Baseline Mutations and ctDNA Dynamics as Prognostic and Predictive Factors in ER-Positive/HER2-Negative Metastatic Breast Cancer Patients

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ABSTRACT

Purpose: Prognostic and predictive biomarkers to cyclin-dependent kinases 4 and 6 inhibitors are lacking. Circulating tumor DNA (ctDNA) can be used to profile these patients and dynamic changes in ctDNA could be an early predictor of treatment efficacy. Here, we conducted plasma ctDNA profiling in patients from the PEARL trial comparing palbociclib+fulvestrant versus capecitabine to investigate associations between baseline genomic landscape and on-treatment ctDNA dynamics with treatment efficacy.

Experimental Design: Correlative blood samples were collected at baseline [cycle 1-day 1 (C1D1)] and prior to treatment [cycle 1-day 15 (C1D15)]. Plasma ctDNA was sequenced with a custom error-corrected capture panel, with both univariate and multivariate Cox models used for treatment efficacy associations. A prespecified methodology measuring ctDNA changes in clonal mutations between C1D1 and C1D15 was used for the on-treatment ctDNA dynamic model.

Results: 201 patients were profiled at baseline, with ctDNA detection associated with worse progression-free survival (PFS)/overall survival (OS). Detectable *TP53* mutation showed worse PFS and OS in both treatment arms, even after restricting population to baseline ctDNA detection. *ESR1* mutations were associated with worse OS overall, which was lost when restricting population to baseline ctDNA detection. *PIK3CA* mutations confer worse OS only to patients on the palbociclib+fulvestrant treatment arm. ctDNA dynamics analysis ($n = 120$) showed higher ctDNA suppression in the capecitabine arm. Patients without ctDNA suppression showed worse PFS in both treatment arms.

Conclusions: We show impaired survival irrespective of endocrine or chemotherapy-based treatments for patients with hormone receptor–positive/HER2-negative metastatic breast cancer harboring plasma *TP53* mutations. Early ctDNA suppression may provide treatment efficacy predictions. Further validation to fully demonstrate clinical utility of ctDNA dynamics is warranted.

Introduction

Cyclin-dependent kinases 4 and 6 inhibitors (CDK4/6i) in combination with endocrine therapy (ET) is the mainstay treatment for patients with hormone receptor–positive (HR^+), HER2-negative ($HER2^-$) metastatic breast cancer (MBC), both in endocrine-sensitive (1–3) and resistant scenarios (4–6). However, efficacy of this combination was not originally tested in comparison with chemotherapy, an optional standard treatment in pretreated patients. The GEICAM/2013–02 PEARL study (ClinTrials.gov reference NCT02028507) was a multicenter randomized phase III clinical trial that enrolled patients with aromatase inhibitor (AI)-resistant $HR^+/HER2^-$ MBC. Overall, the trial concluded there was no superiority of palbociclib plus ET over capecitabine in AI-resistant patients,

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Translational Relevance

Our results show that in patients with hormone receptor–positive/HER2-negative metastatic breast cancer, circulating tumor DNA (ctDNA) detection, likely reflecting higher tumor burden, is a poor prognostic biomarker. In addition, detection of plasma *TP53* mutations confer impaired survival irrespective of endocrine or chemotherapy-based treatments, even after correction for known relevant clinicopathologic variables and ctDNA detection itself. We suggest this observation should be added to prognostic models and taken into account when designing therapeutic strategies for this population. Other plasma mutations in relevant genes like *PIK3CA* and in particular *ESR1*, might be associated with worse long-term outcomes. Our study also shows early ctDNA suppression can select optimal responders in both treatment arms and adds evidence that ctDNA dynamics might be a clinically useful tool for treatment efficacy predictions, although further validation would be required.

although a more favorable safety profile was found for patients treated with CDK4/6i compared with capecitabine (7), reinforcing the evidence that CDK4/6i should be considered upfront for the treatment of $HR^+/HER2^-$ MBC patients, but prognostic and predictive biomarkers to better select patients likely to benefit from these treatments is an area of intense research.

Plasma circulating tumor DNA (ctDNA) is shed into circulation by tumor cells in small fragments (typically around 143–145 bp), thought mainly to derive from tumor necrosis and apoptosis mechanisms (8). In theory, the pool of plasma ctDNA should better capture the temporal and spatial heterogeneity of the tumor across a patient cancer history, serving as an ideal genetic source for biomarkers.

Work using ctDNA assays in plasma from patients randomized to palbociclib or placebo in addition to fulvestrant in the PALOMA-3 trial showed high ctDNA fraction; *TP53* mutations and *FGFR1* amplifications were associated with worse outcome with no interaction between treatments (9). Mutations in other functionally important genes in MBC like *PIK3CA* and *ESR1* have not shown clinical significance for treatment decisions on CDK4/6i in patients with similar clinical situation to those enrolled in PEARL (9, 10).

Dynamic changes in ctDNA as a result of therapeutic pressure have been proposed as a tool for early treatment efficacy predictions, and work conducted in patients treated with CDK4/6i combinations had previously reported high on-treatment ctDNA as an adverse biomarker predicting worse progression-free survival (PFS; refs. 11–14), although different technologies and ctDNA change calculations have been used, preventing standardization of a methodology. Exploratory work in limited cohorts of MBC patients treated with different targeted therapies sought to compare various strategies to optimize a cutoff for predictions, proposing an optimal method to further explore in larger cohorts (15).

In this work, we conducted plasma ctDNA profiling in patients treated in cohort 2 of the PEARL trial to investigate associations between baseline genomic landscape and on-treatment plasma ctDNA dynamics with efficacy.

Materials and Methods

Clinical trial and patients

The GEICAM/2013–02 (PEARL) clinical trial was a phase III, multicenter, international, open-label, randomized study that enrolled two successive cohorts of patients with AI-resistant $HR^+/HER2^-$ MBC. Cohort 1 included 296 patients randomized to palbociclib plus exemestane versus capecitabine. Upon discovery of *ESR1* mutations conferring resistance to further AI (16), cohort 2 was added and included 305 patients randomized to palbociclib plus fulvestrant versus capecitabine, as described before (7). Eligible patients for both cohorts had to be postmenopausal women with AI-resistant HR^+ $HER2^-$ MBC, defined as recurrence while on or within 12 months after the end of adjuvant treatment or progression while on or within 1 month after the end of treatment for advanced disease. Patients must have an Eastern Cooperative Oncology Group (ECOG) performance status of 0 or 1 and measurable disease according to the RECIST (version 1.1) or at least one lytic or mixed bone lesion. One prior line of chemotherapy for MBC was permitted. Exclusion criteria included prior treatment with CDK4/6i, mTOR or PI3K inhibitors, capecitabine, or visceral crisis. The study was conducted in compliance with the Declaration of Helsinki. GEICAM/2013–02 (PEARL) clinical trial (NCT02028507) research protocol was approved by every site's institutional review board and every country's regulatory agency. All patients signed written informed consents.

For this work, we focused on patients from cohort 2. A schematic view of samples used is given in **Fig. 1**. Blood samples were collected for ctDNA analysis from -7 days to cycle 1-day 1 (C1D1) for baseline prognostic analysis and cycle 1-day 15 (C1D15) when available. A total of 246 different patients with C1D1 were considered. 13 patients had DNA concentrations too low (under 0.10 ng/μL) so were discarded. From remaining 233 patients, 22 did not have an additional follow-up sample at C1D15 to have a paired analysis, so were also discarded. From remaining 211 patients, 10 failed as result of mislabeling during aliquoting, resulting in a total of 201 final different patients included in the analysis, all with a baseline and at least 1 follow-up sample.

Sample processing

Correlative blood samples were collected from each PEARL study cohort 2 patient at two extraction times: baseline (C1D1) and prior to treatment on C1D15 (±3 days).

For the ctDNA analysis, three 10-mL blood samples were collected directly in Cell-Free DNA BCT tubes (Streck Corporate) by venepuncture. Blood was mixed with the preservative in the BCT by gentle inversion and plasma processed within 2 hours after extraction. The procedure, specified in the PEARL study Sample Management Manual, consists of a single 20-minute centrifugation step at 1,600 g and immediate transfer of plasma into labelled cryovials and storage at −80°C.

Circulating plasma DNA was extracted from 4 mL of plasma sample using the MagMAX Cell-Free DNA Isolation Kit (Thermo Scientific, catalog no. A29319) on a KingFisher Flex Purification System as per the manufacturer's instructions. Extracted cell-free DNA was stored at −20°C until further use.

ctDNA sequencing

Plasma DNA was captured for targeted sequencing using the Nonacus Cell3 Target Cell-Free DNA Target Enrichment System protocol (Birmingham, UK), with 1.8 to 50.1ng (average of 23.5 ng) plasma DNA with no additional fragmentation. The DNA was tagged using dual index Unique Molecular Identifier (UMI) adapters at a concentration of 15 μmol/L for DNA input of >20 to 50 ng or 7.5 μmol/L for ≤20 ng input prior to six or eight cycles of PCR respectively. Libraries were then pooled by equal mass and hybridized overnight with a custom capture panel designed to target the coding regions of the 21 most commonly mutated genes in breast cancer

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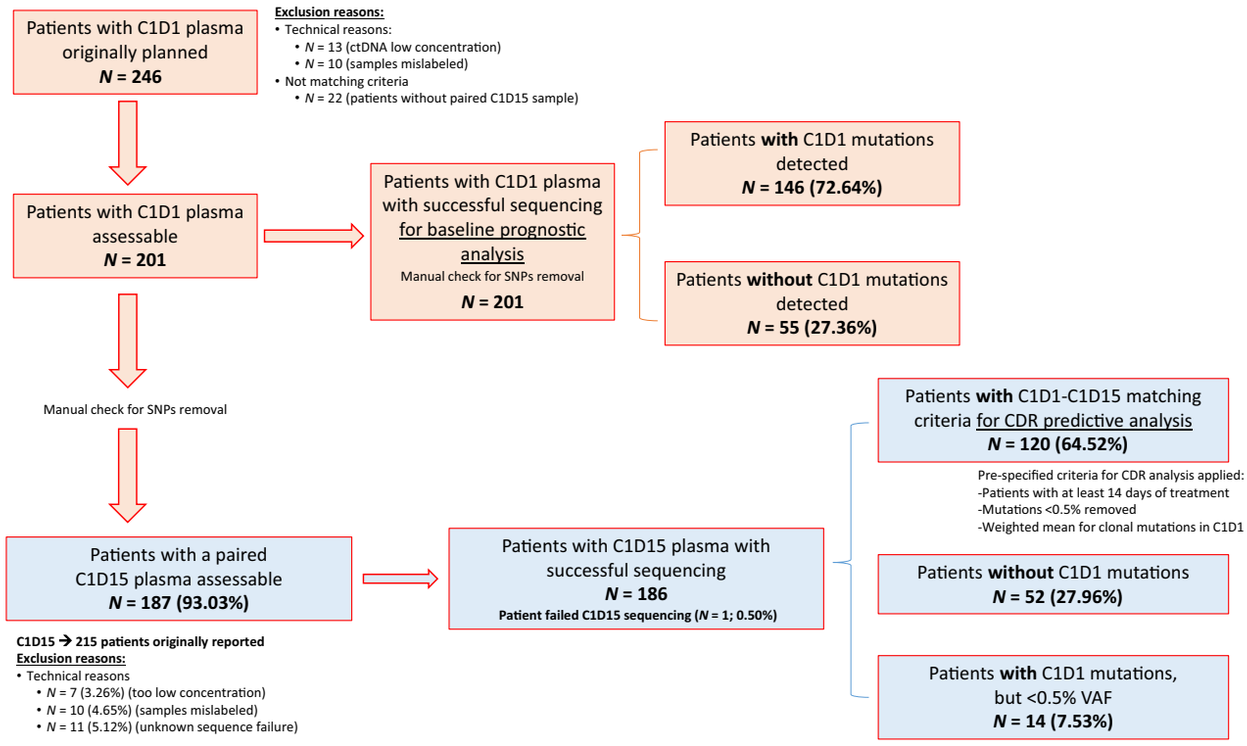

Figure 1.

CONSORT diagram of the study population and samples. Blood samples were collected for ctDNA analysis from -7 days to C1D1 for baseline prognostic analysis and C1D15 when available. A total of 246 different patients with C1D1 were considered. 13 patients had DNA concentrations too low (under 0.10 ng/μL) so were excluded. From remaining 233, 22 did not have an additional follow-up sample at C1D15 to have a paired analysis, so were also excluded. From remaining 211, 10 samples were excluded as result of mislabeling during aliquoting, resulting in a total of 201 final different patients included in the analysis, all with a baseline and at least 1 follow-up sample.

(Supplementary Table S1). Post capture hybridisation, libraries were cleaned and amplified using 13 cycles of PCR according to the manufacturer's instructions. Amplified libraries were sequenced on an Illumina NovaSeq6000 (Illumina, San Diego, CA) in the Centre for Molecular Pathology at The Royal Marsden Hospital NHS Foundation Trust (London, United Kingdom), with 100 bp paired-end reads and v1.5 chemistry according to the manufacturer's instructions. 4 Gb of sequence data was allocated for plasma DNA samples, resulting in mean unique deduplicated UMI consensus depth of 1978x and 1620x for C1D1 and C1D15 samples, respectively.

Data analysis

A bespoke pipeline (MDIMSv4) developed in-house by the Translational Research Group at The Royal Marsden Hospital NHS Foundation Trust was used to analyze the ctDNA data.

Briefly, base calls were demultiplexed using bcl2fastq2 (v2.20.0). Reads had adapters removed with Picard (v2.23.8) and were aligned to the reference genome (GRCh37/Hg19) using BWA-MEM (v0.7.17). fgbio (v1.3.0) was used to collapse reads from the same original molecule using UMIs. UMI families were defined as containing ≥3 molecules with the same UMI. UMI families containing <3 reads were excluded from further analysis. Overlapping forward and reverse reads were hard clipped with ClipBam (fgbio). Picard was used for quality control (QC). BAM files were base recalibrated using GATK (v4.1.9.0) and finally variant calling was performed with VarDict (v1.8.2) without germline subtraction. Likely germline calls based on a Variant Population Frequency in general population in the Genome Aggregation Database (https://gnomad.broadinstitute.org/) >0.0001 were removed from the dataset. Analysis and filtering of the data using custom R scripts of potential germline mutations based on variant allele frequency (VAF) and oncogenic status evaluated with restriction to oncogenic/likely oncogenic variants. Remaining variants were manually checked using Integrative Genome Viewer (IGV; Broad Institute and the Regents of the University of California). Variant calling has been validated to a sensitivity and specificity of 94.74% [95% confidence interval (CI), 73.97%–99.87%] and 100.00% (95% CI, 99.15%–100.00%) at 0.125% VAF and/or ≥5 alt reads, assuming 50 ng assay input and optimal sequencing. For follow up samples, previously discovered variants were reported at ≥2 reads, ≥0.05%.

ctDNA dynamics analysis

Additional to the QC requirements, for the predictive on-treatment ctDNA dynamics analysis, a prespecified criteria had to be met. A minimum of 14 days of treatment in the first cycle was required. Variants with a VAF <0.5% in C1D1, set to allow an above limit of detection to allow a dynamic range for on-treatment ctDNA analysis, were excluded. A ctDNA ratio (CDR) for C1D1–C1D15 was calculated per patient as the weighted mean (mean of each individual C1D15 VAF divided by the corresponding individual C1D1 VAF for each SNV or indel matching the definition above) for potentially clonal mutations at C1D1. Clonal mutations were defined as mutations with VAF > 0.5 of the maximum VAF for a patient. Alternative methodologies for

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calculating an optimal cutoff were also explored in this work. Briefly, a CDR based on the log-fold change [ln(C1D15+0.1)−ln(C1D1+0.1)] as a way to take into account the relative differences and a second alternative CDR using only the mutation with the maximum allele frequency on C1D1 for the ratio.

Statistics

For survival analysis, PFS was defined as the time from randomization to the first documented progressive disease (PD) based on the investigators' assessments, using RECIST v1.1, or death from any cause, whichever occurs first. Overall survival (OS) analysis was exploratory in nature, given the noncontrolled treatments upon progression on PEARL. OS was defined as the time from the date of randomization to the date of death from any cause.

Cox regression models were used in all PFS and OS analysis conducted in this study, both univariate and multivariate adjusting for known clinicopathologic variables known to be relevant in the metastatic setting (site of disease, sites of metastasis, prior chemotherapy for MBC, prior sensitivity to HT, ECOG). A P value <0.05 was considered for statistical significance.

For response analysis, objective response (OR) was defined as a complete response (CR) or partial response (PR) out of the patients who had measurable disease and Clinical Benefit Rate (CBR) as the sum of OR and stable disease (SD) according to RECIST v1.1, recorded from randomization until disease progression or death due to any cause. Tumor response was assessed on the basis of the investigator's assessment according to RECIST v1.1, and this assessment was performed at baseline and every 8 weeks (±7days) using the same method of measurement. The best response across treatment was recorded.

The optimal cutoff was selected using the minimum P value method. The procedure selects the cutoff that minimizes the significance level of the log-rank test with comparison of the two groups defined by the cutoff. The selection of the candidate cutoffs was studied only in the central range of the variable, between the 20 per cent and the 80 per cent quantile of the distribution of the continuous variable. The P value of the log-rank test using a maximally selected cutoff was adjusted for the method proposed by Lausen and Schumacher (17), with an additional permutation test assessing statistical significance of the model based in the statistic of the log-rank test (17). A Cox regression model was calculated with the binary variable obtained with the optimal cut point method, and the potential overestimation of the log-HR was corrected by a shrinkage factor c as proposed by Verweij and Van Houwelingen (18). A bootstrap resampling proposed by Hollander and Schumacher was used to calculate an adequate confidence interval of the log-HR (19). The statistical significance of the log-HR was valuated with a permutation test, repeating the whole cutpoint selection process with randomly permuted survival times and censoring (17). R v4.1.1 was used as statistical software.

Data sharing statement

The complete set of results used in the analysis, for all patients involved, is available on Supplementary Table S2 and Supplementary Table S3.

The clinical database of this study is available upon request from the corresponding author.

Results

Demographic analysis

A total of 201 patients with baseline sample for prognostic analysis were included, 107 (53%) treated in the palbociclib plus fulvestrant arm and 94 (47%) in the capecitabine arm. Our study population did not differ in baseline characteristics with the overall PEARL population (Supplementary Table S4). Analysis of classical demographic characteristics showed no significant differences between treatment, excepting more patients with multiple sites of metastasis in the capecitabine arm (76.6% vs. 59.81%; $P = 0.02$), but without differences in visceral versus non-visceral disease between arms (Supplementary Table S5). Similarly, PFS in this population was not significantly different between treatment arms [median PFS (mPFS) 7.82 vs. 9.26 months, m; HR, 0.97; 95% CI, 0.7–1.35; $P = 0.86$; $P_{adj} = 0.96$; Supplementary Fig. S1A]. Exploratory OS also showed nonsignificant differences between treatments [median OS (mOS) 23.95 vs. 30.03 m; HR, 1.24; 95% CI, 0.74–2.06; $P = 0.41$; $P_{adj} = 0.45$; Supplementary Fig. S1B].

Baseline prognostic analysis

From total 201 patients, 146 (73%) had at least one mutation identified in their baseline sample (i.e., ctDNA detection), with 55 (27%) without any mutation detected (Supplementary Table S6 includes a full summary of all mutations detected at baseline for the 146 patients with ctDNA detection). Baseline genomic landscape did not differ between treatments arms (Supplementary Table S7) with *ESR1*, *PIK3CA* and *TP53* being the genes most commonly mutated in both arms (**Fig. 2A**). PFS was significantly worse in patients with ctDNA detection versus those with no detection (mPFS 7.23 m vs. 14.75m; HR, 1.85; 95% CI, 1.24–2.76; $P < 0.01$; **Fig. 2B**). OS was also significantly worse in patients with ctDNA detection versus no detection (mOS 23.26 m vs. not reached; HR 5.22; 95% CI, 2.08–13.08; $P < 0.01$; **Fig. 2C**).

Baseline PFS prognostic analysis. *TP53*, *ESR1* and *PIK3CA*

Overall, patients with a detectable *TP53* mutation had worse PFS than those with *TP53* mutation not detected (*TP53* ND) with mPFS 4.4 m versus 10.9 m (HR, 1.84; 95% CI, 1.28–2.63; $P < 0.01$; $P_{adj} < 0.01$; **Fig. 3A**). In addition, patients harboring more than one *TP53* mutation had worse PFS (mPFS 3.55 m) and showed an increased HR (1.98; 95% CI, 0.8–4.9) than those with only one mutation (mPFS 5.26 m; HR, 1.82; 95% CI, 1.25–2.64), when compared with *TP53* ND (mPFS 10.91 m; $P < 0.01$; $P_{adj} = 0.02$; **Fig. 3B**). In the palbociclib plus fulvestrant arm, PFS was worse for patients with *TP53* mutations detected vs. not detected (mPFS 4.83 vs. 10.18m; HR, 1.74; 95% CI, 1.08–2.78; $P = 0.03$; $P_{adj} = 0.06$; **Fig. 3C**). In the capecitabine arm, PFS was also worse for patients with *TP53* mutations detected vs. not detected (mPFS 3.78 vs. 11.99 m; HR, 2.07; 95% CI, 1.17–3.67; $P = 0.02$; $P < 0.05$; **Fig. 3D**). Adverse PFS conferred by multiple versus one versus *TP53* ND was also apparent when analyzing separately patients on palbociclib and fulvestrant or capecitabine but with limited numbers (Supplementary Fig. S2A and S2B). There was no interaction between *TP53* mutations and treatment randomization ($P = 0.69$; $P_{adj} = 0.63$).

Given that patients with ctDNA detection had been previously found to have significantly worse PFS compared with those without any mutation detected, we then repeated the analysis considering ctDNA detection as a surrogate of higher tumor burden and more aggressive biology and potential confounding variable; therefore, we repeated analysis removing patients without ctDNA detection. Analysis within the population with ctDNA detection also showed patients with *TP53* mutation had worse PFS than those with *TP53* ND with mPFS 4.4 m vs. 9.26 m (HR, 1.48; 95% CI, 1–2.17; $P = 0.05$; $P_{adj} = 0.10$; Supplementary Fig. S3A).

No significant differences were found in PFS by *ESR1* or *PIK3CA* mutations in analysis for both complete population and restricted to

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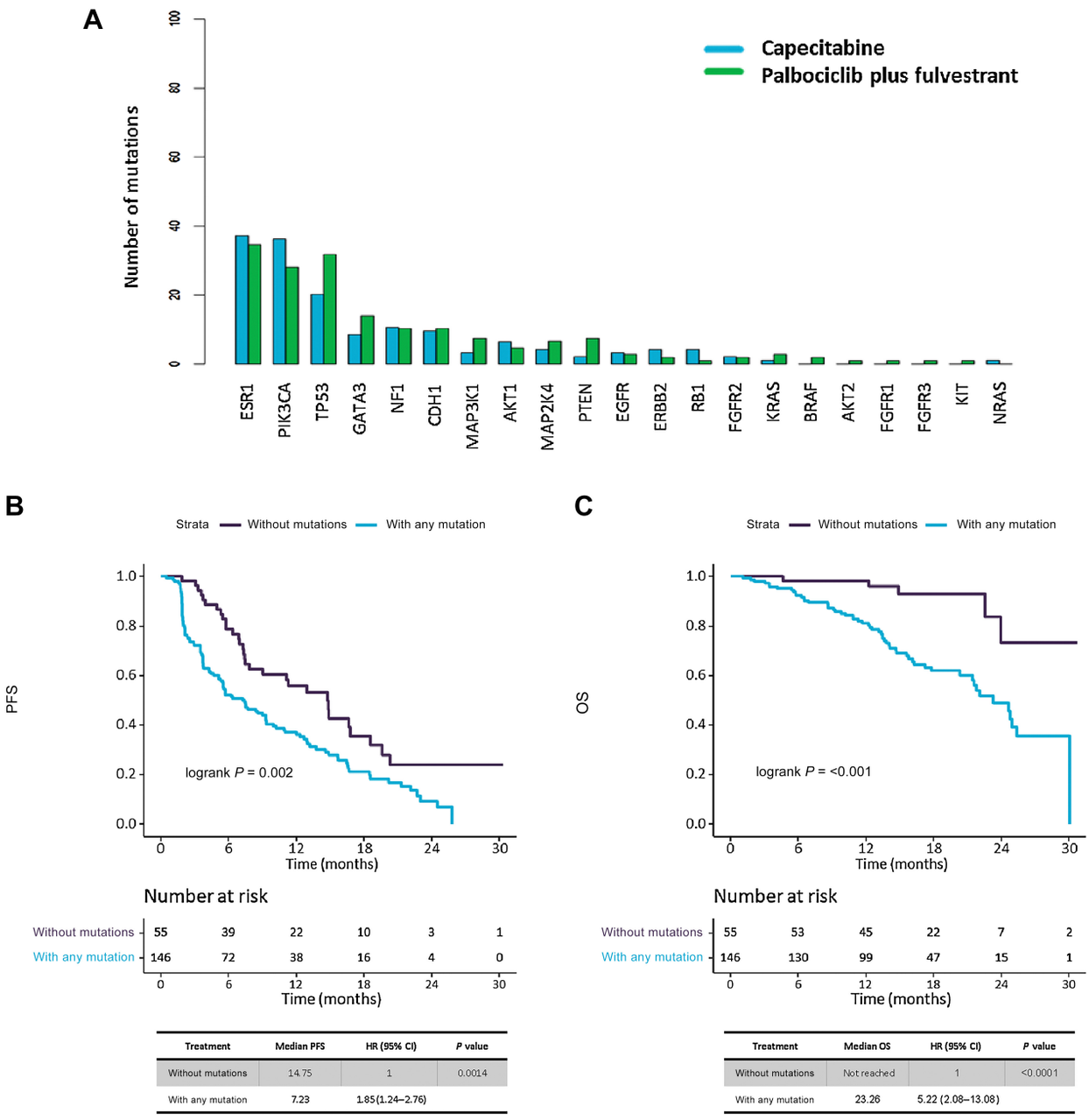

Figure 2.

Mutation distribution across study population, and analysis of ctDNA detection effect in survival. **A,** Bar plot showing distribution of mutations in study population, by mutated genes and treatment arms. **B** and **C,** Kaplan–Meier survival curves, including risk table (time in months, m), to measure presence of any mutation (ctDNA detection) effect in survival, among patients with and without any mutation detected. **B,** PFS, and (**C**) OS.

ctDNA detection. A summary of baseline *ESR1* and *PIK3CA* mutations can be found on Supplementary Table S8. A summary of results for univariate and multivariate PFS analyses can be found on Supplementary Table S9.

Baseline OS prognostic analysis. *TP53*

Patients with a detectable *TP53* mutation had worse OS than those with *TP53* ND with mOS 16.13 m versus 30.03 m (HR, 2.89; 95% CI, 1.74–4.81; $P = 0.0001$; $P_{adj} = 0.0004$; **Fig. 4A**). Patients harboring more than one *TP53* mutation had worse OS (mOS 13.47m) and showed an increased HR (4.79; 95% CI, 1.43–16.04) than those with only one *TP53* mutation (mOS 21.42m; HR, 2.76; 95% CI, 1.64–4.67) when compared with *TP53* ND (mOS 30.03m; $P < 0.001$; $P_{adj} = 0.001$; **Fig. 4B**). The adverse OS risk conferred by *TP53* mutation was maintained in patients treated with palbociclib plus fulvestrant (15.61 m vs. not reached; HR, 2.81; 95% CI, 1.44–5.49; $P < 0.01$; $P_{adj} = 0.04$; **Fig. 4C**) and patients treated with capecitabine (22.08 m vs. 30.03; HR, 2.86; 95% CI, 1.29–6.38; $P = 0.01$; $P_{adj} = 0.04$; **Fig. 4D**).

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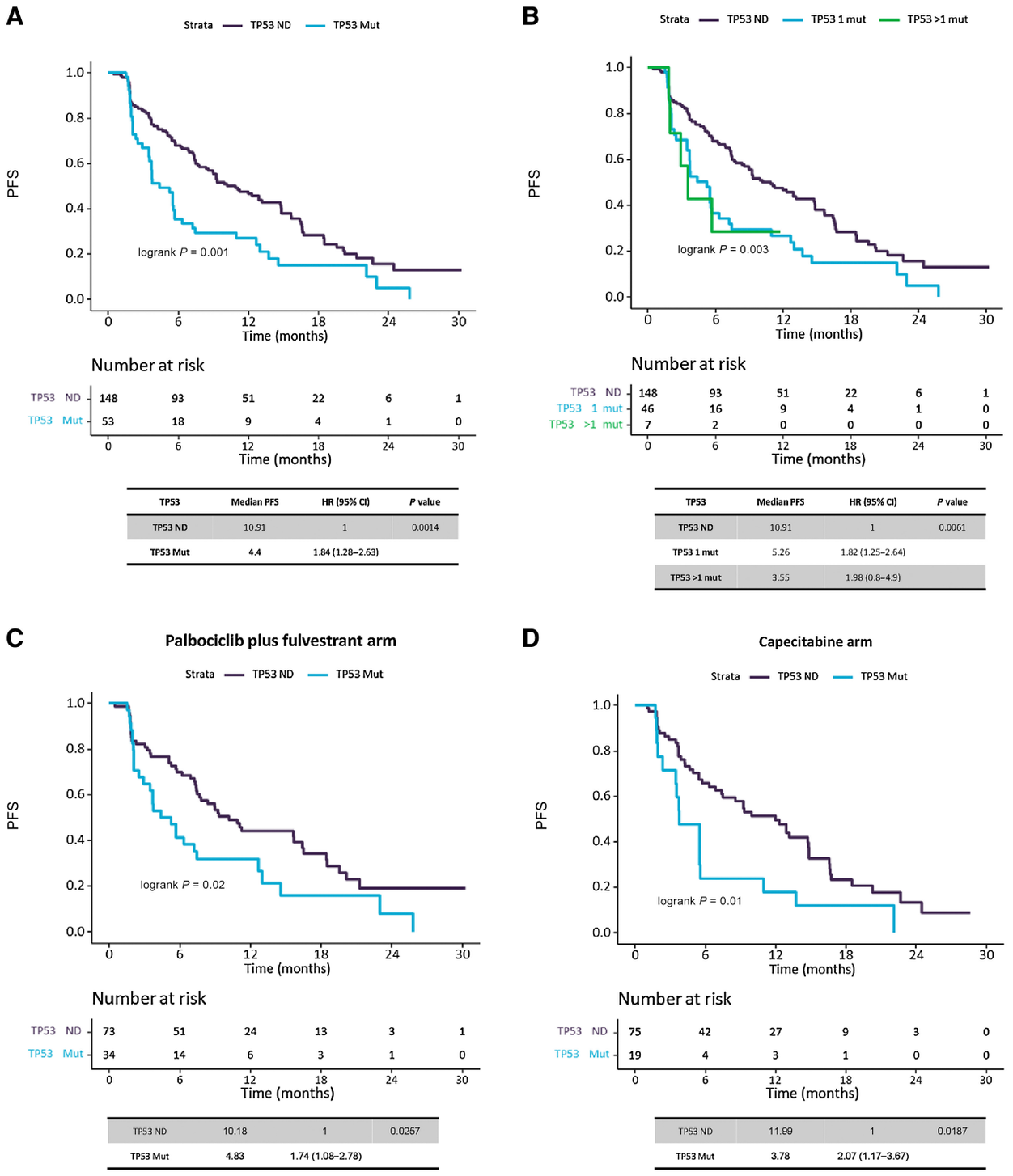

TP53	Median PFS	HR (95% CI)	*P* value
TP53 ND	10.91	1	0.0014
TP53 Mut	4.4	1.84 (1.28–2.63)	

TP53	Median PFS	HR (95% CI)	*P* value
TP53 ND	10.91	1	0.0061
TP53 1 mut	5.26	1.82 (1.25–2.64)	
TP53 >1 mut	3.55	1.98 (0.8–4.9)	

TP53 ND	10.18	1	0.0257
TP53 Mut	4.83	1.74 (1.08–2.78)	

TP53 ND	11.99	1	0.0187
TP53 Mut	3.78	2.07 (1.17–3.67)	

Figure 3.

Analysis of *TP53* mutation effect in PFS. Kaplan–Meier survival curves, including risk table (time in months, m), to measure the effect of *TP53* mutations in PFS. **A,** Survival analysis on *TP53* gene mutations, regardless treatment, by presence/absence of mutations. **B,** Survival analysis on *TP53* gene mutations, regardless treatment, by number of mutations (0 mutations, 1 mutation or more than 1 mutations). **C,** Survival analysis on *TP53* gene mutations, in palbociclib plus fulvestrant treated patients, by presence/absence of mutations. **D,** Survival analysis on *TP53* gene mutations, in capecitabine treated patients, by presence/absence of mutations.

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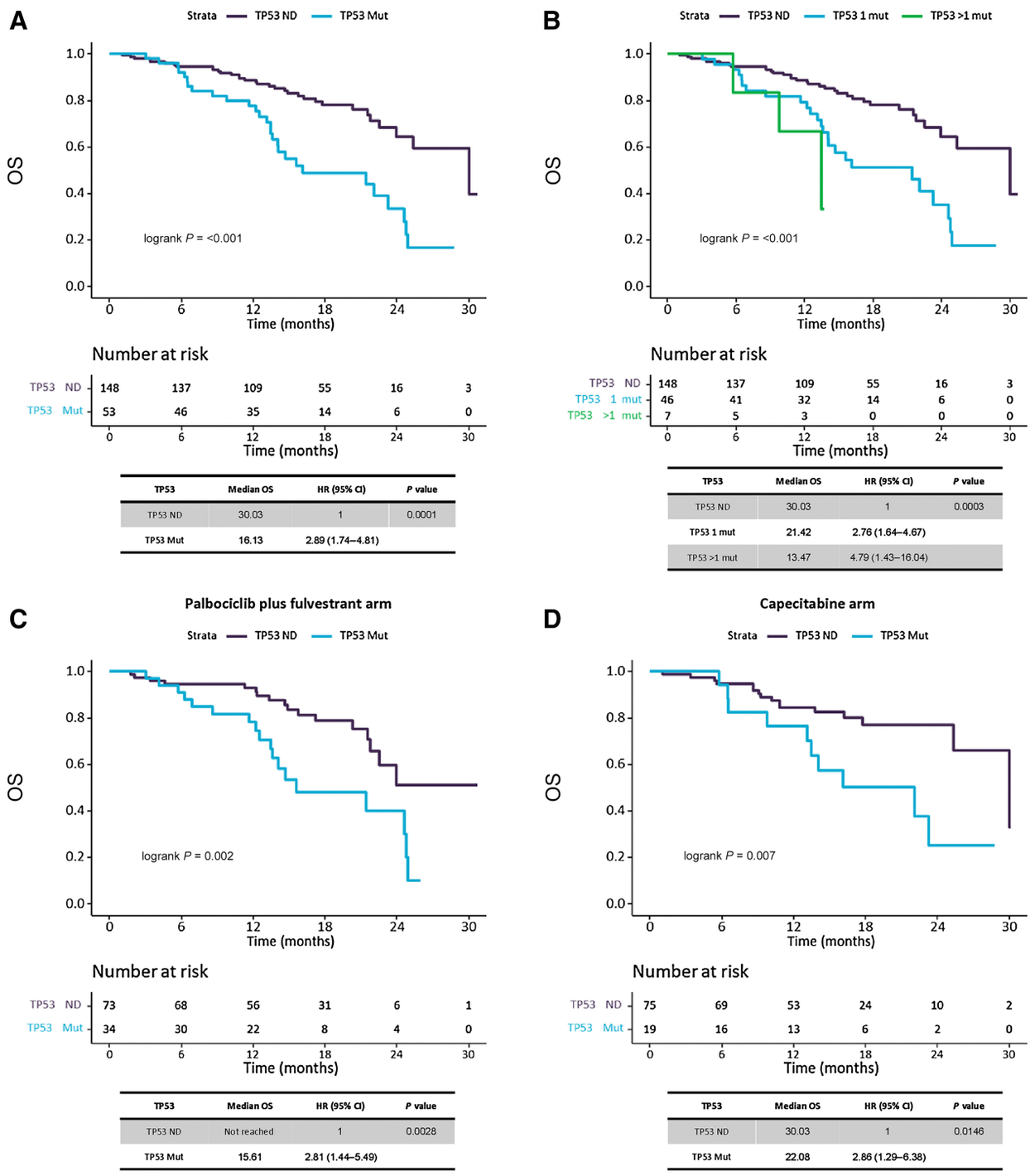

TP53	Median OS	HR (95% CI)	*P* value
TP53 ND	30.03	1	0.0001
TP53 Mut	16.13	2.89 (1.74–4.81)	

TP53	Median OS	HR (95% CI)	*P* value
TP53 ND	30.03	1	0.0003
TP53 1 mut	21.42	2.76 (1.64–4.67)	
TP53 >1 mut	13.47	4.79 (1.43–16.04)	

TP53	Median OS	HR (95% CI)	*P* value
TP53 ND	Not reached	1	0.0028
TP53 Mut	15.61	2.81 (1.44–5.49)	

TP53	Median OS	HR (95% CI)	*P* value
TP53 ND	30.03	1	0.0146
TP53 Mut	22.08	2.86 (1.29–6.38)	

Figure 4.

Analysis of *TP53*, *ESR1* and *PIK3CA* mutation effect in OS. Kaplan–Meier survival curves, including risk table (time in months, m), to measure the effect of *TP53* mutations in OS. **A,** Survival analysis on *TP53* gene mutations, regardless treatment, by presence/absence of mutations. **B,** Survival analysis on *TP53* gene mutations, regardless treatment, by number of mutations (0 mutations, 1 mutation or more than 1 mutations). **C,** Survival analysis on *TP53* gene mutations, in palbociclib plus fulvestrant treated patients, by presence/absence of mutations. **D,** Survival analysis on *TP53* gene mutations, in capecitabine treated patients, by presence/absence of mutations. (*Continued on the following page.*)

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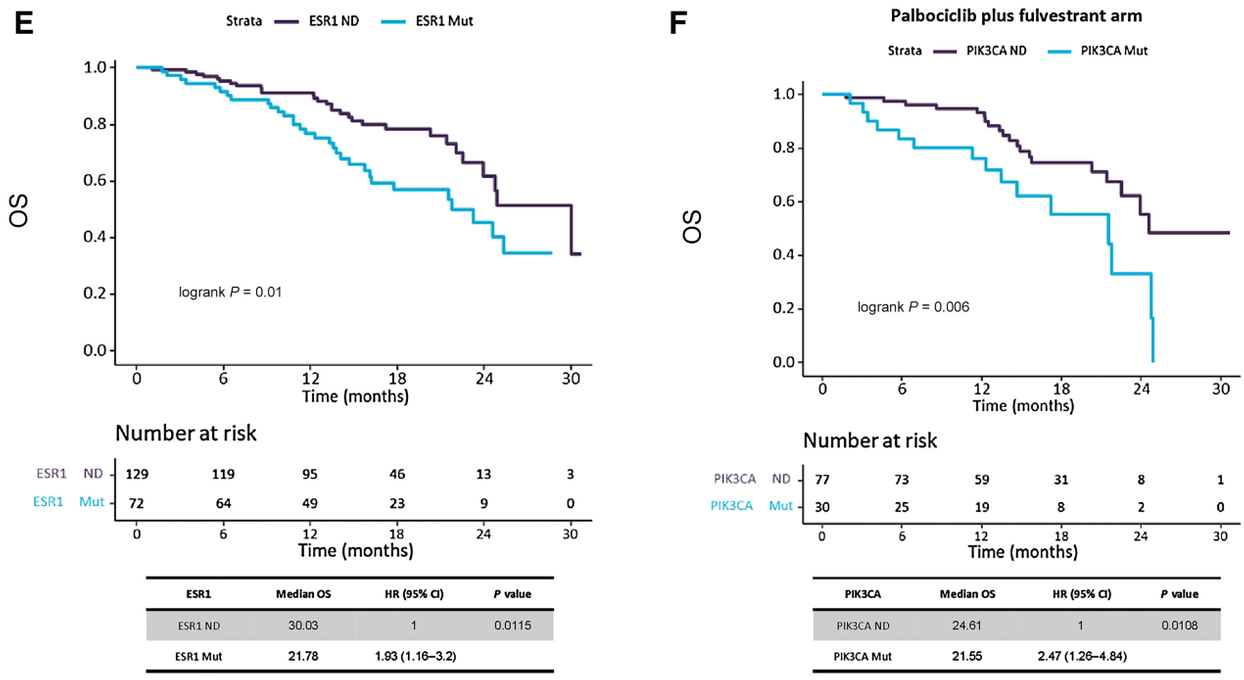

Figure 4.
(*Continued.*) **E,** Survival analysis on *ESR1* gene mutations, regardless treatment, by presence/absence of mutations. **F,** Survival analysis on *PIK3CA* gene mutations, in palbociclib plus fulvestrant treated patients, by presence/absence of mutations.

Interaction test for OS between treatment and *TP53* mutations was not significant ($P = 0.96$; $P_{adj} = 0.87$).

Acknowledging the impact of ctDNA detection also in OS, we repeated our OS analysis in the population with ctDNA detection. This analysis confirmed patients with *TP53* mutation had worse OS than those with *TP53* ND (mOS 16.13 m vs. 30.03m; HR, 2.06; 95% CI, 1.21–3.5; $P < 0.01$; $P_{adj} = 0.02$; Supplementary Fig. S3B), also confirming worse OS and increased HR for patients harboring more than one *TP53* mutations (mOS 13.47m; HR, 3.31; 95% CI, 0.98–11.19) than those with only one mutation (mOS 21.42m; HR, 1.97; 95% CI, 1.14–3.4), when compared with *TP53* ND (mOS 30.03m; $P = 0.02$; $P_{adj} = 0.03$; Supplementary Fig. S3C).

Baseline OS prognostic analysis. *ESR1* and *PIK3CA*

Patients with a detectable *ESR1* mutation were also found to have worse OS than those with *ESR1* ND with mOS 21.78 m versus 30.03 m (HR, 1.93; 95% CI, 1.16–3.2; $P = 0.01$; $P_{adj} = 0.04$; **Fig. 4E**). This observation was maintained when stratifying by number of mutations, as patients with more than one *ESR1* mutation showed worse OS with an increased HR (mOS 21.55 m; HR, 2.45; 95% CI, 1.27–4.72) than those with only one mutation (mOS 24.61 m; HR, 1.67; 95% CI, 0.92–3.01), when compared with *ESR1* ND (mOS 30.03 m; $P = 0.02$, $P_{adj} = 0.07$). The magnitude of OS differences was less evident when analysis was repeated by treatment allocation, with palbociclib plus fulvestrant (21.55 m vs. 24.77m; $P = 0.09$, $P_{adj} = 0.11$) or capecitabine (23.26 m vs. 30.03 m; $P = 0.04$, $P_{adj} = 0.13$). Interaction test for OS between treatment and *ESR1* was not significant ($P = 0.71$; $P_{adj} = 0.77$).

Analysis within population with ctDNA detection did not show differences in OS for presence of *ESR1* mutation ($P = 0.47$; $P_{adj} = 0.99$; Supplementary Fig. S4A).

Patients with a detectable *PIK3CA* mutation were found to have similar OS than those with *PIK3CA* ND in the overall population ($P = 0.14$). Interestingly, and unlike *TP53* and *ESR1*, differences in OS appeared to be restricted to patients on palbociclib plus fulvestrant with mOS 21.55 m vs. 24.61 m (HR, 2.47; 95% CI, 1.26–4.84; $P = 0.01$; $P_{adj} = 0.02$; **Fig. 4F**), maintained when exploring number of mutations detected [*PIK3CA* > 1 mutation detected (mOS 12.32 m; HR, 7.16; 95% CI, 2.78–18.47) and *PIK3CA* 1 mutation detected (mOS 21.78; HR, 1.74; 95% CI, 0.79–3.83)] and compared with *PIK3CA* ND (mOS 24.61 m; $P < 0.01$; $P_{adj} = 0.03$). Interaction test for OS between treatment and *PIK3CA* was significant in the univariate ($P = 0.04$) and marginally significant in the multivariate analysis ($P = 0.05$).

Analysis within the population with ctDNA detection confirmed no overall influence in OS for *PIK3CA* mutations ($P = 0.71$, $P_{adj} = 0.75$) but confirmed opposite OS trend for *PIK3CA* mutations when stratifying by treatment. In capecitabine treatment arm, patients with *PIK3CA* mutations showed higher OS than those in palbociclib and fulvestrant, resulting in a significant interaction test for OS in this selected population between treatment and *PIK3CA* ($P < 0.01$; $P_{adj} < 0.01$; Supplementary Fig. S4B–S4D).

A summary of results for univariate and multivariate OS analyses in *ESR1* and *PIK3CA* mutations can be found in Supplementary Table S10.

Predictive ctDNA dynamic analysis

A total of 120 patients met prespecified criteria for assessing ctDNA dynamics, C1D1-C1D15 CDR analysis, 64 (53%) in the palbociclib plus fulvestrant arm and 56 (47%) in the capecitabine arm. From the overall baseline population, 81 (40%) patients were not included in the CDR analysis because of baseline ctDNA not detected ($n = 52$; 64%), baseline mutations only <0.5% VAF ($n = 14$; 17%), not having a paired

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C1D15 sample (n = 14; 17%) and having a C1D15 sample failing sequencing (n = 1; 1%; **Fig. 1**). Patients for CDR analysis had significantly more ECOG 1 than non-CDR patients, without other clinicopathologic differences (Supplementary Table S11). Significant differences in survival were found between CDR and non-CDR population (mPFS 6.21 m vs. 14.75 m, respectively) with increased HR in CDR population (HR, 1.78; 95% CI, 1.25–2.52; $P < 0.001$; Supplementary Fig. S1C), consistent with the fact that 81% of the non-CDR had no baseline ctDNA detection or very low frequency mutations (<0.5% VAF). There were no differences in genomic landscape between CDR and non-CDR populations (Supplementary Table S12).

CDR and PFS

Median CDR between C1D15–C1D1 calculated with prespecified methodology was significantly different between treatment arms, with patients treated with capecitabine having a lower CDR (i.e., more profound ctDNA suppression) than those on palbociclib plus fulvestrant (median CDR 0.07 vs. 0.21; $P < 0.01$; **Fig. 5A**). Furthermore, a greater proportion of patients treated with capecitabine had a CDR = 0 (i.e., complete ctDNA suppression under limit of detection) compared with those on palbociclib plus fulvestrant (23.21% vs. 7.81%, respectively; $P = 0.02$).

We then tested how our proposed optimal methodology could be used to predict PFS in each treatment arm. In the palbociclib plus fulvestrant arm optimal cutoff was defined at CDR = 0.0247, with HR 2.14 (95% CI, 0.92–5), $P = 0.06$ (**Fig. 5B**). In the capecitabine arm optimal cutoff was 0.0127, with HR 2.37 (95% CI, 0.96–5.83), $P = 0.05$ (**Fig. 5C**).

Analysis of potential detrimental effect of *TP53* mutations in patients with ctDNA suppression, although limited in number, showed no significant differences in survival in both treatment arms with overall improved survival in patients with *TP53* mutations and ctDNA suppression, suggesting patients achieving early ctDNA drop surpassed the detrimental effect of baseline *TP53* mutations (palbociclib plus fulvestrant: mPFS 14.55 vs. 16.53 m; HR, 1; 95% CI, 02–5; $P = 0.99$ and capecitabine: mPFS 8.25 vs. 16.66 m; HR, 2.71; 95% CI, 0.52–14.06; $P = 0.27$).

A weighted mean for clonal mutations at C1D1 was the best methodology for predictions in both treatment arms. Although inferior, alternative methodologies explored for CDR calculations and predictions showed overall relatively similar results (Supplementary Table S13).

CDR and response

Response data were available in 118 patients of the 120 total patients included into the longitudinal predictive ctDNA analysis. OR defined as a CR or PR and CBR as the sum of OR and SD according to RECIST v1.1, were evaluated according to CDR values for all patients included in the study.

OR was 18% (all responses recorded as PR), and CBR was 73.7%. Patients under PD represented 26.3%.

We found a significant association of greater CDR suppression overall in patients with CBR ($N = 87$) versus those progressing ($N = 31$) with CDR 0.1 versus 0.2, respectively ($P = 0.03$), but differences were not maintained when separated per treatment ($P = 0.12$ for palbociclib plus fulvestrant and $P = 0.29$ for capecitabine), however with limited sample size (Supplementary Fig. S5).

Discussion

We show that a significant proportion of patients with $HR^+/HER2^-$ MBC have a detectable *TP53* mutation in plasma and this confers them a significantly worse outcome compared with those patients where the mutation was not detected. Patients with *TP53* mutations had very poor survival in both endocrine-based or chemotherapy treatment arms, extending results reported in patients from PALOMA-3 trial treated with endocrine-based therapies (9), and suggest aggressive behaviour irrespectively of treatment, highlighting the need of designing clinical strategies to improve outcomes in this population (20). We believe *TP53* mutations should be incorporated in future prognostic and predictive models for $HR^+/HER2^-$ MBC given this deleterious effect could be sufficiently strong to prevent applicability of models not incorporating this information. In addition, in our exploratory OS analysis, *TP53* mutations also conferred patients a worse OS. Although subsequent treatments were not controlled upon progression on PEARL, we now have evidence that both PFS and OS did not differ between treatment arms (21) suggesting consistency of our OS impact finding. Interestingly, we found that multiple *TP53* mutations confer even worse outcomes, an observation that would need further validation. We acknowledge a potential limitation for these findings is the fact that we did not perform germline correction for our sequencing and that some *TP53* mutations identified could be resulting from clonal hematopoiesis (22, 23). Given the high incidence of *TP53* mutations in breast cancer, clonal hematopoiesis may have played some contamination effect in our conclusions. However, we sought to take some pre-planned actions to minimize this effect. Apart from usual QC, known population-level germline variants were eliminated as per variant population frequency. We further performed analysis and filtering of the remaining data using custom R scripts of potential germline mutations based on VAF and only oncogenic/likely oncogenic variants were included in our analysis. *TP53* mutations are present in 5% to 10% of clonal hematopoeiesis of indeterminate potential (CHIP) (24, 25) and maximum estimates of the incidence of detectable CHIP with ctDNA in patients with MBC are 25% patients (26). Therefore, only 1.25% to 2.5% of patients in our study would be expected to have potential *TP53* CHIP mutations and most of them would have been filtered by our analysis methods. We therefore believe CHIP would have had a minimal effect in our conclusions. We acknowledge germline white blood cell DNA sequencing would have helped optimize CHIP substraction, but unfortunately this was not possible in our study. Other frequent mutations present or acquired in this population, like *ESR1* or *PIK3CA* mutations, were particularly relevant in the exploratory OS analyses. *ESR1* mutations were found to confer worse survival matching previous observations (16, 27, 28). In addition, a detrimental effect of *PIK3CA* mutations was also found for OS on those patients treated with palbociclib and fulvestrant. In particular, this reinforces the growing evidence that *PIK3CA* mutations detected in MBC seem to confer worse outcomes as opposed to detection in early settings, where it has been traditionally associated with better outcomes (29). Proportion of patients with *ESR1* mutation was slightly higher in or study compared with previous reports in studies conducted in tissue samples, likely reflecting the effectiveness of ctDNA assays to pick up this particular mutation, often polyclonal and present at the sub-clonal level.

For our early on-treatment predictions, we applied a previously reported (15) prespecified methodology for our CDR calculations, consisting on a weighted mean for VAFs in genes considered potentially clonal in the pretreatment sample. We show that this methodology can select patients deriving an optimal benefit for both treatment arms of our study, adding evidence to previous publications identifying early ctDNA dynamics as a potentially useful tool for efficacy predictions (11–14).

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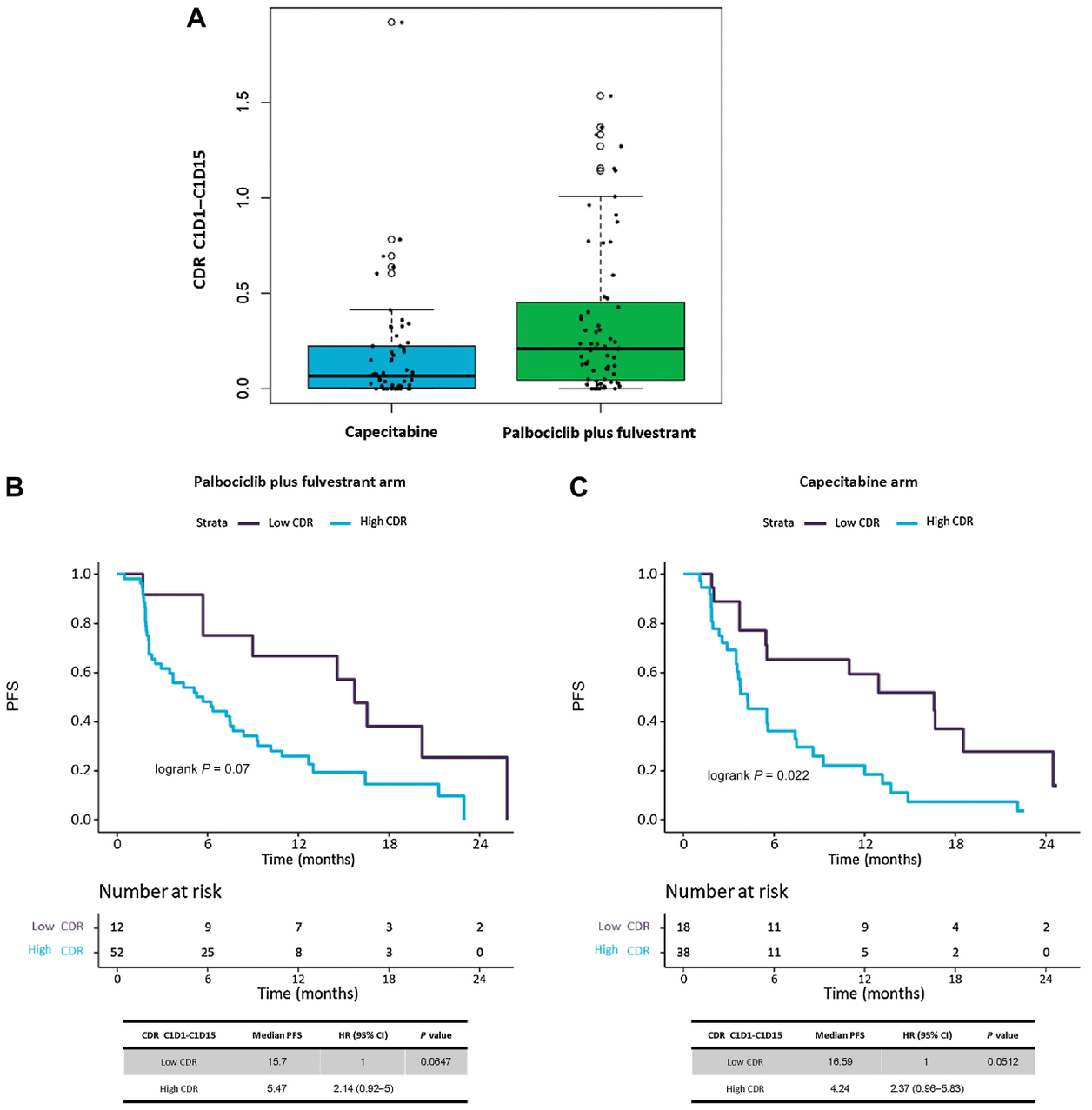

CDR C1D1-C1D15	Median PFS	HR (95% CI)	*P* value
Low CDR	15.7	1	0.0647
High CDR	5.47	2.14 (0.92–5)	

CDR C1D1-C1D15	Median PFS	HR (95% CI)	*P* value
Low CDR	16.59	1	0.0512
High CDR	4.24	2.37 (0.96–5.83)	

Figure 5.

Longitudinal predictive ctDNA analysis by use of median CDR methodology, calculated at C1D15 timepoint. **A,** Boxplot graph showing distribution of CDR C1D15 values in patients by treatment arms, palbociclib plus fulvestrant vs. capecitabine. **B** and **C,** Kaplan–Meier survival curves, including risk table (time in months, m), to measure the effect of CDR (Low vs. High) in PFS by treatment arm, (**B**) in palbociclib plus fulvestrant treated patients and (**C**) in capecitabine treated patients. Median CDR between C1D15–C1D1, calculated with prespecified methodology, offered a CDR value optimal cutoff of 0.0247 for palbociclib plus fulvestrant arm, while it was of 0.0127 for the capecitabine treatment arm. Those optimal cut-off values were used to create Low versus High CDR groups, in both treatment arms.

We acknowledge our CDR results have to be considered exploratory and further validation is required. Our panel may seem limited to broadly explore ctDNA dynamics, but our methodology seeks to measure early dynamics in relevant mutations with enough representation in patients' blood circulation to be reliably measured for change, an important issue not fully addressed in other similar reports. We also acknowledge our approach is potentially less sensitive than a tumor-informed approach, but given we focus on change, we think the critical point is having a reliable quantification of mutation abundance (i.e., VAF in our study), and we sought to accomplish this by focusing on relevant genes and performing a deep error-corrected sequencing. We believe our CDR results are limited, yet we attempted to provide rigorous data. An optimal cutoff for treatment efficacy predictions is in nature context-dependent and will vary between clinical setting,

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treatment, and algorithm used to measure the ctDNA change. However, it is interesting to notice that optimal cutoffs found in PEARL with our methodology are in line with those reported in PALOMA-3 (11) and previous reports with subsets of patients treated with targeted therapies (15), suggesting an important early ctDNA suppression often in the range of 0 to 0.1 CDR is required to select those patients deriving the greatest benefit. We notice that this may not apply to patients treated with immunotherapies, where less ctDNA suppression may be sufficient to select good responders (30). We hypothesize that an important degree of cytostatic or cytotoxic effect must be achieved with both endocrine-based or chemotherapy treatments to predict long term benefit, whereas for patients on immunotherapy microenvironment plays an important role in controlling proliferation and less cytostatic or cytotoxic effect reflected in higher CDR might be sufficient to select optimal responders.

ctDNA suppression on day 15 was significantly more pronounced in patients treated with capecitabine versus those on palbociclib and fulvestrant, suggesting chemotherapy cytotoxicity might result in faster suppression.

Interestingly, patients not having any detectable baseline mutation had significantly better survival compared with those with evaluable mutations. This is expected and adds evidence towards not having a detectable mutation probably reflecting low tumor burden or proliferation and therefore better prognosis independent of the treatment received.

In summary, our work shows impaired PFS irrespective of endocrine or chemotherapy-based treatments for patients with $HR^{+}/HER2^{-}$ MBC harboring plasma *TP53* mutations. Our findings also suggest presence of plasma *ESR1* mutations could be detrimental in the long term for these patients and adds additional evidence that early ctDNA suppression may be clinically helpful for long-term treatment efficacy predictions, suggesting an optimal methodology that helps selecting patients deriving the most benefit. Further validation to fully demonstrate clinical utility of ctDNA dynamics is warranted.

Authors' Disclosures

J. Pascual reports personal fees and nonfinancial support from AstraZeneca; personal fees from Novartis; and nonfinancial support from Pfizer outside the submitted work. M. Gil-Gil reports personal fees from Pfizer, Novartis; and other support from Lilly outside the submitted work. C. Zielinski reports grants from AstraZeneca, Pfizer, Servier, Novartis, Eli Lilly, Roche, BMS, MSD, Takeda, Daiichi Sankyo, Celgene, Halozyme, Amgen, and Boehringer Ingelheim during the conduct of the study; in addition, C. Zielinski has a patent for HerVaxx issued and licensed to Imugene. E.M. Ciruelos Gil reports personal fees from Lilly, Roche, AstraZeneca, Daiichi Sankyo, Seagen, Gilead, Novartis, Pfizer, and MSD during the conduct of the study. M. Muñoz-Mateu reports personal fees from Roche, Gilead, Segean, AstraZeneca, Gilead, and Lilly outside the submitted work. B. Bermejo reports personal fees from Pfizer; grants from Novartis, AstraZeneca, Daiichi Sankyo, and MSD outside the submitted work. M. Margeli Vila reports grants from Pfizer; other support from Novartis, Gylead, PiereFabre, Lilly, and Daiichi Sankyo outside the submitted work. A. Antón reports personal fees from Pfizer during the conduct of the study and personal fees from Gilead, Eli Lilly, Daiichi Sankyo, Seagen, and AstraZeneca outside the submitted work. Y. Liu reports other support from Pfizer outside the submitted work. M. Hubank reports other support from Guardant Health; personal fees from Roche, Qiagen, Novartis, GSK, Bayer, Incyte, Jansenn, and AstraZeneca outside the submitted work. N.C. Turner reports has received advisory board honoraria from AstraZeneca, Lilly, Pfizer, Roche/Genentech, Novartis, GlaxoSmithKline, Repare Therapeutics, Relay Therapeutics, Zentalis, Gilead, Inivata, Guardant, Exact Sciences; and research funding from AstraZeneca, Pfizer, Roche/Genentech, Merck Sharpe and Dohme, Guardant Health, Invitae, Inivata, Personalis, and Natera. M. Martín reports personal fees from Pfizer, Puma, Roche, Novartis, Daiichi Sankyo, Seagen, Lilly, and AstraZeneca during the conduct of the study; and personal fees from SANOFI outside the submitted work. No disclosures were reported by the other authors.

Authors' Contributions

J. Pascual: Conceptualization, data curation, formal analysis, funding acquisition, investigation, visualization, methodology, writing–original draft, project administration, writing–review and editing. **M. Gil-Gil:** Resources, investigation, writing–review and editing. **P. Proszek:** Data curation, formal analysis, methodology, writing–review and editing. **C. Zielinski:** Investigation, writing–review and editing. **A. Reay:** Data curation, formal analysis, methodology, writing–review and editing. **M. Ruiz-Borrego:** Resources, investigation, writing–review and editing. **R. Cutts:** Data curation, formal analysis, methodology, writing–review and editing. **E.M. Ciruelos Gil:** Resources, investigation, writing–review and editing. **A. Feber:** Data curation, formal analysis, methodology, writing–review and editing. **M. Muñoz-Mateu:** Resources, investigation, writing–review and editing. **C. Swift:** Data curation, formal analysis, methodology, writing–review and editing. **B. Bermejo:** Resources, investigation, writing–review and editing. **J. Herranz:** Data curation, formal analysis, methodology, writing–original draft, writing–review and editing. **M. Margeli Vila:** Resources, investigation, writing–review and editing. **A. Antón:** Resources, investigation, writing–review and editing. **Z. Kahan:** Resources, investigation, writing–review and editing. **T. Csöszi:** Resources, investigation, writing–review and editing. **Y. Liu:** Funding acquisition, writing–review and editing. **D. Fernandez Garcia:** Data curation, formal analysis, methodology, writing–original draft, project administration, writing–review and editing. **I. Garcia-Murillas:** Data curation, formal analysis, methodology, writing–review and editing. **M. Hubank:** Conceptualization, methodology, writing–review and editing. **N.C. Turner:** Conceptualization, formal analysis, funding acquisition, methodology, writing–original draft, project administration, writing–review and editing. **M. Martín:** Conceptualization, resources, data curation, supervision, funding acquisition, investigation, methodology, writing–original draft, project administration, writing–review and editing.

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Note

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