaluations [9, 10]. However, the identification of early prognostic biomarkers under HT could help tailoring monitoring and an early change in therapy.

The search for circulating biomarkers, easily accessible by blood sampling (as opposed to the biopsy of a metastasis) could be a preferred route especially for patients who cannot be biopsied. Circulating cell-free tumor DNA (cfDNA) is a specific marker of tumor disease. Many studies, particularly in lung and colorectal cancers but also in breast cancer (BC), have shown that predictive factors of response to treatments such as *KRAS*, *EGFR*, or *PI3KCA* mutations could be detected in cfDNA [11–13]. Circulating biomarkers such as cfDNA may also allow a global evaluation of all the metastatic sites, at the time of treatment initiation and during treatment, without the need for invasive biopsies and the difficulties associated with the specific requirements of bone metastases [7, 14, 15]. The detection of specific mutations on cfDNA could thus be proposed as a dynamic surrogate endpoint for clinical efficacy of a given treatment, allowing a short-term prediction of the long-term treatment outcome.

We prospectively evaluated in a bicentric clinical trial, the early prognostic value of cfDNA *PIK3CA* detection and dynamics in a prospective population of locally advanced or mBC patients receiving first-line HT to explore the prognostic value of baseline and early changes of cfDNA *PIK3CA* mutations.

Methods

Study design and patients

Between June 2012 and January 2014, 39 patients with ER+/HER2− metastatic or locally advanced BC treated with first-line HT in 2 comprehensive cancer centers were prospectively included in a dedicated clinical trial (NCT01612871) aiming at the identification of circulating biomarkers associated with prognosis under HT. Serial blood sampling was performed before HT initiation (M0), at 4 weeks (M1), 3 months (M3), and 6 months (M6) after HT initiation, and at tumor progression (Fig. 1). Patients were followed until progression or until the end of the study (2 years follow-up). Considering the natural history of ER+/HER2− mBC and the frequency of non-measurable disease in this subgroup, 1-year progression-free survival was used as primary endpoint. As part of the study evaluated the prognostic impact of biological markers, this manuscript adheres to the REMARK guidelines [16, 17].

Plasma isolation and cfDNA*PIK3CA* determination

Plasma samples were obtained from blood collected in EDTA K2 tubes before 3 h after blood draw by centrifugation at 1500×*g* for 15 min and were stored at − 80 °C until use. Plasma samples were processed and stored in our respective Biological Resources Centers (Biobank number BB-0033-00059), then centralized for subsequent analysis. Once thawed at room temperature, stored plasma were centrifuged at 16,000×*g* for 15 min at + 4 °C and cfDNA was isolated from the supernatant using the QiaAmp circulating nucleic acid isolation kit (Qiagen, Courtaboeuf, France) according to the manufacturer's instructions. After cfDNA extraction, quality of the cfDNA was evaluated with an Agilent High Sensitivity DNA kit and the Bioanalyzer 2100

Fig. 1 Design and key eligibility criteria
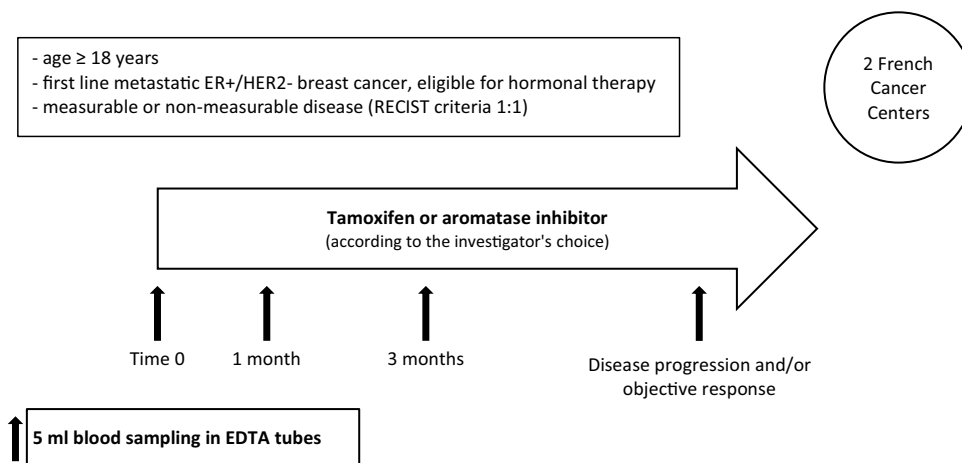
instrument (Agilent Technologies, Les Ulis, France). Subsequently, the circulating cfDNA concentration was quantified by fluorometry with the Qubit 2.0 Fluorometer (Life Technologies, Saint-Aubin, France). Mutation detection was performed using droplet digital PCR on a QX100™ system (Bio-Rad, Marnesla Coquette, France) according to a previously published method [18]. Briefly, a 20 µL reaction mixture containing 10 µl of 2× ddPCR Supermix for probes with no dUTP (Bio-Rad), 5 µL of extracted cfDNA, 900 nM of each specific forward and reverse primer, and 250 nM of each FAM-labeled mutant probe and HEX-labeled wild-type probe were carried out. After droplet formation using 70 µL of generation oil (Bio-Rad) and the entire volume of the PCR mix, 40 µL of droplets was transferred for thermal cycling as follows: 95 °C for 10 min, 40 cycles of 94 °C for 30 s then 60 °C for 60 s followed by 98 °C for 10 min and cooling at 4 °C. The temperature ramp increment was 2 °C/s for all steps. The assays targeted wild-type *PIK3CA* and mutations p.E542K, p.E545K in exon 9, and p.H1047R in exon 20. Primers and TaqMan hydrolysis probes were custom designed and provided by Bio-Rad (sequences available upon request). Genomic DNA from the T84 cell line (c.1624G > A, p. E542K), MCF-7 cell line (c.1633G > A, p.E545K), and LS174T cell line (c.3140 A > G, P.H1047R) were used as positive *PIK3CA* mutation controls. DNA from the LNCaPcell line (wild-type *PIK3CA*) was the negative *PIK3CA* mutation control. For each series of ddPCR assays, a non-template control was carried out.

Data analysis was performed using the QuantaSoft v1.7.4 software. Thresholds for the detection of positive and negative droplets were set manually based on results from non-template control wells and negative *PIK3CA* mutation control (LNCaP cell line). All data were evaluated above the threshold. All experiments were performed in duplicate. First, concentration of amplified fragments, FAM-labeled or HEX-labeled were calculated (copies/reaction) based on the Poisson distribution and the quantification of mutated and wild-type alleles was estimated with the following equation: $\lambda = -\ln(1 - P)$, where λ is the average number of target DNA molecules per replicate reaction and P is the fraction of positive end point reactions. Finally, cfDNA target concentration was calculated as copies/reaction and cfDNA concentrations were reported as number of copies/mL of plasma. The *PIK3CA* mutant allele fraction (MAF % = number of mutated copies/µl/total number of copies/µl) for each sample was determined by using the QuantaSoft software V1.7.4 (Bio-Rad). To assess the limit of detection of the three assays, isogenic reference DNAs with known mutant allele frequency were used (Horizon Diagnostics, Cambridge, United Kingdom). Based on confidence intervals for Poisson parameters, a sample was considered positive for a targeted mutation if the average mutant copies detected was four copies and above per reaction.

Statistical analyses

Data were presented by frequencies and percentages for categorical variables and by medians and ranges for continuous variables. The association between clinicopathological factors and *PIK3CA* mutations at baseline was performed by the Chi-square test or the Fisher's exact test. All survival times were calculated from the date of inclusion to the progression or the death. Patients progression-free or alive were censored at the time of their last follow-up. Progression-free survival rates were analyzed using the Kaplan–Meier method and presented with 95% confidence intervals (95% CI). For univariate analyses, the log-rank test was used for categorical variables and the Cox proportional hazards model for continuous variables. Multivariate analyses were performed using the Cox proportional hazards model including significant covariates indicated by $p < 0.10$ in univariate analyses and hazard ratios with 95% confidence intervals were presented for each covariate in the models.

Results

Patients' characteristics

The main clinical characteristics of the study population are summarized in Table 1. The patients' median age at inclusion was 63 years (range 40–86). Most patients (34/39, 87.2%) received an aromatase inhibitor as first-line HT (letrozole, $n = 32$, tamoxifen, $n = 5$, anastrozole, $n = 1$ and exemestane, $n = 1$ patient). Patients mainly presented with a non-measurable disease ($n = 28$, 71.8%) and 27 had bone metastases precluding a relevant evaluation of prognostic factors for response.

Response and survival

The overall objective response rate was 24.3% (2 complete responses, 5.4%; 7 partial responses, 18.9%), while 70.3% of the patients achieved stable disease. The one- and 2-year progression-free survival (PFS) rates were 67.6% [95% CI (50.0; 80.1)] and 32.4% [95% CI (18.2; 47.5)], respectively, with 25 progression events (64.1%) by the end of the study.

cfDNA *PIK3CA* determination and clinicopathological correlates

Plasma sample results were available for 36, 35, 33, and 30 patients at M0, M1, M3, and M6, respectively. *PIK3CA* mutations were present in 10 (27.8%), 5 (14.3%), 3 (9.1%), and 4 (13.3%) cases at M0, M1, M3, and M6, respectively. M0 cfDNA*PIK3CA* mutations were detected in the helical domain in 6 (60%) samples (c.1624G > A, p. E542K, ($n = 4$),

Table 1 Patients' characteristics at baseline

	$N = 39$
Age at inclusion (years), median (range)	63 (40–86)
Time from initial diagnosis to inclusion (months), median (range)	7.8 (0.5–337.1)
Time from initial diagnosis to metastases (months), median (range)	3.2 (0–336.0)
Missing	1
Measurable disease (RECIST 1.1)	11 (28.2%)
Body Mass Index at inclusion, median (Range)	25.6 (18.9–43.8)
Missing	9
ECOG Performance status	
0	28 (73.7%)
1	10 (26.3%)
Missing	1
Number of metastatic sites, *n* (%) 0 (LABC)	
0	1 (2.6%)
1	23 (59.0%)
2	8 (20.5%)
3	5 (12.8%)
4	2 (5.1%)
Metastases localization (*n* = 38, 1 patient with LABC), *n* (%)	
Bone	27 (71.1%)
CNS	0
Cutaneous	3 (7.9%)
Lymph nodes	11 (28.9%)
Serous	0
Liver	4 (10.5%)
Lung	12 (31.6%)

RECIST response evaluation criteria in solid tumors, *LABC* locally advanced, unresectable, breast cancer, *CNS* central nervous system

c.1633G > A, p.E545K, ($n = 2$)) and in 4 (40%) samples in the catalytic kinase domain (c.3140 A > G, P.H1047R). The MAF ranged from 1.35% to 76.7%. M0 cfDNA *PIK3CA* mutation detection was significantly associated with the presence of bone metastases (10/10 patients with detectable mutated *PIK3CA* cfDNA vs. 14/25 patients without detectable mutated *PIK3C A*cfDNA, $p = 0.0146$; Table 2). To evaluate the correlation between cfDNA parameters and tumor burden, we analyzed the correlations between all the evaluated cfDNA parameters and the number of metastatic sites as an indirect reflect of tumor burden (0–1 sites, $N = 24$ vs. 2+ sites, $N = 15$). No statistically significant correlation was seen with any of the evaluated parameters (data not shown).

Univariate and multivariate analyses of prognostic factors

In univariate analyses (Supplementary Tables 1 and 2), the only clinical variable close to the significance threshold was the presence of lymph node metastases ($p = 0.0615$). Baseline cfDNA concentration and baseline *PIK3CA* MAF (%) were found to be significantly associated with PFS in univariate analysis, while a trend was seen for 1 month *PIK3CA* MAF (%, $p = 0.069$) and cfDNA concentration evolution (1 month—baseline).

While the presence of a cfDNA *PIK3CA* mutation in the M0 sample was not significantly associated with PFS (rate at 12 months: 70% [95% CI (32.9; 89.2)] versus 70.8% [95% CI (48.4; 84.9)]), the persistence of a detectable circulating mutation at M1 was highly correlated with a poorer PFS (rate at 12 months: 40% [95% CI (5.2; 75.3)] versus 76.7% [95% CI (57.2; 88.1)], $p = 0.0053$) (Fig. 2, Supplementary Table 1).

In a second step, considering the number of recorded PFS events, we performed four multivariate analyses using the lymph node metastases variable and the selected cfDNA variables (Supplementary Table 3). Lymph node metastases remain an independent prognostic factor in three models among the four models, and the model taking into account lymph node metastases and persistence of mutated *PIK3CA* cfDNA at 1 month was associated with higher hazard ratios.

No correlation was reported between cfDNA *PIK3CA* mutation detection, whatever the time point, and the tumor response.

Table 2 Clinicopathological correlates with *PIK3CA* mutations, baseline

Baseline *PIK3CA* mutations	No	Yes	*p* value
WHO performance status (*n* = 35)			0.6936
0	19 (76%)	7 (70%)	
1	6 (24%)	3 (30%)	
Metastases localization (*n* = 35)			
Bone metastases			0.0146
No	11 (44%)	0	
Yes	14 (56%)	10 (100%)	
SNC metastases			NA
No	25 (100%)	10 (100%)	
Yes	0	0	
Cutaneous metastases			0.1902
No	24 (96%)	8 (80%)	
Yes	1 (4%)	2 (20%)	
Lymph nodes metastases			1.0000
No	17 (68%)	7 (70%)	
Yes	8 (32%)	3 (30%)	
Serous metastases			NA
No	25 (100%)	10 (100%)	
Yes	0	0	
Liver metastases			1.0000
No	23 (92%)	9 (90%)	
Yes	2 (8%)	1 (10%)	
Lung metastases			1.0000
No	16 (64%)	7 (70%)	
Yes	9 (36%)	3 (30%)	

WHO World Health Organization

Discussion

In metastatic breast cancer (mBC), the evolutionary process under therapeutic pressure and genomic instability leads to the emergence of clonal populations harboring mutations associated with resistance to anticancer therapies [8]. At the same time, identification of the different clonal populations by multiple biopsies of the different metastatic sites is difficult, especially in ER+/HER2− mBC. Indeed, they frequently develop bone metastases, a metastatic site associated with technical issues for molecular analyses. Liquid biopsy appears, in this context, a sensitive and specific, minimally invasive method to identify and monitor mutations of interest [15, 19, 20].

The clinical impact of the tissue *PIK3CA* status remains unknown in different breast cancer histologic types, molecular subtypes as well as under various therapeutic strategies, such as in first-line HT [21]. For example, in an ancillary analysis of the MONALEESA-2 phase III study evaluating the first-line association of CDK4/6 inhibitor ribociclib and

letrozole, ribociclib treatment benefit was maintained irrespective of the *PIK3CA* mutation status [22].

At the same time, identification of circulating cfDNA *PIK3CA* mutations has recently been reported to be associated with clinical behavior and response to anti-PI3K targeted therapies. In the randomized phase III BELLE-2 trial evaluating the impact of buparlisib, a PI3K inhibitor, associated with fulvestrant, tissue-based evaluation of the PI3K pathway activation (based on *PI3KCA* activating mutations or loss of PTEN expression) was not predictive of buparlisib efficacy [14]. However, in the same population, a significant difference in PFS was reported in the buparlisib group versus the placebo group in patients with detectable cfDNA *PIK3CA* mutations, while there was no difference between the groups of patients with no detectable cfDNA *PIK3CA* mutations. At the same time, the overlap between tissue and cfDNA *PIK3CA* mutations detection was partial in this study, as for the 446 patients with known tumor and cfDNA *PIK3CA* status, overall concordance was only 77%, emphasizing the evolutionary process occurring during metastasis.

Recently, the SOLAR-1 study showed alpelisib, a selective PI3K alpha-specific inhibitor, associated with fulvestrant nearly doubled median PFS (11.0 vs. 5.7 months) in HR+/HER2− advanced breast cancer patients with a PIK3CA mutation compared to fulvestrant alone [23]. Alpelisib is now FDA-approved for women with a PIK3CA mutation in HR+/HER2− advanced breast cancer and the use of liquid biopsy will become widespread for women with advanced BC, thus prompting the interest for identification of reliable, minimally invasive, biomarkers of sensitivity/resistance in this population.

In our dedicated prospective clinical trial, in a first-line HT setting, the 4-week persistence or occurrence of a cfDNA *PIK3CA* mutation, and not the baseline cfDNA *PIK3CA* mutation status, appeared highly correlated with PFS. Evolution of specific cfDNA mutations could increase the prognostic value of this biomarker. Our results are in accordance with those recently reported by O'Leary and colleagues [24]. In their ancillary analysis of the pivotal PALOMA-3 trial evaluating the impact of the addition of palbociclib to fulvestrant, 22% of the baseline plasma samples were positive for a *PIK3CA* mutation in ctDNA. No statistically significant association was seen between the baseline *PIK3CA* status in ctDNA and PFS in patients treated with palbociclib and fulvestrant. However, assessment of the effect of the dynamic changes in cfDNA showed a decrease of mutated *PIK3CA* (and not ESR1) ctDNA level after 15 days of palbociclib and fulvestrant treatment, strongly correlated with PFS.

Early identification of a population of mBC harboring persistent cfDNA *PIK3CA* mutations could allow the future evaluation, in a selected population with an unfavorable prognosis, of anti-PI3K targeted therapies combined with HT and/or CDK4/6 inhibitors, considering the

Fig. 2 Progression-free survival (PFS) depending on the presence (black curve) or absence (gray curve) of a *PIK3CA* activating mutation at baseline (**a**) and after 1 month of treatment (**b**)
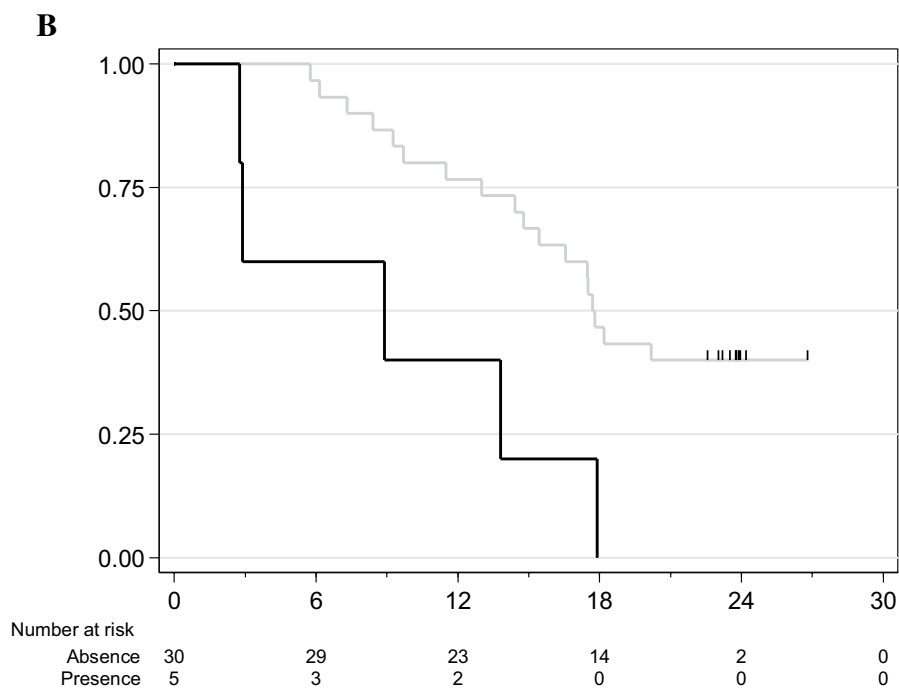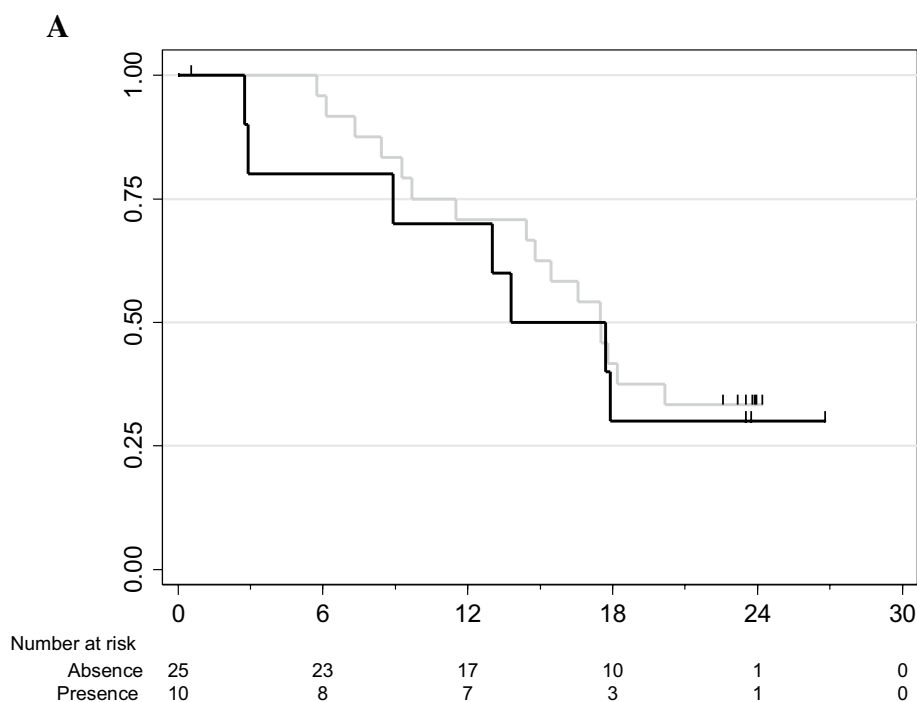
reported synergistic effect of these two classes of drugs [25, 26]. Indeed, focus on relevant biomarker identification on early samples could allow, in the better scenario case, an early adaptation of the treatment, as the aim of a prognostic and/or predictive biomarker is to allow a better identification of a given population early in the therapeutic process. Later time samplings, while of scientific interest, appear less useful considering this tailoring concept.

Although the analysis of early changes in mutation content of cfDNA seems an attractive biomarker [27], there is no consensus on the best technical approach. Digital PCR is an accurate and rapid methodology, easy to implement

in the clinical setting and that compares favorably in terms of sensitivity and cost to the next generation sequencing. In addition, compared to standard real-time PCR, digital PCR can achieve a higher sensitivity and an absolute quantification of targeted mutations.

Our study has some limitations, among which the relatively small number of patients, precluding a global statistically valid multivariate analysis as, considering the number of events, it would have violated the 10 events per 1 variable rule [28], inducing greater risks of bias. To circumvent this limitation, we performed four separate multivariate analyses. In these multivariate analyses, of the considered cfDNA variables, persistence of mutated *PIK3CA* cfDNA at 1 month was associated with the better hazard ratios and appeared the more consistent considering the clinical impact and the published literature on cfDNA value under treatment. Also, only hotspot mutations in the *PIK3CA* gene were assayed. However, these hotspot mutations are expected to represent 80% of the total *PIK3CA* gene mutations in HR+/HER2− mBC [29]. These results need to be confirmed in a separated data set including a greater number of patients and events.

Conclusion

Our study provides a proof of concept and attests of the feasibility of cfDNA analysis for patients with ER+ locally advanced or metastatic BC. Early identification of this population with cfDNA *PIK3CA* mutations allows the identification of a population affected by an unfavorable prognosis. Dedicated and ancillary studies of anti-PI3K targeted therapies are warranted in order to determine the optimal time point to evaluate this new biomarker.

Compliance with ethical standards

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