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Phase I study of ribociclib (CDK4/6 inhibitor) with spartalizumab (PD-1 inhibitor) with and without fulvestrant in metastatic hormone receptor-positive breast cancer or advanced ovarian cancer

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ABSTRACT

Background Preclinical evidence suggests that cyclin-dependent kinases 4 and 6 (CDK4/6) inhibitors enhance antitumor immunity. We conducted a phase I trial of ribociclib (CDK4/6 inhibitor) plus spartalizumab (PD-1 inhibitor) in patients with hormone receptor (HR)-positive/HER2-negative metastatic breast cancer (MBC) or advanced ovarian cancer (AOC). The combination was also evaluated with fulvestrant in MBC.

Methods In Cohort A, ribociclib was administered on Days 1–21 (28-day cycle) starting at 400 mg, and spartalizumab at 400 mg on Day 1. Dose escalation was followed by expansion in AOC. Fulvestrant was added (Cohort B) with a safety run-in followed by expansion in MBC. Primary objectives were to determine the maximum tolerated dose (MTD) and recommended phase 2 dose (RP2D), and safety and tolerability of the combinations.

Results 33 patients enrolled (n=18, Cohort A; n=15, Cohort B). The RP2D of ribociclib in both cohorts was 600 mg. Treatment-related adverse events in >20% of patients in either cohort were neutropenia, fatigue, anemia, thrombocytopenia, hypertransaminasemia, maculopapular rash, fatigue, and nausea. Hypertransaminasemia occurred in 66.7% (AST) and 46.7% (ALT) of patients in Cohort B, including 46.7% and 40.0%, respectively, of grade 3 or 4 events. Two confirmed partial responses were observed (13.3%) in Cohort B, in patients with low baseline serum thymidine kinase activity, coupled with an increase on-treatment. Peripheral blood flow cytometry across patients demonstrated on-target drug binding with increases in PD-1 occupancy and activated $CD8^+$ T cells during treatment, irrespective of response. PD-L1-positivity, tumor-infiltrating lymphocytes, or tumor mutational burden did not correlate with progression-free survival (PFS). Several copy-number variations detected with next-generation sequencing correlated with PFS.

Conclusions Ribociclib with spartalizumab and fulvestrant showed limited efficacy and elevated hepatotoxicity, precluding further development. Correlative analyses revealed treatment-induced immunological effects, and genomic alterations associated with PFS.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Cyclin-dependent kinases 4 and 6 (CDK4/6) inhibitors upregulate genes involved in antigen presentation and T cell inflammatory pathways and, in preclinical models, have demonstrated synergistic activity with immune checkpoint blockade.

WHAT THIS STUDY ADDS

⇒ In this phase Ib study, ribociclib (CDK4/6 inhibitor) plus spartalizumab (PD-1 inhibitor) with and without fulvestrant in patients with advanced ovarian and HR-positive/HER2-negative breast cancer showed limited efficacy and elevated hepatotoxicity, precluding further development. While the differential contribution of PD-1 inhibition to CDK4/6 inhibition was unclear in this nonrandomized trial, correlative analyses suggested that this treatment combination has the potential to enhance antitumor immunity as noted by changes detected in peripheral immune cell subsets.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ With emerging data for PD-1 inhibition as treatment for high-risk HR-positive/HER2-negative early-stage breast cancer, elucidating the underlying mechanisms of toxicity of the triplet combination is imperative to understand if CDK4/6 and PD-1 inhibitors can be safely administered sequentially with endocrine therapy in the adjuvant setting.

INTRODUCTION

Cyclins and cyclin-dependent kinases 4 and 6 (CDK4/6) play an important role in cellular proliferation by mediating cell cycle

progression through phosphorylation and inactivation of Rb, thus allowing transcription of genes that promote transition from G1 into the S phase.[1] Dysregulation of the cyclin D-CDK4/6-Rb signaling pathway is frequently detected across cancer types and has been associated with oncogenesis in breast cancer (predominantly luminal subtype)[2–4] and ovarian[5–7] cancer, indicating that CDK4/6 inhibition may represent a promising therapeutic target in these patients.

In recent years, inhibitors of CDK4/6 have transformed the treatment landscape of hormone receptor (HR)-positive/HER2-negative breast cancer by demonstrating improved clinical outcomes when added to endocrine therapy in patients with metastatic disease.[8–19] Ribociclib is one of three CDK4/6 inhibitors that are currently approved in combination with endocrine therapy as first-line treatment or after progression on prior endocrine therapy in patients with HR-positive/HER2-negative metastatic breast cancer (MBC). Results from the randomized phase III MONALEESA-2 trial showed that the addition of ribociclib to letrozole resulted in significantly longer progression-free survival (PFS) and overall survival (OS) compared with letrozole plus placebo in postmenopausal women who had not received prior therapy for advanced HR-positive/HER2-negative breast cancer.[15 16 20] Similarly, MONALEESA-7 compared the addition of ribociclib or placebo to endocrine therapy (goserelin and either a non-steroidal aromatase inhibitor or tamoxifen) in pre-/perimenopausal women with previously untreated HR-positive/HER2-negative MBC, and showed a significant increase in rates of both PFS and OS favoring the ribociclib arm.[18] In these trials, the overall response rate of ribociclib with endocrine therapy was approximately 41% (51–53% among patients with measurable disease). In addition, combination of ribociclib and the selective estrogen receptor degrader (SERD) fulvestrant extended PFS and OS in patients with treatment-naïve advanced disease and in those who had experienced progression on one prior line of endocrine therapy in the phase III MONALEESA-3 trial,[13 14] supporting the use of fulvestrant and CDK4/6 inhibition as either first-line or second-line treatment for HR-positive/HER2-negative MBC. Here, the reported overall response rate of the combination of ribociclib and fulvestrant was 32% in all patients, and 41% among those with measurable disease.

In addition, aberrant expression of cyclins and CDKs in ovarian cancer and its association with resistance to conventional cytotoxic therapies (eg, amplification of CCNE1 in high-grade serous ovarian cancer and resistance to platinum) has supported targeting CDK4/6 in this tumor type.[21] In a phase II study in patients with heavily pretreated ovarian cancer, median PFS with single-agent palbociclib was 3.7 months, with 30% of patients remaining progression-free at 6 months.[22] In LACOG 1018, the combination of letrozole and palbociclib yielded a median PFS of 4.2 months and clinical benefit rate of 72% in HR-positive (defined as >10%) recurrent high-grade serous or endometrioid ovarian cancer, fallopian tube cancer, or peritoneal cancer, suggesting potential antitumor activity of CDK4/6 inhibition in this patient population.[23]

Preclinical studies have demonstrated that the biological effects of CDK4/6 inhibition extend beyond cell cycle arrest and senescence by promoting antitumor immunity through different mechanisms. CDK4/6 inhibition enhances tumor antigen presentation by upregulating tumor cell expression of genes involved in antigen presentation (eg, major histocompatibility complex (MHC) class I molecules) via interferon signaling in response to an increase in intracellular levels of double-stranded RNA, and induces suppression of immunosuppressive regulatory T cells.[24] CDK4/6 inhibition has also been described to increase T cell tumor infiltration, upregulate T cell inflammatory pathways (eg, IFN-gamma, granzyme B, and PD-L1/-L2 ligands), and promote immunologic memory.[24–27] This suggests that combination of CDK4/6 inhibition and immune checkpoint blockade may be efficacious in tumors considered refractory to immunotherapy, such as ovarian or HR-positive/HER2-negative breast cancer.

In immunocompetent murine models, the combination of abemaciclib and a PD-L1 inhibitor has shown synergistic activity, with prolonged tumor regression and enhanced antigen presentation via upregulation of MHC class I and II on both tumor and myeloid cells.[24 25] Similarly, delayed tumor progression and improved survival have been observed in vivo with the addition of a PD-1 inhibitor to either palbociclib or trilaciclib compared with either agent alone.[26 28] These studies provide rationale supporting the combination of CDK4/6 inhibitors and immune checkpoint blockade.

We conducted an investigator-initiated phase I study of ribociclib with spartalizumab (previously known as PDR001), a humanized IgG4 monoclonal antibody directed against PD-1, to determine the maximum tolerated dose (MTD), recommended phase 2 dose (RP2D), and safety and tolerability of the combination in patients with HR-positive/HER2-negative MBC or advanced ovarian cancer (AOC). A separate cohort was also included to explore the safety and tolerability of ribociclib and spartalizumab combined with fulvestrant in HR-positive/HER2-negative MBC.

PATIENTS AND METHODS

Eligibility criteria

Patients with histologically confirmed HR-positive/HER2-negative MBC per ASCO/CAP guidelines[29] or advanced epithelial ovarian, fallopian tube, or peritoneal cancer (all histologies and tumor grades allowed) were eligible for the trial. Enrolment to expansion Cohort A (RP2D of ribociclib plus spartalizumab) was limited to patients with ovarian cancer, and the safety run-in and expansion Cohort B of ribociclib, spartalizumab, and fulvestrant was conducted in patients with HR-positive/HER2-negative MBC (online supplemental figure 1).

There was no restriction on the number of prior lines of hormonal therapy (prior fulvestrant allowed, except in expansion Cohort B) or chemotherapy in the advanced setting. For patients with breast cancer, postmenopausal status was required for female participants (GnRH agonist allowed), and men were eligible if a GnRH agonist was administered for at least 4 weeks prior to study entry and for the duration of protocol therapy. Participants with AOC were required to have platinum-resistant disease, defined as disease relapse within 2–6 months of prior platinum-based chemotherapy. Prior CDK4/6 inhibition was not permitted in the expansion cohorts but was allowed in the escalation and safety run-in if prior ribociclib (if given) had not required any dose reductions. Treatment with prior PD-1/PD-L1/CTLA-4 inhibitor was prohibited for all participants.

Patients aged 18 years or older with Eastern Cooperative Oncology Group performance status (ECOG PS) 0 or 1 at baseline, with adequate bone marrow, hepatic, and renal function, and QTcF interval at screening ≤450 ms were enrolled. Measurable disease per RECIST V.1.1[30] was required for the dose expansion cohorts (evaluable disease allowed for the dose escalation and safety run-in).

Key exclusion criteria included history of allogeneic bone marrow or solid organ transplant; participants requiring systemic treatment with immunosuppressive medication or chronic systemic steroid therapy (other than physiologic doses of steroids or replacement-dose steroids for adrenal insufficiency); active autoimmune disease (except vitiligo, type I diabetes mellitus, residual hypothyroidism due to autoimmune condition only requiring hormone replacement, psoriasis not requiring systemic treatment, or conditions not expected to recur in the absence of an external trigger); untreated or active CNS metastases; concurrent use of strong inhibitors or inducers of CYP3A4/5 or CYP2C8, or medications known to prolong QT interval; any grade ≥2 Common Terminology Criteria for Adverse Events (CTCAE) toxicity (except grade 2 peripheral neuropathy or ototoxicity and any grade alopecia, which were allowed); known HIV infection or active hepatitis B or C infection; or participants with gastrointestinal disease that could significantly alter absorption of the study drug.

Study design

This was a single-institution, open-label, phase I trial with a dose escalation to determine the MTD and RP2D of ribociclib plus spartalizumab, followed by an expansion phase to further investigate the safety and preliminary activity of ribociclib plus spartalizumab in AOC, and of ribociclib plus spartalizumab and fulvestrant in HR-positive/HER2-negative MBC. The primary objectives of the study were to determine the MTD, RP2D, and safety and tolerability of the combination of ribociclib plus spartalizumab with and without fulvestrant.

The dose escalation phase followed a traditional 3+3 design to evaluate the safety of ribociclib plus spartalizumab. Once the RP2D of ribociclib with spartalizumab was determined, two expansion cohorts opened. Expansion Cohort A assessed ribociclib plus spartalizumab in patients with AOC (target sample size of 12 patients). Cohort B assessed the combination of ribociclib, spartalizumab, and fulvestrant; first, in a safety run-in of 6–12 patients with HR-positive/HER2-negative MBC, followed by a dose expansion with the potential to enrol a maximum of 24 patients (online supplemental figures 1,2).

Study treatment

Ribociclib was administered orally on Days 1–21 of a 28-day cycle. The starting dose (dose level 1) of ribociclib during the dose escalation was 400 mg daily. Ribociclib dose modifications were per protocol according to a prespecified algorithm; one dose escalation to 600 mg daily (dose level 2) and one dose reduction (dose level −1) to 300 mg daily were allowed. Spartalizumab was administered at 400 mg intravenously on Day 1 of each 28-day cycle. At least six patients were to be treated at the MTD/RP2D of ribociclib and spartalizumab. The MTD was defined as the highest dose level at which fewer than 33% of patients experienced a dose-limiting toxicity (DLT).

After establishing the MTD and RP2D, patients enrolled to expansion Cohort A were treated at the RP2D of ribociclib in combination with spartalizumab. For patients in Cohort B, fulvestrant was administered at a dose of 500 mg intramuscular on Days 1 and 15 of Cycle 1, and then on Day 1 of each 28-day cycle thereafter. Patients enrolled to the safety run-in of the triplet were treated at the RP2D of ribociclib (dose level 1) and spartalizumab, in combination with fulvestrant. If ≥2 DLTs were observed in the first six treated patients, one dose reduction of ribociclib (dose level −1) was allowed and up to six patients treated at that dose level. After establishing the safety of the triplet, patients enrolled to expansion Cohort B were treated at the RP2D of ribociclib identified in the safety run-in, with spartalizumab and fulvestrant. Treatment was administered until disease progression, unacceptable toxicity, patient withdrawal of consent, or decision of the treating investigator to discontinue protocol therapy.

Data were monitored throughout the study by the DF/HCC Data and Safety Monitoring Committee. This trial was registered with the U.S. National Institutes of Health (ClinicalTrials.gov identifier: NCT03294694).

Safety and efficacy assessments

All participants who received at least one dose of any study drug were considered evaluable for toxicity. Safety assessments were conducted weekly for the first cycle (28 days), and every 4 weeks thereafter. Adverse events (AE) were assessed according to the National Cancer Institute CTCAE V.4.0. A DLT was defined as an AE occurring during the first 28-day cycle of treatment that met one of the criteria specified in Section 5.4 of the protocol. Participants who received less than 75% of the ribociclib dose during the first cycle of treatment (missed doses due to reasons other than DLT) were considered unevaluable

for DLTs, and another participant(s) enrolled for DLT evaluation following the 3+3 design.

Participants who received at least one cycle of therapy and who underwent tumor response assessment(s) were considered evaluable for efficacy. Response was evaluated per central review using RECIST V.1.1 criteria.[30] Tumor assessments were performed every two cycles (8 weeks) during the first 24 weeks, and then every three cycles (12 weeks). Confirmation of tumor response at least 4 weeks later was required to deem a confirmed complete response (CR) or partial response (PR).

Correlative analyses

Baseline research tumor tissue biopsies were required prior to initiating protocol therapy for all participants with safely accessible disease who enrolled on the dose expansion cohorts. A second research biopsy after approximately 7 weeks of protocol therapy was required for patients who had undergone the baseline biopsy. A third biopsy at the time of disease progression was optional for all patients enrolled on the study. All research biopsies were optional for participants enrolled on the dose escalation and safety run-in cohorts, and strongly encouraged if archival tissue was not available. Research blood draws were performed in all participants for isolation of peripheral blood mononuclear cells (PBMCs) and collection of serum (for thymidine kinase 1 activity (TKa) assay) at baseline, on Day 1 of each cycle, and at the time of progression.

Tumor-infiltrating lymphocytes

Tumor-infiltrating lymphocytes (TILs) were evaluated and reported according to the recommendations of the International TILs Working Group.[31] A 5 µm section of formalin-fixed paraffin-embedded (FFPE) tissue was stained with hematoxylin and eosin for quantitative assessment of stromal and intratumoral TILs. Scoring was performed by a trained breast pathologist at the Brigham and Women's Hospital (BWH) Pathology Core. Stromal TILs (sTILs) were grouped into <1%, 1–9%, 10–49%, ≥50%, and "Unknown" for descriptive presentation. Considering the distribution of scores of sTILs in the present study, sTILs were dichotomized by absence versus presence of sTILs (<1% vs ≥1%), and low versus intermediate/high sTILs (<10% vs ≥10%) for stratified presentation of efficacy, as well as for Cox regression analyses to assess differences in PFS between groups. For intratumoral TILs (iTILs), the low prevalence of iTILs ≥1% precluded comparisons between groups.

PD-L1 immunohistochemistry

PD-L1 immunohistochemistry (IHC) was performed at the BWH Pathology Core following standardized internal protocol for Ventana oSP142 testing, the only FDA-approved companion diagnostic test for the treatment of MBC (indication for atezolizumab in combination with *nab*-paclitaxel in patients with metastatic triple-negative breast cancer that was PD-L1-positive, defined as ≥1% tumor-infiltrating immune cells staining positive for PD-L1[32]) at the time of selection of the PD-L1 assay for the present study. All IHC was performed using antibody PD-L1 clone SP142 (Abcam ab2284632) at 1:50 dilution; following EDTA antigen retrieval (Ventana CC1 950-500), the staining was visualized via a Ventana HRP multimer/DAB detection system on the Ventana Benchmark Ultra automated staining platform. Scoring of PD-L1 on tumor and immune cells was performed by a trained breast pathologist. PD-L1 positivity was defined as staining of ≥1% of immune-infiltrating cells. PD-L1 status was coded as a binary variable: 0=<1% and 1=≥1%.

OncoPanel testing

FFPE tumor specimens were analyzed using OncoPanel to detect somatic mutations, copy-number variations (CNVs), and structural variants (SVs). Genomic testing was performed centrally at the Center for Advanced Molecular Diagnostics at BWH (Boston, Massachusetts), a Clinical Laboratory Improvement Amendments (CLIA)-certified laboratory environment, according to published methods.[33–35] A total of 200 ng of DNA was used for library preparation (with a low input threshold of 50 ng). DNA was analyzed using a solution-phase Agilent SureSelect hybrid capture kit and an Illumina HiSeq 2500 sequencer, with the following exonic coverage across OncoPanel versions: V.1.0, 753 334 bases; V.2.0, 826 167 bases; V.3.0, 1 315 078 bases). Data were analyzed by an internally developed bioinformatics pipeline, as previously described.[36]

Tumor mutation burden (TMB) was computed by counting the number of nonsynonymous somatic mutations per megabase of exonic sequence, summing this number for each patient, and normalizing each patient sum by the size of the exonic bait-set of the panel used. False discovery rate adjustment for TMB analyses was not performed given the limited number of comparisons.

For correlation of the presence of a particular CNV or SNV with a binary variable, an OR was calculated, and significance was tested using Fisher's exact test. For correlation between TMB and best overall response, linear regression was performed with the levels of response encoded as integers: 0=progressive disease (PD), 1=stable disease (SD) <6 months, 2=SD >6 months, and 3=PR. For correlation of TMB with binary variables, a Wilcoxon rank-sum test was used. For correlation of TMB with PFS, TMB was coded as a binary variable: high TMB, ≥10 mutations/megabase (mut/Mb); low TMB, <10 mut/Mb.

Flow cytometry

PBMCs collected from patients immediately before starting treatment (baseline) and during treatment (Cycle 2 Day 1) were analyzed by flow cytometry. Samples were kept frozen at −80°C until the time of analysis. Samples were thawed in warm RPMI Complete media, washed, incubated for 5 min in 0.1 mg/mL DNase I (Sigma-Aldrich #10104159001), washed again, then incubated for 15 min at 4°C in staining media (2% FBS and

1 mM EDTA in PBS) with the following antibody panel: CD45RO BV650 (Clone UCHL1, BioLegend 304231), HLA-DR BV570 (Clone L243, BioLegend 307637), CD38 BV711 (Clone HIT2, BioLegend 303527), CD16 BV786 (Clone 3G8, BioLegend 302045), CD14 BV605 (Clone M5E2, BioLegend 301834), CD45RA FITC (Clone HI100, BioLegend 304148), CX3CR1 BV421 (Clone 2A9-1, BioLegend 341619), KLRG1 PerCP-Cy5.5 (Clone 14C2A07, BioLegend 368612), CD4 BV510 (Clone SK3, BD Biosciences 562971), CD3 PE-CF594 (Clone UCHT1, BD Biosciences 562280), PD1 PE (Clone EH12.2h7, BioLegend 329905), CD8 PE-Cy7 (Clone HIT8a, BioLegend 300914). At the end of the incubation, the cells were washed and fixed with 1% formalin, then analyzed by flow cytometry using a Sony SP6800 Spectral Analyzer. Gating for cell populations of interest was performed with FlowJo software. Changes in cell populations before and after treatment were compared using a paired Wilcoxon signed-rank test.

Thymidine kinase 1 activity

Thymidine kinase (TK) is an enzyme that plays a key part in DNA replication.[37] The expression and activity of TK are strongly linked to the cell cycle and its presence or absence is an indicator of cell proliferation.[38] TK diffuses from proliferating cells to the bloodstream, and TKa levels in serum or plasma can serve as a pharmacodynamic marker of drug effects on cell proliferation, including CDK4/6 inhibitors. Multiple studies have demonstrated that higher baseline serum TKa levels, and lack of suppression of TKa from baseline to C1D15, denote poorer prognosis and resistance to endocrine therapy in combination with palbociclib or ribociclib.[39 40] PD-L1 binds to and activates PD-1 receptors on T cells, stopping T cell proliferation. PD-1 inhibition blocks this inhibitory interaction and triggers rapid T cell proliferation and activation. In patients with metastatic melanoma treated with immune checkpoint inhibition, lower pretreatment TKa levels combined with an increase between pretreatment and early on-treatment TKa levels have been correlated with durable responses and longer survival.[41]

TKa was analyzed retrospectively using the DiviTum TKa assay (Biovica International, Uppsala, Sweden). TKa was determined using a refined ELISA-based method according to the manufacturer's instructions (www.biovica.com) and was performed at Biovica's CLIA-certified, CAP-accredited laboratory in San Diego, California. DiviTum TKa is a multistep end-point ELISA assay involving a cascade of enzymatic reactions and one antibody binding reaction. During the DiviTum TKa test, a patient's serum sample is combined with a reaction mixture containing the substrate bromodeoxyuridine (BrdU). Since BrdU is a substrate analog to thymidine, TK from the sample phosphorylates the BrdU to its monophosphate, BrdUMP. The BrdUMP is then phosphorylated to BrdUTP and incorporated into a DNA/RNA hybrid, bound to the 96-well microplate solid surface using a reverse transcriptase DNA polymerase. An alkaline phosphatase-conjugated anti-BrdU antibody is added to the reaction product after washing. The amount of phosphatase conjugate bound to the DNA is determined by a colorimetric reaction, turning the substrate from colorless to yellow, and the absorbance reading indicates the TKa level in the sample. Calibrators with predetermined nominal values are used to generate a standard curve by which the optical density (OD) readings from patient samples are converted to TKa expressed as DiviTum Units (DuA). To evaluate changes in TKa at the different time points, fold -change between on-therapy and baseline TKa values was calculated.

Statistical methods and analyses

Descriptive statistics were used to summarize patient demographics, safety, and efficacy measurements. Objective response rate (ORR) was defined as the proportion of patients with CR or PR as the best overall response. PFS was defined as the time from study entry to the first documented evidence of disease progression (per RECIST V.1.1[30] or documented clinical progression) or death from any cause, whichever occurred first. Participants alive without disease progression at the end of the study were censored at the date of last radiographic tumor assessment. Kaplan-Meier curves were used to summarize PFS, and 95% CIs were reported. Cox regression was also used to explore associations between covariates of interest and PFS. The proportional hazards assumption was checked for all models. All data were generated using R V.4.1.0.

Correlative analyses were exploratory. Statistical analyses were conducted in R (package V.3.2.7 or V.4.1.0). For the purposes of all correlative analyses, survival analysis was performed using Kaplan-Meier estimation and differences in survival were tested using a log-rank test.

RESULTS

Study population

A total of 33 patients enrolled to the study between November 2017 and February 2020. 10 patients were treated in the dose escalation phase (3 at dose level 1; 7 at dose level 2), 9 of whom had HR-positive/HER2-negative MBC and 1 had AOC. Eight patients with AOC were enrolled on expansion Cohort A. Six patients with MBC received the triplet in the safety run-in, followed by nine additional patients who enrolled on expansion Cohort B.

The median age in the overall study cohort was 54.6 years (range, 32.6–71.5). 20 patients (60.6%) had received prior therapy for advanced disease, including 15 (45.5%) treated with prior CDK4/6 inhibitor and 18 (54.5%) with chemotherapy (median of 4 prior lines of chemotherapy; range, 1–10). Among patients on Cohort B, 20.0% patients (3/15) had received prior fulvestrant. Patient characteristics are summarized in table 1. The median numbers of cycles of study treatment received in Cohorts A and B were 2 (range, 1–4) and 4 (range, 1–14), respectively.

Table 1 Patient baseline characteristics

Characteristic	No. of patients (%)				
	Ribociclib+spartalizumab (Cohort A)		Ribociclib+spartalizumab+fulvestrant (Cohort B)		
	Dose escalation (n=10)	Dose expansion (n=8)	Safety run-in (n=6)	Dose expansion (n=9)	All patients (n=33)
Age, years					
Median	56.7	60.6	52.2	41.5	54.6
Range	51.2–70.9	32.6–71.5	35.3–66.2	34.4–58.2	32.6–71.5
Sex					
Female	10 (100%)	8 (100%)	6 (100%)	9 (100%)	33 (100%)
Race					
White	8 (80.0%)	8 (100%)	6 (100%)	7 (77.8%)	29 (87.9%)
Black/African American	1 (10.0%)	0 (0%)	0 (0%)	0 (0%)	1 (3.0%)
Asian	1 (10.0%)	0 (0%)	0 (0%)	1 (11.1%)	2 (6.1%)
More than one race	0 (0%)	0 (0%)	0 (0%)	1 (11.1%)	1 (3.0%)
Ethnicity					
Non-Hispanic	10 (100%)	7 (87.5%)	1 (16.7%)	9 (100%)	30 (90.9%)
Hispanic	0 (0%)	0 (0%)	4 (66.7%)	0 (0%)	1 (3.0%)
Unknown	0 (0%)	1 (12.5%)	1 (16.7%)	0 (0%)	2 (6.1%)
Tumor type					
Ovarian	1 (10.0%)	8 (100%)	0 (0%)	0 (0%)	9 (27.3%)
Breast	9 (90.0%)	0 (0%)	6 (100%)	9 (100%)	24 (72.7%)
PD-L1 IHC status (SP142)					
Positive (≥1% IC)	5 (50.0%)	2 (25.0%)	4 (66.7%)	3 (33.3%)	14 (42.4%)
Negative (<1% IC)	4 (40.0%)	6 (75.0%)	1 (16.7%)	6 (66.7%)	17 (51.5%)
Unknown	1 (10.0%)	0 (0%)	1 (16.7%)	0 (0%)	2 (6.1%)
Stromal tumor-infiltrating lymphocytes					
<1%	3 (30.0%)	4 (50.0%)	2 (33.3%)	4 (44.4%)	13 (39.4%)
1–9%	2 (20.0%)	1 (12.5%)	2 (33.3%)	5 (55.6%)	10 (30.3%)
10–49%	2 (20.0%)	3 (37.5%)	0 (0%)	0 (0%)	5 (15.2%)
≥50%	1 (10.0%)	0 (0%)	1 (16.7%)	0 (0%)	2 (6.1%)
Unknown	2 (20.0%)	0 (0%)	1 (16.7%)	0 (0%)	3 (9.1%)
ECOG PS					
0	7 (70.0%)	3 (37.5%)	6 (100%)	8 (88.9%)	24 (72.7%)
1	3 (30.0%)	5 (62.5%)	0 (0%)	1 (11.1%)	9 (27.3%)
Sites of disease					
Bone	8 (80.0%)	0 (0%)	5 (83.3%)	6 (66.6%)	19 (57.6%)
Liver	9 (90.0%)	1 (12.5%)	2 (33.3%)	3 (33.3%)	15 (45.5%)
Lymph nodes	4 (40.0%)	5 (62.5%)	2 (33.3%)	4 (44.4%)	15 (45.5%)
Lung	5 (50.0%)	0 (0%)	1 (16.7%)	4 (44.4%)	10 (30.3%)
Breast/chest	0 (0%)	0 (0%)	1 (16.7%)	4 (44.4%)	5 (15.2%)
Soft tissue	0 (0%)	1 (12.5%)	0 (0%)	1 (11.1%)	2 (6.1%)
Peritoneum/pleura	6 (60.0%)	6 (75.0%)	1 (16.7%)	1 (11.1%)	14 (42.4)
Other	1 (10.0%)	5 (62.5%)	0 (0%)	1 (11.1%)	7 (21.2%)
Prior therapy					
Neoadjuvant	3 (30.0%)	3 (37.5%)	1 (16.7%)	2 (22.2%)	9 (27.3%)

Continued

Table 1 Continued

Characteristic	No. of patients (%)				
	Ribociclib+spartalizumab (Cohort A)		Ribociclib+spartalizumab+fulvestrant (Cohort B)		
	Dose escalation (n=10)	Dose expansion (n=8)	Safety run-in (n=6)	Dose expansion (n=9)	All patients (n=33)
Adjuvant	7 (70.0%)	7 (87.5%)	2 (33.3%)	6 (66.7%)	22 (66.7%)
Metastatic/recurrent	10 (100%)	3 (37.5%)	6 (100%)	1 (11.1%)	20 (60.6%)
Prior endocrine therapy in neo-/adjuvant setting					
Yes	9 (90.0%)	3 (37.5%)	2 (33.3%)	7 (77.8%)	21 (63.6%)
AI	5 (55.6%)	3 (100%)	0 (0%)	4 (57.1%)	8 (38.1%)
Tamoxifen	8 (88.9%)	0 (0%)	2 (100%)	3 (42.9%)	11 (52.4%)
Fulvestrant	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Tamoxifen followed by AI	0 (0%)	0 (0%)	0 (0%)	3 (42.9%)	3 (14.3%)
No	1 (10.0%)	5 (62.5%)	4 (66.7%)	2 (22.2%)	12 (36.4%)
Prior endocrine therapy in advanced setting					
Median no. prior lines of endocrine therapy for advanced disease (range)*	2 (1–4)	1 (1–1)	2 (1–4)	1 (1–1)	2 (1–4)
Yes	9 (90.0%)	1 (12.5%)	6 (100%)	1 (11.1%)	17 (51.5%)
AI	9 (100%)	1 (100%)	5 (83.3%)	0 (0%)	14 (82.4%)
Tamoxifen	1 (11.1%)	0 (0%)	2 (33.3%)	1 (100%)	4 (23.5%)
Fulvestrant	7 (77.8%)	0 (0%)	3 (50.0%)	0 (0%)	10 (58.8%)
No	1 (10.0%)	7 (87.5%)	0 (0%)	8 (88.9%)	16 (48.5%)
Prior CDK4/6 inhibitor therapy in neo-/adjuvant settting					
Yes	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
No	10 (100%)	8 (100%)	6 (100%)	9 (100%)	33 (100%)
Prior CDK4/6 inhibitor therapy in advanced setting					
Yes	9 (90.0%)	0 (0%)	6 (100%)	0 (0%)	15 (45.5%)
No	1 (10.0%)	8 (100%)	0 (0%)	9 (100%)	18 (54.5%)
No. prior lines of chemotherapy for advanced disease					
Median (range)	4.5 (2–10)	4 (3–5)	3.5 (1–6)	0 (0–0)	4 (1–10)
0	0 (0%)	6 (75.0%)	0 (0%)	9 (100%)	15 (45.5%)
1	0 (0%)	0 (0%)	2 (33.3%)	0 (0%)	2 (6.1%)
2	3 (30.0%)	0 (0%)	0 (0%)	0 (0%)	3 (9.1%)
≥3	7 (70.0%)	2 (25.0%)	4 (66.7%)	0 (0%)	13 (39.4%)

*One patient in Cohort B, expansion received leuprolide acetate in combination with tamoxifen.
AI, aromatase inhibitor; CDK4/6, cyclin-dependent kinases 4 and 6; IHC, immunohistochemistry; ECOG PS, Eastern Cooperative Oncology Group performance status.

Determination of MTD and RP2D

Among the first three patients treated at dose level 1 of ribociclib with spartalizumab, none experienced DLT; thus, dose level 2 was explored. A total of seven patients received ribociclib at dose level 2 in combination with spartalizumab, with one of seven patients experiencing DLTs (grade 3 atrial fibrillation, grade 3 atrial flutter, and grade 4 neutropenia). The RP2D of ribociclib in combination with spartalizumab was determined to be dose level 2, 600 mg daily (21 days-on/7 days-off).

Among six patients who enrolled to the safety run-in of ribociclib, spartalizumab, and fulvestrant, none experienced DLT, allowing expansion Cohort B to open. However, due to the elevated rate of grade 3–4 hepatotoxicity observed with the triplet combination and limited antitumor activity in patients treated with ribociclib and

spartalizumab alone, the decision was made to permanently close the trial to accrual.

Safety

Treatment-related adverse events (TRAEs) grade 2 or higher occurred in 100% of patients in the dose escalation (n=10/10) and dose expansion (n=8/8) phases of Cohort A. The most common TRAE was neutropenia, which occurred in 80% of patients (n=8/10) in the dose escalation and 62.5% of patients (n=5/8) in the dose expansion (table 2). Other grade 2 or higher TRAEs observed in ≥20% of patients in the dose expansion of ribociclib plus spartalizumab were as follows: decreased white blood cells (75.0%, 6/8), anemia (62.5%, 5/8), elevated ALT (50.0%, 4/8), elevated AST (37.5%, 3/8), fatigue (37.5%, 3/8), thrombocytopenia (37.5%, 3/8), maculopapular rash (37.5%, 3/8), diarrhea (25.0%, 2/8), vomiting (25.0%, 2/8), hypokalemia (25.0%, 2/8), and hypophosphatemia (25.0%, 2/8). Grade 3–4 TRAEs were observed in 75.0% of patients treated in the dose expansion of the doublet, including four (50.0%) patients who experienced G3–4 decreased white blood cells, four (50.0%) patients who experienced G3 neutropenia, one (12.5%) G3 febrile neutropenia, three (37.5%) G3 ALT elevation, two (25.0%) G3 AST elevation, one (12.5%) G4 thrombocytopenia, two (25.0%) G3–4 maculopapular rash, and one (12.5%) G3 diarrhea.

In Cohort B, TRAEs grade 2 or higher occurred in 100% of patients in both the safety run-in phase (n=6/6) and the dose expansion (n=9/9). Neutropenia was the most common TRAE, which occurred in 100% of patients (6/6) in the safety run-in and 77.8% of patients (7/9) in the dose expansion. Grade 3 neutropenia occurred in four of six patients (66.7%) in the safety run-in and six of nine patients (66.7%) in the dose expansion (table 2). Elevated liver enzymes were common among patients treated with ribociclib, spartalizumab, and fulvestrant. Grade 2 or higher elevated ALT occurred in 66.7% of patients (n=4/6) in the safety run-in and 66.7% of patients (n=6/9) treated in the dose expansion of Cohort B, with 50.0% (3/6) and 33.3% (3/9) of G3–4 elevation, respectively. Grade 2 or higher elevated AST occurred in 50% of patients (n=3/6) in the safety run-in and 44.4% of patients (n=4/9) in the dose expansion, all of which were G3–4 (table 2). One patient (16.7%) in the safety run-in and one patient (11.1%) in the expansion of the triplet experienced G2 hypothyroidism attributed to spartalizumab (online supplemental tables 1, 2). Immune-related TRAEs of special interest attributed to spartalizumab (±ribociclib/fulvestrant) are listed in online supplemental table 2. No G5 events were reported in the study.

Dose adjustments and management recommendations for toxicities attributable to ribociclib and/or spartalizumab followed protocol-specified guidelines. Regarding hepatotoxicity possibly related to ribociclib and spartalizumab, per investigator attribution, treatment with both drugs was held for G2/3 AST, ALT, or isolated total bilirubin elevation, and permanently discontinued for G4 events or concurrent G2 or higher bilirubin elevation. In the overall cohort, ALT elevation attributed to spartalizumab (±ribociclib/fulvestrant) led to dose interruption in 5/14 patients, with subsequent recovery in 4 cases; 4/5 patients resumed ribociclib, 2 of whom also restarted spartalizumab and experienced recurrent ALT elevation. AST elevation attributed to spartalizumab (±ribociclib/fulvestrant) led to dose interruption in 3/10 patients, all of whom recovered and resumed ribociclib, 1 in combination with spartalizumab who subsequently developed recurrent AST elevation. There was one case of concurrent bilirubin (G2) and AST/ALT elevation (G3) in a patient enrolled in Cohort B.

Dose reductions of ribociclib occurred in eight patients (24.2%), 1 patient in Cohort A and seven patients in Cohort B. Six patients (18.2%) in Cohort B discontinued study therapy due to protocol-specified unacceptable toxicity; three patients (9.1%), one in Cohort A and two in Cohort B, discontinued per physician decision due to intolerable toxicity (not specified by protocol to require discontinuation); and three patients (9.1%), all in Cohort A, discontinued per patient decision due to intolerable toxicity.

Efficacy of ribociclib plus spartalizumab with and without fulvestrant

Of the 15 patients with HR-positive/HER2-negative MBC treated at the RP2D of ribociclib with spartalizumab and fulvestrant, two confirmed PRs (13.3%) were observed with a duration of 7.3 and 7.4 (ongoing at last follow-up) months. SD ≥24 weeks was observed in another two patients, resulting in a clinical benefit rate of 26.7% among all patients with MBC who received the triplet. No responses were confirmed among MBC or AOC patients in Cohort A (table 3) (figure 1A–C).

The median PFS among all patients enrolled on the trial (n=33) was 2.04 months (95% CI 1.77 to NA) (figure 2A). The median PFS was 1.64 months (95% CI 1.51 to NA) among all MBC patients treated with ribociclib plus spartalizumab (online supplemental figure 3A) and 1.84 months (95% CI 1.84 to NA) among all AOC patients treated with ribociclib plus spartalizumab (online supplemental figure 3B). The median PFS was 9.95 months (95% CI 3.68 to NA) for all MBC patients (n=15) treated with ribociclib, spartalizumab, and fulvestrant (online supplemental figure 3C), and 3.12 months (95% CI 1.74 to NA) among all MBC patients in the study (online supplemental figure 3D).

Outcomes according to PD-L1 status and stromal TILs

Pathologist assessment of sTILs and PD-L1 was performed on the baseline (or, if not available, on-treatment) research biopsy, or archival tissue if a research biopsy could not be safely obtained (online supplemental table 3). Among all MBC patients who enrolled on the study, 45.8% (11/24) had PD-L1-positive tumors, whereas PD-L1 positivity was observed in 33.3% (3/9) of patients with AOC. Of the two patients in the dose expansion of Cohort B who had

Table 2 All treatment-related adverse events occurring in ≥10% of patients

No. of Patients (%)																				
	Ribociclib+spartalizumab (Cohort A)								Ribociclib+spartalizumab+fulvestrant (Cohort B)											
	Dose escalation (n=10)				Dose expansion (n=8)				Safety run-in (n=6)				Dose expansion (n=9)				All Patients (n=33)			
Event	≥G2	G2	G3	G4	≥G2	G2	G3	G4	≥G2	G2	G3	G4	≥G2	G2	G3	G4	≥G2	G2	G3	G4
Any event (max. grade per patient)	10 (100)	5 (50)	4 (40)	1 (10)	8 (100)	2 (25)	4 (50)	2 (25)	6 (100)	1 (16.7)	4 (66.7)	1 (16.7)	9 (100)		8 (88.9)	1 (11.1)	33 (100)	8 (24.2)	20 (60.6)	5 (15.2)
Neutrophil count decreased	8 (80)	4 (40)	3 (30)	1 (10)	5 (62.5)	1 (12.5)	4 (50)		6 (100)	2 (33.3)	4 (66.7)		7 (77.8)	1 (11.1)	6 (66.7)		26 (78.8)	8 (24.2)	17 (51.5)	1 (3)
Alanine aminotransferase increased	1 (10)		1 (10)		4 (50)	1 (12.5)	3 (37.5)		4 (66.7)	1 (16.7)	2 (33.3)	1 (16.7)	6 (66.7)	3 (33.3)	2 (22.2)	1 (11.1)	15 (45.5)	5 (15.2)	8 (24.2)	2 (6.1)
Aspartate aminotransferase increased	1 (10)		1 (10)		3 (37.5)	1 (12.5)	2 (25)		3 (50)		2 (33.3)	1 (16.7)	4 (44.4)		4 (44.4)		11 (33.3)	1 (3)	9 (27.3)	1 (3)
Fatigue	4 (40)	4 (40)			3 (37.5)	3 (37.5)			3 (50)	2 (33.3)	1 (16.7)		1 (11.1)	1 (11.1)			11 (33.3)	10 (30.3)	1 (3)	
Anemia	2 (20)	1 (10)	1 (10)		5 (62.5)	3 (37.5)	2 (25)		1 (16.7)	1 (16.7)							8 (24.2)	5 (15.2)	3 (9.1)	
White blood cell decreased	1 (10)		1 (10)		6 (75)	2 (25)	2 (25)	2 (25)	1 (16.7)	1 (16.7)							8 (24.2)	3 (9.1)	3 (9.1)	2 (6.1)
Nausea	1 (10)	1 (10)			1 (12.5)	1 (12.5)			2 (33.3)	2 (33.3)			2 (22.2)	2 (22.2)			6 (18.2)	6 (18.2)		
Platelet count decreased	2 (20)	2 (20)			3 (37.5)	2 (25)		1 (12.5)	1 (16.7)	1 (16.7)							6 (18.2)	5 (15.2)		1 (3)
Rash maculopapular	1 (10)	1 (10)			3 (37.5)	1 (12.5)	1 (12.5)	1 (12.5)					2 (22.2)	2 (22.2)			6 (18.2)	4 (12.1)	1 (3)	1 (3)
Anorexia	2 (20)	2 (20)			1 (12.5)	1 (12.5)							1 (11.1)	1 (11.1)			4 (12.1)	4 (12.1)		
Dehydration	2 (20)	1 (10)	1 (10)		1 (12.5)	1 (12.5)							1 (11.1)	1 (11.1)			4 (12.1)	3 (9.1)	1 (3)	

Table 3 Efficacy endpoints

Endpoint	No. of patients (%)				
	Ribociclib+spartalizumab (Cohort A)		Ribociclib+spartalizumab+fulvestrant (Cohort B)		
	Dose escalation (n=10)	Dose expansion (n=8)	Safety run-in (n=6)	Dose expansion (n=9)	All patients (n=33)
Confirmed ORR, No. (%, 95% CI)	0 (0%)	0 (0%)	0 (0%)	2 (22.2%)	2 (6.1%)
Confirmed CBR, No. (%, 95% CI)	0 (0%)	0 (0%)	2 (33.3%)	2 (22.2%)	4 (12.1%)
Ongoing responders, No. (%)	0 (0%)	0 (0%)	0 (0%)	1 (11.1%)	1 (3%)
BOR, No. (%)					
Confirmed CR	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Confirmed PR	0 (0%)	0 (0%)	0 (0%)	2 (22.2%)	2 (6.1%)
SD	1 (10%)	3 (37.5%)	6 (100%)	3 (33.3%)	13 (39.4%)
SD ≥24 weeks	0 (0%)	0 (0%)	2 (33.3%)	0 (0%)	2 (6.1%)
Unconfirmed PR	0 (0%)	0 (0%)	1 (16.7%)	2 (22.2%)	3 (9.1%)
PD	7 (70%)	3 (37.5%)	0 (0%)	4 (44.4%)	14 (42.4%)
Non-evaluable*	2 (20%)	2 (25%)	0 (0%)	0 (0%)	4 (12.1%)
Median DOR, months (95% CI)	NA	NA	NA	7.29 (7.29 to NA)	7.29 (7.29 to NA)
Median PFS, months (95% CI)	1.68 (1.51 to NA)	2.04 (1.84 to NA)	9.95 (3.68 to NA)	13.5 (1.77 to NA)	2.04 (1.77 to NA)

CBR defined as confirmed CR, PR, or SD ≥24 weeks.
*Cases 6, 9, 17, and 23 were considered non-evaluable because they did not have response assessed, or the assessment was unknown
BOR, best overall response; CBR, clinical benefit rate; CR, complete response; DOR, duration of response; ORR, objective response rate; PD, progressive disease; PFS, progression-free survival; PR, partial response; SD, stable disease.

confirmed PR (no prior treatment with CDK4/6 inhibition), both had sTILs<10%; one (6.7%) had PD-L1-positive disease and sTILs ≥1%, and the other had PD-L1-negative disease and sTILs <1% (online supplemental tables 4–6). Median PFS did not differ significantly between patients who had PD-L1-positive or PD-L1-negative tumors (9.95 months vs 1.81 months, p=0.22) (figure 2B), or between patients who had stromal TILs ≥1% or <1% (3.12 months vs 2.04 months, p=0.88) (figure 2C) or ≥10% or <10% (3.12 months vs 2.04 months, p=0.80) (figure 2D).

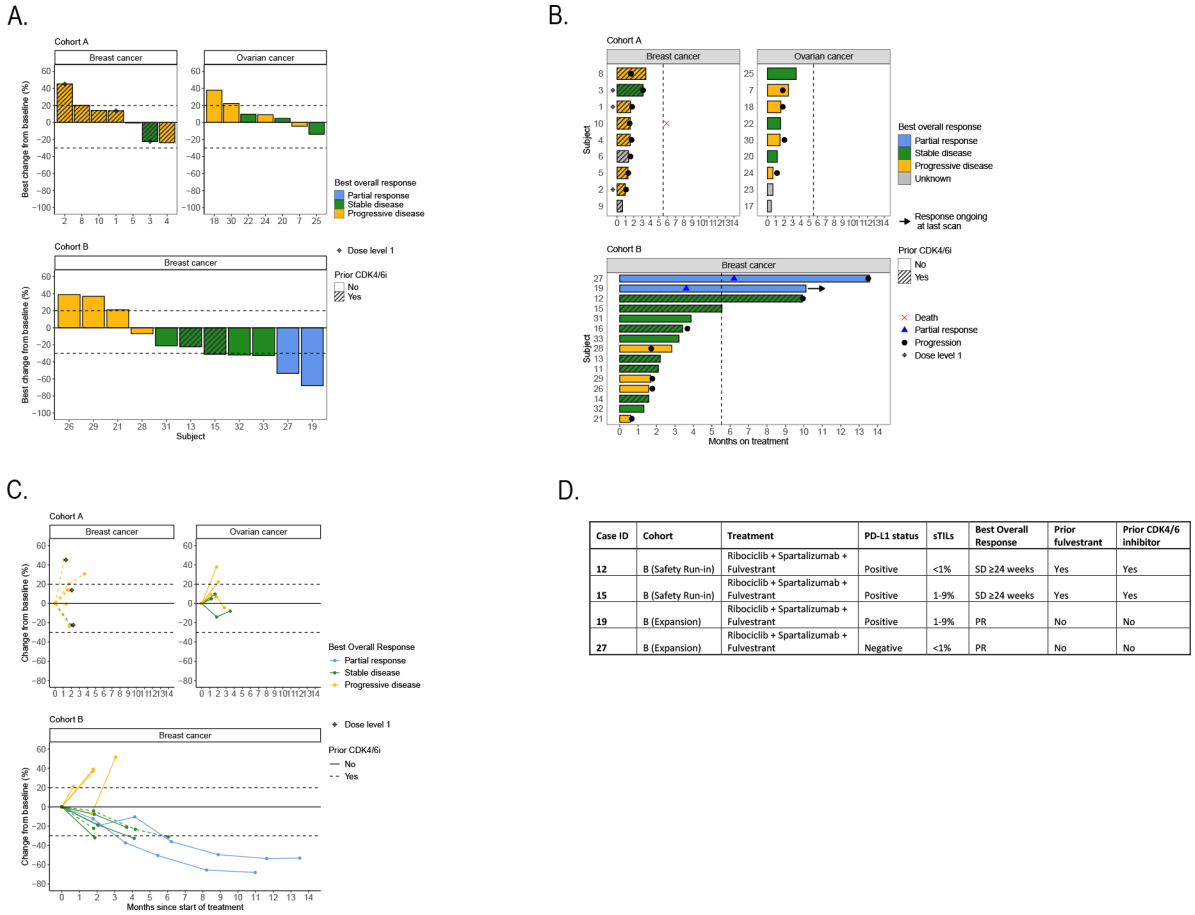
OncoPanel

OncoPanel testing was conducted in tumor samples (n=15; all MBC patients), including 5 from Cohort A (dose escalation, n=3; dose expansion, n=2) and 10 from Cohort B (safety run-in, n=4; dose expansion, n=6). Anatomic sites included breast (n=6), axillary lymph node (n=2), liver (n=2), bone (n=2), chest wall (n=1), ovary (n=1), and adrenal (n=1). In total, this analysis identified 129 single-nucleotide variants (SNVs, 1017 CNVs, and 5 SVs. Mutations in *TP53* were the most common genomic alterations identified, whether considering putative driver mutations (60%), no low amplifications (60%), no low amplifications or single-copy deletions (47%), or only putative driver mutations without low amplifications or single-copy deletions (47%) (figure 3).

High TMB was present in 20.0% (3/15) of tumors. TMB was not correlated with response, PFS, toxicity, PD-L1 status, or prior CDK4/6 inhibitor (online supplemental figure 4A). Moreover, we did not identify any CNVs that correlated with response, toxicity, PD-L1 status, or prior CDK4/6 inhibitor (online supplemental figure 4B). However, some CNVs were negatively associated with PFS, including *RBL2* deletion (p=0.022), *CYLD* deletion (p=0.022), and amplification of *RAF1* and *PPARG* (p=0.016). In contrast, *KMT2A* deletion was associated with improved PFS (p=0.008) (online supplemental figure 4C). Oncogenic missense SNVs in *TP53* and *PIK3CA* were identified in several patients, though there was no significant correlation with PFS (online supplemental figure 4D).

Correlation between peripheral immune cell populations and outcomes

The gating strategy for analysis of PBMCs is shown in figure 4A. There were no significant changes in the percentages of major immune cell populations ($CD14^+$ monocytes, $CD16^+$ NK cells, $CD3^+$ total T cells, $CD4^+$ T



Case ID	Cohort	Treatment	PD-L1 status	sTILs	Best Overall Response	Prior fulvestrant	Prior CDK4/6 inhibitor
12	B (Safety Run-in)	Ribociclib + Spartalizumab + Fulvestrant	Positive	<1%	SD ≥24 weeks	Yes	Yes
15	B (Safety Run-in)	Ribociclib + Spartalizumab + Fulvestrant	Positive	1-9%	SD ≥24 weeks	Yes	Yes
19	B (Expansion)	Ribociclib + Spartalizumab + Fulvestrant	Positive	1-9%	PR	No	No
27	B (Expansion)	Ribociclib + Spartalizumab + Fulvestrant	Negative	<1%	PR	No	No

Figure 1 Characteristics of objective response. (**A**) Best % change in target lesion from baseline; (**B**) time to and duration of best overall response; (**C**) characteristics of objective response in all patients; (**D**) clinicopathologic characteristics of patients with confirmed clinical benefit. For patients with a decrease in target lesions and best response of "progressive disease" (participants 4, 7, and 28), the best response is due to progression in non-target lesion(s) and/or appearance of new lesions. For patients with >30% decrease in target lesions and best response of "stable disease" (participants 15, 32, and 33), the partial response was unconfirmed.*Participants 6, 9, and 23 from Cohort A dose escalation, and participant 17 from Cohort A dose expansion, are not shown in this plot because they did not have any postbaseline target lesions assessed, or the assessment was unknown. *Participants 11, 12, 14, and 16 (from Cohort B safety run-in) are not shown in plot above because they did not have target lesions at baseline. All these patients had stable disease. PR, partial response; SD, stable disease.

cells, or $CD8^+$ T cells) before and after the initiation of treatment, independent of hepatotoxicity(figure 4B). Consistent with observations from other tumor types,[42] PD-1 blockade was associated with an increase in activated $CD8^+$ T cells during treatment as defined by coexpression of CD38 and HLA-DR ($p<0.0001$). Similarly, expression of the differentiation marker CX3CR1 was significantly increased on $CD4^+$ and $CD8^+$ T cells, as previously observed in patients with non-small cell lung cancer treated with PD-1 blockade[43] (figure 4C).

We first confirmed that PD-1^+ $CD8^+$ T cells were present in peripheral blood at baseline in all patients. We then confirmed that spartalizumab was bound to its target by showing a decrease in surface accessibility of PD-1 on peripheral blood T cells in on-treatment blood samples. We observed a decrease in PD-1 detectability on $CD3^+$ T cells after initiation of therapy due to occlusion of the PD-1 epitope by spartalizumab ($p<0.0001$) (figure 4D). Several patients from this study had been previously analyzed by single-cell TCR clonotype analysis in peripheral blood and showed an increase in the frequency of proliferating clonotypes upon treatment, consistent with PD-1 blockade inducing T cell reactivation in blood.[44]

Thymidine kinase 1 activity

TKa was measured in 130 plasma samples from 33 patients. Baseline TKa levels were high (median=1136 DuA, mean=1451 DuA, range 168–5637 DuA) among the overall cohort, likely reflecting the heavily pretreated, advanced disease status of this study population as compared with previously reported median baseline TKa levels in patients with HR-positive/HER2-negative MBC treated with 0–1 prior systemic therapies (175 DuA;[40] 255 DuA[45]). The fold change in TKa levels between baseline and C2D1 was evaluated; the average fold change was 1.29, with a median of 1.07 (range 0.41–3.81).

Patients with confirmed PR exhibited both low baseline TKa values (average: 382.0 DuA), defined as TKa value below the median, and a two-fold or greater increase (average: 2.90-fold change) in TKa from baseline to

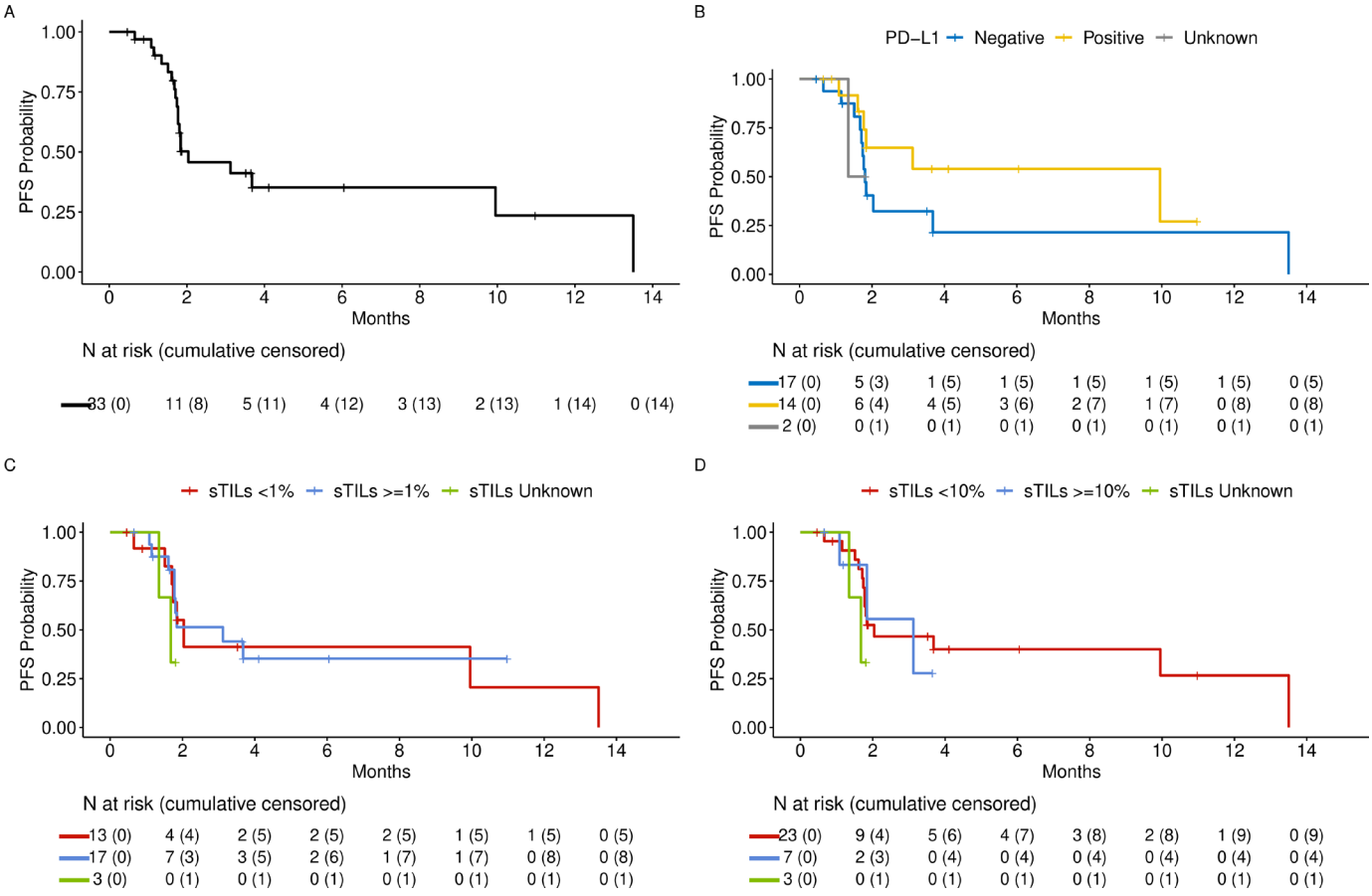


Figure 2 Progression-free survival (PFS). (**A**) PFS among the overall cohort (median PFS: 2.04 months; 95% CI 1.77 to NA). (**B**) PFS among patients with PD-L1-positive (median PFS: 9.95 months; 95% CI 1.84 to NA) and PD-L1-negative disease (median PFS: 1.81 months; 95% CI 1.71 to NA), p=0.22; (**C**) PFS among patients with stromal tumor-infiltrating lymphocytes (sTILs) ≥1% (median PFS: 3.12 months; 95% CI 1.77 to NA) and sTILs <1% (median PFS: 2.04 months; 95% CI 1.74 to NA), p=0.88; (**D**) PFS among patients with sTILs ≥10% (median PFS: 3.12 months; 95% CI 1.84 to NA) and sTILs <10% (median PFS: 2.04 months; 95% CI 1.77 to NA), p=0.80.

on-treatment (C2D1) (online supplemental table 7). One of these patients, with bone-only disease and no prior CDK4/6 inhibitor, had baseline TKa of 174 DuA (the second lowest value in this cohort) and the largest on-treatment TKa increase after one cycle of therapy (663 DuA; 3.8-fold change). TKa levels continued to increase after two cycles (1069 DuA; 6.1-fold change). Partial response occurred at Cycle 6, at which point the tumor volume decrease per RECIST V.1.1 criteria was 36%; the greatest decrease during the study was 54% and PFS was 411 days. This patient experienced G2 ALT elevation (probable attribution to spartalizumab), as well as G2 maculopapular rash (possibly related to spartalizumab). At progression, TKa level was 666 DuA.

The other patient with confirmed PR, also not previously treated with a CDK4/6 inhibitor, had baseline TKa of 590 DuA with an early on-treatment increase after one cycle (1173 DuA; 2.0-fold change). The greatest tumor reduction during the study was 68%. The patient ultimately discontinued protocol therapy due to toxicity. Only one other patient (with prior exposure to CDK4/6 inhibitor) met the criteria of low baseline TKa value plus on-therapy TKa increase of two-fold or greater; baseline TKa value was 296 DuA, which increased 2.6-fold to 758 DuA after the first cycle. After 8 weeks, a target lesion decrease of 22.5% was noted (figure 1A); however, PD was observed after 95 days.

DISCUSSION

In the present study, based on promising preclinical data for combined inhibition of CDK4/6 and PD-1, we evaluated the safety and preliminary efficacy of ribociclib plus spartalizumab in patients with HR-positive/HER2-negative MBC or AOC. A separate cohort was also included to explore the safety and activity of ribociclib with spartalizumab and fulvestrant in HR-positive/HER2-negative MBC. Due primarily to the high rate of grade 3–4 hepatotoxicity observed with the addition of endocrine therapy to the CDK4/6 and PD-1 inhibitors, the study was closed early after 33 patients (9 with AOC, 24 with HR-positive/HER2-negative MBC) enrolled. We observed limited activity (including two PRs) among HR-positive/HER2-negative MBC patients treated with the triplet; efficacy did not vary significantly based on PD-L1 or sTILs. No responses were observed in patients with HR-positive/

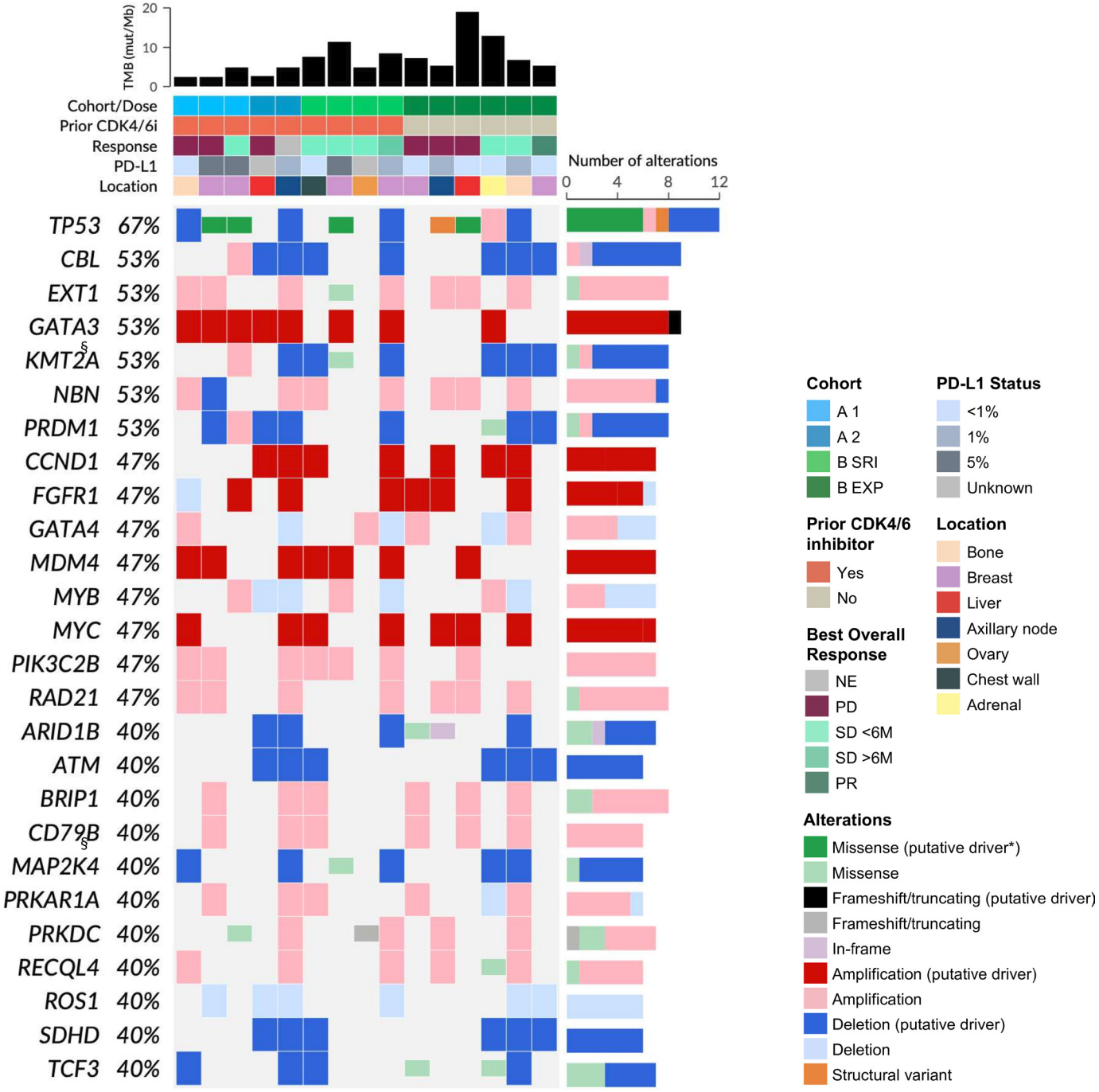


Figure 3 Genomic alterations per targeted NGS panel testing. Most frequently altered genes (SNVs, CNVs, or SVs) in tumors analyzed with OncoPanel.

HER2-negative MBC or AOC treated with the doublet of ribociclib and spartalizumab.

Immune checkpoint inhibitors have shown mixed results in patients with HR-positive/HER2-negative MBC. We previously conducted a phase II trial in which patients with HR-positive/HER2-negative MBC were randomized to receive eribulin with or without pembrolizumab. This study failed to demonstrate a significant improvement in clinical outcomes with the addition of PD-1 inhibition to chemotherapy. Median PFS was 4.1 months (95% CI 3.5 to 6.2) with the combination compared with 4.2 months (95% CI 3.7 to 6.1) with eribulin alone (p=0.33), and ORR was 27% (95% CI 14.9% to 42.8%) and 34% (95% CI 20.5% to 49.9%), respectively.[46] In addition, median OS was 14.3 months with eribulin plus pembrolizumab and 13.1 months with eribulin monotherapy (p=0.84).[47] In the single-arm phase II KELLY trial, ORR was 40.9% (95% CI 26.3% to 56.8%) and median PFS was 6.0 months (95% CI 3.7 to 8.4) among patients with HR-positive/HER2-negative MBC treated with eribulin plus pembrolizumab.[48]

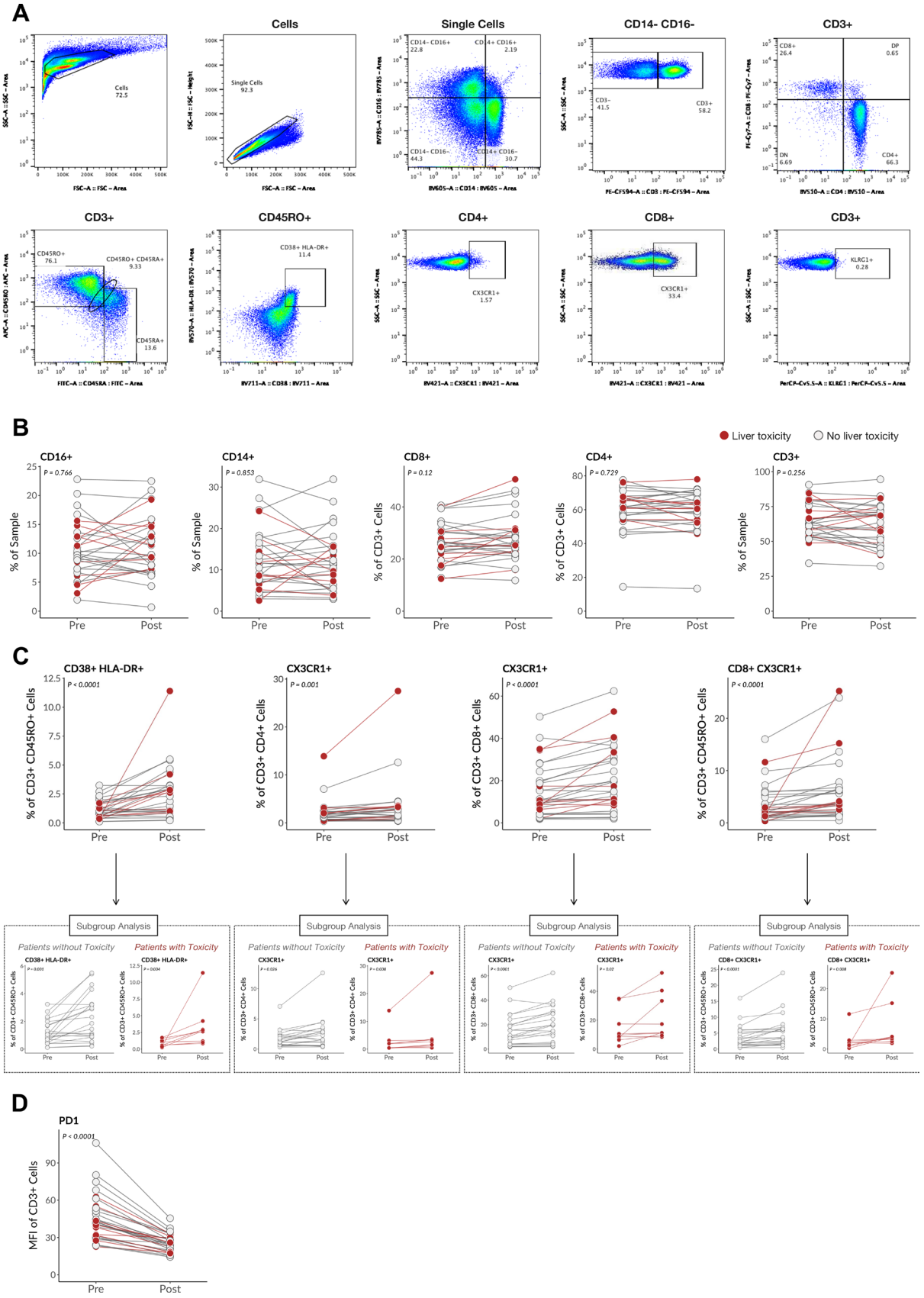


Figure 4 Flow cytometry analysis of PBMCs. (**A**) Flow cytometry gating strategy. CD14+, monocytes; CD14- FSC/SSC low CD16+, NK cells; CD3+, total T cells; CD4+, CD4 T cells; CD8+, CD8+ T cells, CD45RO+, antigen-experienced T cells. Labels above each graph indicate the gated population of cells being analyzed. (**B**) Changes in peripheral immune cell populations before and after initiation of treatment. (**C**) Changes in T cell phenotype during treatment in patients with and without hepatic toxicity. (**D**) Decreased detection of PD-1 on T cells after initiation of therapy.

Severe toxicity has now been reported across different trials exploring PD-1 inhibition in combination with CDK4/6 inhibition and endocrine therapy. The NEWFLAME phase II study evaluating nivolumab with abemaciclib and fulvestrant or letrozole in patients with HR-positive/HER2-negative MBC was discontinued early due to safety concerns. Grade 3 or higher hepatotoxicity was observed in 59% (10/17) of patients, and interstitial lung disease (ILD)/pneumonitis in 18% (3/17) of patients, including one grade 5 event.[49] Liver biopsies in three patients who experienced hepatotoxicity showed predominant $CD8^+$ T cell lymphocytic infiltration, suggesting immune-mediated hepatitis. Another phase Ib trial exploring abemaciclib plus pembrolizumab with or without anastrozole also reported elevated rates of G3 or higher transaminitis and ILD/pneumonitis, including two treatment-related deaths.[50] Similarly, in HR-positive/HER2-negative primary breast cancer, neoadjuvant treatment with nivolumab, palbociclib, and anastrozole resulted in 29% (6/21) G3–4 hepatotoxicity.[51] In contrast, the PD-L1 inhibitor avelumab was safely combined with fulvestrant and palbociclib in patients with HR-positive/HER2-negative MBC after progression on prior CDK4/6 inhibition in the PACE trial and, while the study was not powered to compare the triplet combination to fulvestrant alone, a 3.3-month absolute difference in median PFS that did not reach statistical significance was observed (8.1 vs 4.6 months; HR 0.75, p=0.23).[52] In another phase 1b/2 study, the incidence of G3–4 hepatotoxicity with letrozole and palbociclib plus pembrolizumab in HR-positive/HER2-negative MBC was 17% (4/23), demonstrating a more favorable safety profile despite combination with PD-1 inhibition.[53] It remains unclear if there are mechanistic differences between PD-1 and PD-L1 inhibitors that impact the toxicity profiles observed with these triplet combinations with CDK4/6 inhibitors and endocrine therapy. This underscores the need for improved strategies for detection of biomarkers of toxicity in clinical trials, with prespecified planned biospecimen collections at the time of development of adverse events of special interest (eg, liver biopsy and blood collection at onset of hepatic toxicity).

Elucidating the underlying mechanisms of both antitumor activity and toxicity with these combinations is key to help inform future therapeutic strategies for patients with HR-positive/HER2-negative breast cancer. In the present study, TMB did not correlate with clinical response. This contrasts with our recent findings in patients with metastatic TNBC, in whom high TMB (≥10 mut/Mb) was associated with response to anti-PD-1/PD-L1 agents as monotherapy or in combination with chemotherapy.[54] Moreover, in the phase II NIMBUS trial, patients with HER2-negative MBC (70% of whom had HR-positive breast cancer) with high TMB (≥9 mut/Mb) were treated with nivolumab (anti-PD-1) in combination with ipilimumab (anti-CTLA-4). The ORR was 13.3% in the overall cohort, and 60% among patients with TMB ≥14 mut/Mb.[55] The limited number of confirmed responses and relatively few patients with high TMB in the present study may have impacted the ability to detect a significant association.

In the present study, certain genomic alterations were associated with shorter PFS, including deletion of *RBL2* and *CYLD*. *RBL2* encodes the retinoblastoma-like protein 2 (RBL2), also called p130. RBL2/p130 is a key member of the DREAM complex that binds to cell cycle genes homology region (CHR) promoters to inhibit the expression of genes involved in cell cycle progression in response to p53 activation.[56 57] In patients with non-small cell lung cancer registered with the Pan-lung TCGA,[2] heightened expression of *RBL2* and concomitant reduced expression of target *AURKA/B* pathway genes were associated with improved survival in patients with *p53* wild-type but not *p53*-mutant disease.[58] CYLD is a tumor suppressor that deubiquitinates p53 in response to genotoxic stress, thus facilitating its stabilization and activation.[59] In vitro, *CYLD* downregulation promotes the survival and migration of breast cancer cells through NF-κB activation. In breast cancer patients, reduced *CYLD* expression has been identified as an independent poor prognostic factor.[60] In the present study, the same four patients had deletion of *RBL2* and *CYLD*. This could have resulted in a loss of p53-mediated regulation of the cell cycle, which could reduce the effectiveness of CDK4/6 inhibitor-based therapy.[61] All four patients had received prior CDK4/6 inhibition and developed rapid disease progression on study.

In contrast, *KMT2A* deletion was associated with improved PFS. *KMT2A* encodes lysine methyltransferase 2A (KMT2A), a transcriptional coactivator that regulates gene expression during early development and hematopoiesis. Chromosomal rearrangements in *KMT2A* are associated with the development and progression of leukemia.[62 63] In patients with various solid tumors, *KMT2A* mutations have been associated with improved PFS during immune checkpoint inhibitor treatment.[64] Findings from the present study are hypothesis-generating, and more detailed mechanistic studies will be required to understand the functional consequences of the CNVs we identified.

Treatment induced a significant increase in the fraction of circulating $CD3^+CD45RO^+$ T cells that expressed the activation markers CD38, HLA-DR, and CX3CR1. These findings suggest that spartalizumab combined with ribociclib, with or without fulvestrant, induces T cell reinvigoration, even in patients without an objective response to therapy. As we previously reported, single-cell analysis of a subset of patients from this study for whom matched blood and TILs were collected revealed that on-treatment samples showed increased cycling T cells, some of which matched TCR clonotypes present in the tumor.[44] Similar results of peripheral blood T cell reactivation by PD-1 blockade in the absence of clinical benefit have been reported in other disease settings such as pancreatic ductal adenocarcinoma,[65] raising the question as to what other factors might be needed to convert a reinvigorated T cell response into durable tumor control. In the

previously noted NEWFLAME trial, the authors reported a decrease in PD-1-positive effector T-regs in PBMCs compared with pretreatment values in patients who experienced hepatotoxicity. In our study, subgroup analyses according to occurrence of hepatotoxicity did not reveal differences in the patterns of change in peripheral immune cell subsets. In future studies, it would be beneficial to track TCR clonotypes of these activated T cells to determine whether any of the activated T cell clonotypes in peripheral blood are shared between tumor and liver. Single-cell transcriptional profiling of biopsies of inflamed liver may also reveal pathways distinct from the antitumor immune response that may offer opportunities to selectively target the toxicity without impairing anticancer efficacy.

High baseline TKa levels, as observed in most patients enrolled on our study, have been associated with highly proliferative tumors and/or greater disease burden.[66 67] Multiple studies have suggested that tumor burden impacts the efficacy of PD-1 inhibitors, where patients with smaller and fewer metastases have improved responses and survival compared with patients with greater baseline tumor burden across multiple cancer types.[68] Low baseline TKa levels likely reflect slower tumor growth and/or lesser disease burden. An early on-treatment TKa increase could indicate a proliferative burst of immune cell proliferation in response to anti-PD-1 treatment. The dual concept of requiring both low tumor burden and a specific-fold increase in PD-1^{+} Ki67^{+} CD8^{+} T cells for response to PD-1 blockade has been previously described in the literature.[68 69] Additional work is needed to correlate pretreatment TKa levels with tumor burden, and on-therapy TKa rise with an increase in proliferating T cells, to develop a more precise algorithm to predict outcomes using specific TKa fold change values.

Here, both patients with confirmed PR and longest duration of therapy (11.1 and 13.7 months) had low baseline TKa levels and a two-fold or greater increase in TKa at C2D1, suggesting that this may be the minimum required threshold to indicate sufficient antitumor immune activity. Overall, in the few patients on this study who had a durable response, the initial rise in TKa was later followed by a decrease in TKa which correlated with tumor shrinkage/response, whereas in patients with treatment-resistant and progressive disease, TKa levels steadily increase over time. It is important to note that the first on-treatment blood draw (C2D1) was drawn after the 1-week holiday of ribociclib (21 days-on/7 days-off schedule); blood collected at C1D15 may more accurately predict the effects of combined CDK4/6 and PD-1 inhibition on T cell proliferation and likelihood of response to therapy. It is challenging to discern in this trial the effects on TKa of increased immune cell proliferation in response to PD-1 inhibition, increased tumor burden due to disease progression, suppression of TKa in tumor cells that are arrested by CDK4/6 inhibition, and decrease in TKa that reflects decrease in tumor burden. Although CDK4/6 inhibition may have increased antitumor immunity, this cannot be definitively concluded from the TKa data from our study.

The toxicity observed with the combination of PD-1 inhibition, CDK4/6 inhibition, and endocrine therapy precludes further clinical development of this combinatorial strategy. With the recently reported improvements in pathologic complete response observed with the addition of PD-1 inhibition to neoadjuvant chemotherapy in high-risk early-stage HR-positive/HER2-negative breast cancer (followed by adjuvant PD-1 inhibitor or placebo plus endocrine therapy) in the KEYNOTE-756[70] and CheckMate 7FL[71] phase 3 trials, and the increased invasive disease-free survival with adjuvant CDK4/6 inhibition observed in the monarchE[72–75] and NATALEE[76] trials in this patient population, it will be imperative to understand the underlying mechanisms of toxicity of the triplet combination, and if PD-1 inhibition and CDK4/6 inhibitors can be safely administered sequentially with endocrine therapy in the adjuvant setting if the immune checkpoint inhibitor trials also demonstrate clinically meaningful improvements in long-term outcomes. While the development of optimal sequential strategies should take into consideration the pharmacokinetic profile of CDK4/6 inhibitors (eg, mean plasma half-life: ribociclib, 30–55 hours;[77] abemaciclib, 18 hours[78]) and PD-1 inhibitors (eg, spartalizumab, 11–41 days;[79] pembrolizumab, 22 days[80]), the potential for late onset of immune-mediated toxicity, even months after discontinuation of therapy, may limit the ability to target CDK4/6 after neoadjuvant immune checkpoint inhibition if the treatment paradigm for early-stage HR-positive/HER2-negative breast cancer changes. Until the mechanisms that drive toxicity are fully elucidated and there are additional clinical trial data regarding sequential use of PD-1 and CDK4/6 inhibition, decisions regarding systemic therapy may need to be individualized based on the estimated magnitude of benefit of each approach.

It is important to note some limitations of our study, including the relatively small sample size of patients treated in each cohort and limited number of baseline and on-treatment tumor biopsies collected on study. Results should be interpreted with caution, taking into consideration potential confounding factors, such as PD-L1 status, TILs, and prior exposure to CDK4/6 inhibition. Thus, the findings are hypothesis-generating and require further validation.

In conclusion, the combination of ribociclib plus spartalizumab with and without fulvestrant induced elevated hepatotoxicity with limited efficacy in patients with HR-positive/HER2-negative MBC and AOC. Correlative analyses suggest that this treatment combination has the potential to enhance antitumor immunity as noted by changes detected in peripheral immune cell subsets, and sequential treatment in mouse models suggests that these positive immune effects may be preserved even if PD-1 blockade is administered after CDK4/6 inhibition is stopped.[44] Additional studies to further elucidate the effects of this combination on the tumor immune

microenvironment and validation of genomic alterations and serum TKa dynamics in patients treated with CDK4/6 inhibition and immune checkpoint inhibition with endocrine therapy are needed to help inform future therapeutic strategies.

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Ethics approval This study involves human participants. The study followed the Declaration of Helsinki and Good Clinical Practice guidelines, and approval was obtained from the Dana-Farber/Harvard Cancer Center (DF/HCC) IRB (DFCI #17-285). All patients provided written informed consent before the initiation of any study-related procedures or treatments.

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