

Biomarkers Associating with PARP Inhibitor Benefit in Prostate Cancer in the TOPARP-B Trial

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ABSTRACT

PARP inhibitors are approved for treating advanced prostate cancers (APCs) with various defective DNA repair genes; however, further studies to clinically qualify predictive biomarkers are warranted. Herein we analyzed TOPARP-B Phase II clinical trial samples, evaluating whole exome and low-pass whole genome sequencing and immunohistochemical assays evaluating ATM and RAD51 foci (testing homologous recombination repair function). *BRCA1/2* germline and somatic pathogenic mutations associated with similar benefit from olaparib; greater benefit was observed with homozygous *BRCA* deletion. Biallelic, but not mono-allelic, *PALB2* deleterious alterations were associated with clinical benefit. In the ATM cohort, loss of ATM protein by immunohistochemistry associated with better outcome. RAD51 foci loss identified tumors with biallelic *BRCA and PALB2* alteration while most ATM- and CDK12-altered APCs had higher RAD51 foci levels. Overall, APCs with homozygous *BRCA2* deletion are exceptional responders; *PALB2* biallelic loss and loss of ATM immunohistochemical expression associated with clinical benefit.

Statement of significance

Not all APCs with DNA repair defects derive similar benefit from PARP inhibition. Most benefit was seen with *BRCA2* homozygous deletions, biallelic loss of *PALB2*, and loss of ATM protein. Loss of RAD51 foci, evaluating homologous recombination repair function, was found primarily in tumors with biallelic *BRCA1/2* and *PALB2* alterations.

Introduction

Metastatic castration-resistant prostate cancer (mCRPC) is enriched for genomic alterations in DNA damage repair (DDR) pathways, including homologous recombination repair (HRR) genes (1-5). DDR gene mutations (DDRm) can render mCRPC vulnerable to poly (ADP-ribose) polymerase inhibitors (PARPi) (6-8). The PARPi Olaparib recently received regulatory approval for the treatment of mCRPC with several DDR gene mutations, based on the results of the PROfound randomized phase III clinical trial (9,10).

We and others have previously reported on phase II trials of different PARPi in men with mCRPC with DDRm; across these studies *BRCA2* alterations associate with higher radiological and PSA response rates and longer progression-free survival (11-14). Similarly, subgroup analyses of the PROfound trial indicate that patients with *BRCA* alterations achieved the most benefit compared to patients with alterations in other genes such as *ATM* or *CDK12* (9,10). While collectively these trials support implementing mCRPC molecular stratification in clinical practice, further clinical qualification is needed for a more precise understanding of PARPi sensitivity in mCRPC (15,16).

TOPARP is an investigator-initiated adaptive Phase II clinical trial evaluating the antitumor activity of single agent olaparib in mCRPC (ClinicalTrials.gov NCT01682772). The results of the second stage of this trial, TOPARP-B, confirmed the antitumor activity of olaparib in mCRPC with various DDRm (11). In TOPARP-B, patients were prospectively screened for DDRm utilizing an investigational targeted next-generation sequencing (NGS) panel. Herein, we

pursued deeper molecular characterization of acquired samples, including whole exome and low-pass whole genome sequencing, and immunohistochemistry (IHC) and immunofluorescence (IF) assays, aiming to identify molecular features that can refine the predictive biomarker suite for patient stratification and to identify patients achieving major benefit from PARP inhibitor treatment.

Results

Patient population and sample disposition for extended molecular analysis

Overall, 98 men received olaparib on TOPARP-B; patient characteristics and clinical outcomes have been previously reported (***Supplementary Table 1***). In 69 men a treatment-naïve (HNPC) diagnostic biopsy was used for NGS testing, while in 29 a fresh mCRPC biopsy was utilized (***Figure 1***). With an additional 14-months of follow up since the primary report, 97/98 patients had experienced an event for rPFS analysis with 93/97 patients having deceased. Median follow-up for those still alive was 29-months. Median rPFS and overall survival were 5.5 (95%CI 4.67-5) months and 12.8 (95%CI 9.9-16.6) months respectively. The *BRCA1/2* altered subgroup (n=32) had the longest median rPFS (8.4 mo, 95%CI 5.5-14.0) and OS (17.7mo, 95%CI 9.0-22.2) (***Supplementary Table 2 and Supplementary Figure 1***). Whole exome sequencing (WES) was performed for 82 cases on available material from tumor biopsies (53 HNPC samples and 29 mCRPC samples) used for targeted NGS (trial prescreening phase) (2 failed sequencing QC due to low quality data); for the remaining

cases, there was no spare tissue/DNA left, or the DNA did not pass quality controls for whole-exome sequencing. For 74 cases, remaining DNA was also sufficient for lpWGS performed for orthogonal copy number profiling. Predefined “qualifying” alterations detected by tumor NGS classified mCRPC into one of five subgroups: *BRCA*, *ATM*, *CDK12*, *PALB2*, and ‘Other’. The qualifying event was an alteration detectable in germline DNA in 30 (30.6%), not detectable in germline DNA in 52 (31.6%), and a tumor homozygous deletion in 16 patients (16.3%). Based on the integration of targeted, WES and lpWGS data, we identified biallelic events in the genes of interest in 64/98 (65.3%) cases (**Supplementary Table 3**). Overall these analyses provide the deepest interrogation, to date, of mCRPC genomics in a prospective trial of PARP inhibition.

Patterns of response in BRCA-altered prostate cancer

Of the 32 patients in the *BRCA1/2* subgroup (*BRCA1* $n=2$, *BRCA2* $n=30$) identified by targeted NGS, 13 had a germline mutation and 19 a tumor-only pathogenic alterations (*BRCA1* mutation $n=1$, *BRCA2* mutation $n=7$, *BRCA2* homozygous deletion $n=11$). The composite response rates (primary endpoint of the TOPARP trial, including confirmed radiological responses, PSA responses or CTC counts conversions) to olaparib in these *BRCA1/2* subgroups were similar for those with germline mutations (10/13, 77%) and those with alterations only detected in tumor DNA (16/19, 84%) (**Table 1** depicts the composite response rate and the individual components).

Overall, in *BRCA1/2* patients, biallelic events were detected in 8/13 (62%) patients with germline mutations and in 16/19 (84%) patients with tumor-only alterations (5/8 with tumour only mutations and all 11 with homozygous deletion which are by definition biallelic). Tumors with *BRCA2* homozygous deletions (n=11) had the best outcomes in the *BRCA* cohort with a median radiographic progression-free survival (rPFS) of 16.4 months vs 5.6 months for patients with deleterious *BRCA1/2* germline mutations (n=13) and vs 8.2 months for patients with *BRCA1/2* somatic mutations only (n=8) (**Table 1, Figure 2A and Figure 2B**). Overall, 7/11 patients with a *BRCA2* homozygous deletion were on trial for over 1-year, with the longest responder experiencing disease progression after over 3-years of olaparib (**Figure 2C**). Median overall survival was 22.2 months from starting olaparib for the *BRCA2* homozygous deletion cohort (n=11), compared to 14.7 months for patients with deleterious *BRCA1/2* germline mutations (n=13) and 14.6 months for patients with *BRCA1/2* somatic mutations only (n=8).

Considering all patients with biallelic loss (n=24, mutation with a detectable second event and homozygous deletions), the median rPFS and median OS for patients with *BRCA1/2* biallelic loss was 9.7 and 18.9 months respectively, compared to a median rPFS and median OS of 5.6 and 14.6 months for those without detectable biallelic loss (n=8, **Table 1, Figure 2B**).

These data suggest that most tumors with *BRCA* mutations are likely to have biallelic loss, even if the most commonly used NGS assays may miss to detect

some events leading to complete loss-of-function, such as complex rearrangements. Yet, our data largely refers to *BRCA2* alterations as only 2 patients had *BRCA1* alterations. mCRPC patients with *BRCA2* homozygous deletion had superior rPFS and OS outcomes from PARP inhibition suggesting that olaparib resistance may be harder to evolve in these tumors.

Molecular profiles in patients responding to PARPi beyond the BRCA subgroup: PALB2.

Among 7 patients with *PALB2* mutations, 6 had germline mutations, of which 4/6 had a detectable second hit inducing biallelic loss. Interestingly, all 4/4 patients responding to olaparib in TOPARP-B, according to the composite response definition in the trial, had a germline mutation with evidence of biallelic loss, whereas in all 3/3 non responders, there was no evidence of a second detectable event based on all the NGS data analyses conducted (**Table 1, Figure 2D**).

Molecular profiles in patients responding to PARPi beyond the BRCA subgroup: ATM.

Overall, 21 men treated on TOPARP-B had *ATM* altered tumors; most of these *ATM* aberrations were only detected in tumor (15 somatic mutations, 1 homozygous deletion), whereas 5 patients had *ATM* germline mutations (**Figure 3A; Supplementary Table 1 and Table 3**). Of 21 cases, 12 (57.1%) *ATM* altered tumors had a detectable second event; these *ATM* altered cases predicted to have tumor biallelic loss cases had longer rPFS (median 9.5 vs 5.2 months; **Figure 3B**) but this did not translate into improved OS (median 13.5 vs

16.6 months; **Figure 3B**). *ATM* protein expression by immunohistochemistry, was completely lost in 15 of these 21 (71%) tumors, however 5 of these 15 IHC-negative tumors with *ATM* mutations had no detectable second genomic event that would cause biallelic loss (**Supplementary Figure 2, Supplementary Table 4**). Interestingly, all 5 patients with germline mutations had tumors with *ATM* loss on IHC (compared to 10 of 16 with tumor-only mutations), with 4 of these 5 meeting the definition of response based on the composite trial endpoint compared to 4/16 with tumor-only mutations (**Table 1**). Moreover, in this *ATM* cohort, *ATM* loss of expression by IHC associated with longer rPFS (median 5.8 months vs 3.7 months) and overall survival (median 17.4 months vs 10.3 months) (**Figure 3C, Supplementary Table 4**). Overall, these data indicate that *ATM* mutations are not always associated with biallelic loss, and that *ATM* loss of IHC expression associates with better outcomes on olaparib, although it remains possible that other background genomic alterations in these tumors are required to sensitize to PARP inhibition (**Figure 3D**).

Molecular profiles in patients responding to PARPi beyond the BRCA subgroup: CDK12.

All detected *CDK12* alterations were restricted to tumor-only mutations; interestingly, in 18 of 20 tumors a second event in the same gene was detected with most of these alterations being biallelic missense or truncating mutations. **Supplementary Figure 3** summarizes outcomes on the TOPARP-B trial for patients in this *CDK12* subgroup; 5 subjects were on treatment for 6-months or more although in the majority of these olaparib was continued despite PSA progression. Overall, these data indicate that despite many *CDK12* altered

tumors appearing to have biallelic events, olaparib has limited antitumor activity by established response criteria in this cohort.

Molecular profiles in patients responding to PARPi beyond the BRCA subgroup: 'Other'.

Supplementary Figure 3 also depicts the patients whose tumors were categorized in the 'Other' cohort that incorporated multiple, less common, remaining gene alterations. Three patients in this cohort were on olaparib >6 months and had deleterious *CHK2*, *ATRX* and *FANCA* aberrations with none of these having PSA progression during the 6-month period and two remaining on olaparib (*CHK2*, *ATRX*) for more than a year. Interestingly, exome sequencing data identified concurrent deleterious *PPP2R2A* frameshift and *POLA2* mutations in the *CHK2* mutated tumor, while the *ATRX* mutated tumor also had a *ZMYM3* mutation which may also have impacted PARP inhibitor sensitivity. No significant antitumor activity was observed among patients with germline mutations in these 'Other' genomic aberrations. Overall, further data are required on the impact of *CHK2*, *ATRX*, *FANCA* and other rare genomic alterations on PARP inhibition sensitivity.

Genomics of mCRPC with DDR alterations on TOPARP-B

Figure 4A depicts TOPARP-B prostate cancer genomic profiles by whole exome sequencing ranked by binary response assessment using the predefined composite endpoint, as well as by rPFS. The genomics of diagnostic, archival, samples are presented separate to those of mCRPC biopsies. Overall, genomic

alterations in AR (54% vs 2%); TP53 (mainly mutations; 32% vs 21%); MYC (mainly amplification; 28.6% vs 13.5%); RB1 (mainly deletions; 28.6% vs 5.8%); PTEN (21% vs 8%); WNT pathway aberrations including APC (14% vs 2%) and CTNNB1 (7% vs 2%); were commoner in CRPC biopsies than diagnostic, pre-treatment, samples but appeared similar to that previously reported for molecularly unselected lethal prostate cancer (17,18). Exploratory analyses comparing genomic copy number data in responders and non-responders identified a significant enrichment for specific genomic loci in responders (**Figure 4B**), including chromosome 3q amplification in *BRCA1/2* altered tumors ($p < 0.01$; **Supplementary Figure 4A**). In the nonBRCA cohorts, those tumors responding to olaparib had significant enrichment for chromosome 15 and 19 loci gains, and focal chromosome 10 locus loss (**Supplementary Figure 4B**) which all also include multiple genes implicated in DNA repair (**Supplementary Table 5**) that warrant further study. Furthermore, since it has been suggested that some DNA repair defects co-occur with other alterations impacting PARP inhibitor sensitivity, we interrogated co-occurrence or mutual exclusivity for common prostate cancer genomic alterations in this TOPARP-B cohort; in tumors with *BRCA2* and *CDK12* alterations we observed trends towards mutual exclusivity for other DDR alterations, and no significant association between *ATM* and *TP53* alterations (**Figure 4C**). Overall, these exploratory data suggest that the identified genomic loci associating with PARP inhibitor sensitivity warrant further study in independent validation PARP inhibitor clinical trials and if validated, genes in these loci will merit further functional study.

Loss of RAD51 foci as a functional biomarker of HRR in mCRPC

Finally, we studied γ H2AX/geminin (GMN) and RAD51/GMN foci by immunofluorescence in the 52 cases for whom tumor tissue from the same biopsy used for NGS was available (**Supplementary Figure 5A**). In all 52 cases, γ H2AX foci were detected in >40% of GMN-positive cells; inter-reader variability was low (**Supplementary Figure 5B**). Overall, 22 of 52 (42%) cases were scored as RAD51 “low”, using a predefined cut-off of 10% of cells having ≥ 5 nuclear RAD51 foci (19-21). All 16 tested (16/16; 100%) prostate cancers with *BRCA1/2* deleterious alterations had low RAD51 scores; this also included all the tumors arising with and without germline mutations and regardless of having detected a biallelic loss (**Figure 5A**). Of the 4 tumors with *PALB2* mutations evaluated for RAD51 foci, the 2 with low RAD51 scores were responders in the trial; both had biallelic loss; neither of the two patients with high RAD51 scores responded to olaparib with neither of these having biallelic loss (**Figure 5A**). Moreover, low RAD51 foci scores associated with response to olaparib: 15 of 22 (68.2%) patients with prostate cancers with low RAD51 foci scores were responders by the trial composite response primary endpoint, compared with 7 of 30 (23.3%) patients with tumors with high RAD51 scores. Patients with low RAD51 foci scores also had longer rPFS (median 9.3 vs 2.9 months) and overall survival (median 17.4 vs 9.5 months) from initiation of olaparib therapy when compared to those with high RAD51 foci scores (**Figure 5B**). These data support, for the first time, the validity of the RAD51 assay in mCRPC.

Interestingly, *ATM* and *CDK12* altered prostate cancers had lower RAD51 foci scores than the prostate cancers in the 'Other' DNA repair genes cohort, although these scores were higher than those in the *BRCA* cohort (**Figure 5A**). In *ATM* mutated tumors, RAD51 foci scores had a median score of 18% (interquartile range: 14-25) compared to tumors in the 'Other' DDR repair gene category that had a median score of 34% (inter-quartile range of 16-46). Full details of the per-patient outcomes of all the TOPARP-B patients for whom sufficient tumor tissue was available for the conduct of this HRR function immunofluorescence assay are depicted in **Figure 5C**. Overall, these data indicate that RAD51 scoring identifies all *BRCA1/2* mutated tumors and tumors with biallelic *PALB2* loss, although not all tumors with a low RAD51 score respond to olaparib. These findings also suggest that some *ATM* and *CDK12* altered tumors have relatively low RAD51 scores but these are higher than those in *BRCA1/2* gene prostate cancers.

Discussion

The TOPARP trial was the first to demonstrate the antitumor activity of PARP inhibition in a subset of prostate cancers with DNA repair defects (11,14); olaparib has now been granted regulatory approval for treating mCRPC with specific, selected, DNA repair gene alterations pre- and post-chemotherapy after one next-generation hormonal agent based on the PROfound trial data (9,10). Rucaparib has also been approved by the Food and Drug Administration (FDA) to treat mCRPC with *BRCA1/2* pathogenic alterations post-chemotherapy in the United States (13). The approvals of olaparib by the

FDA, and the European Medicines Agency (EMA), are quite different, however, with olaparib being approved for mCRPC with genomic alterations in 14 different DNA repair genes by the FDA but being approved only for BRCA related cancers by the EMA. This discordance in approvals underlines why further study of biomarkers that predict clinical benefit to PARP inhibition in this disease is warranted.

In this deeper study of tissues prospectively acquired for biomarker studies in the TOPARP-B trial utilizing exome, low pass whole genome, and immunohistochemical studies, we now discover multiple clinically important findings that can impact patient care. Firstly, we show that most advanced prostate cancers with a detectable *BRCA2* alteration have biallelic loss. *BRCA1/2* altered mCRPC cases all had low RAD51 scores in keeping with loss of HRR function, even for those cases where we could not detect a second inactivating event with our integrative NGS approach. Our data therefore indicate that detection of a monoallelic pathogenic mutation in *BRCA2* should suffice to select patients for PARPi treatment, even in the absence of having detected a second hit by NGS, as indeed targeted panels implemented in clinical practice may not detect some events that could lead to loss-of-function such as complex rearrangements of copy-neutral LOH, particularly in challenging samples with low tumor content. Moreover, we observed that *BRCA2* mutations associated to similarly high response rates regardless of the germline vs somatic origin of the mutation. Of note, 30 patients in the trial had *BRCA2* alterations, compared to 2 with *BRCA1* mutations; consequently, our data for the *BRCA1/2* cohort is largely related to *BRCA2* alterations and

extrapolation to *BRCA1* which are infrequent in prostate cancer should be avoided. (22,23)

We and others have previously reported significant variability in terms of duration of benefit in mCRPC patients with *BRCA1/2* alterations receiving olaparib or other PARP inhibitors. We hypothesized that the exact type of gene alteration may associate with different magnitudes of benefit. We now have compelling evidence that indicates that mCRPC with *BRCA2* homozygous deletions have substantially longer response durations compared to tumors with frameshift or stop-gain mutations (regardless of these being germline or somatic in origin). Impressively, tumors with homozygous deletions had a median rPFS of 16.4 months, which is a considerable period of disease control for this late stage mCRPC setting. We and others have previously reported how prostate tumors (like other cancers) with truncating mutations in *BRCA1/2*, *PALB2* or *RAD51* accumulate secondary reversion mutations while on PARP inhibitors that restore the DNA repair gene reading frame back to normal, converting a truncating mutation into an in-frame indel (24,25); these reversion mutations associate with clinical secondary resistance to PARP inhibition (26,27). Tumors with *BRCA2* homozygous deletion, which account for approximately 5% of all metastatic prostate cancer (2), are unable to generate such secondary resistance mutations and may have exceptional responses based on these tumors finding it much more difficult to evolve resistance by reverting the homologous recombination function.

The partner and localizer of *BRCA2* (*PALB2*) gene is much less commonly altered in advanced prostate cancer so very little published data is as yet available on this subset of mCRPC. The TOPARP-B trial recruited 7 patients with prostate cancers with *PALB2* alterations. Surprisingly, of 7 patients with *PALB2* pathogenic alterations all but one had germline *PALB2* mutations (6/7; 86%) but only 4 of these 7 tumors had detectable biallelic loss. Overall, only the biallelic *PALB2* mutated prostate cancers in this subset appeared to benefit with all 4 men with biallelic loss mCRPC having either a PSA or RECIST response, and 3 of these being on drug >6 months. These data overall indicate that for *PALB2*-altered mCRPC identifying biallelic loss may be clinically important since almost half the tumors in this subset only had mono-allelic loss with this group not appearing to benefit.

In this study, we tested the capacity of RAD51 foci immunofluorescence to identify loss of homologous recombination repair function in advanced prostate cancer in FFPE tumor slides. This assay successfully identified all 16 of 16 cases with BRCA deleterious alterations as well as the *PALB2* mutated tumors with biallelic loss, distinguishing the latter from *PALB2* mutated tumors with mono-allelic loss. This assay may have clinical utility and complement genomic testing in routine clinical practice especially when insufficient FFPE biopsy material is available for next generation sequencing, a common occurrence in both the PROfound and TRITON2 trials. Moreover, this RAD51 assay can help identify less-common genomic variants impacting HRR function that sensitize to PARP inhibition as observed for the TOPARP-B *PALB2* cohort. Further clinical

qualification is needed to optimally define the predictive value of this RAD51 assay for PARP inhibitor treatment.

Interestingly, the results from this RAD51 assay in the TOPARP-B cases indicate that tumors with *ATM* and *CDK12* alterations have higher RAD51 scores than *BRCA1/2* altered tumors with some cases in these sub-groups having scores at or just above the cut-off of 10% of cells having RAD51 foci. However, the responses seen in *ATM* and *CDK12* aberrant advanced prostate cancer with treatment with olaparib administered for >6 months were observed in tumors with high RAD51 scores, indicating that the PARP inhibitor antitumor activity in this subgroup may not be related to complete loss of homologous recombination repair function but perhaps due to the genomic instability in these tumors associated with *ATM* loss (28). Interestingly, unlike *BRCA2* mutation associated cancers, a significant number of the *ATM* aberrant prostate cancers in the TOPARP-B trial did not have detectable biallelic loss (29). Only a small fraction of the *ATM* cohort had deleterious germline mutations with these tumors with germline mutations all having loss of *ATM* protein by immunohistochemistry. The longest responding patient in this *ATM* cohort had homozygous deletion, and interestingly biallelic *ATM* mutations and *ATM* loss by IHC associated with better outcome. While we acknowledge the small numbers of patients (n=21) in the TOPARP-B *ATM* cohort, these data indicate that patients with germline mutations and loss of *ATM* expression by IHC are more likely to respond and have longer duration of benefit. This is supported by the TOPARP-A data where 2 of the 3 *ATM* aberrant prostate cancer patients on olaparib for over a year had germline deleterious mutations (14). Overall, while

the response rate in this subgroup is low and exploratory analyses of the PROfound trial failed to demonstrate a significant benefit in the *ATM* mutated disease subset, we have observed durable tumor responses in some prostate cancers with *ATM* aberrations in keeping with preclinical genomic screen data (30). This, together with data from the PROfound trial, indicate that there is now an urgent need to integrate data from preclinical and clinical studies to better identify which *ATM* aberrant prostate cancers benefit from PARP inhibition.

Most phase II/III trials of PARP inhibitors in prostate cancer conducted to date defined their patient population based on the identification of deleterious aberrations in predefined lists of genes. A major question that remains for this field is whether other relevant genomic alterations co-operate with the primary DNA repair defects, with which we have categorized these advanced prostate cancers, to generate sensitivity to PARP inhibitors like olaparib. Exploratory studies described herein identify multiple putative genomic loci that statistically associate with response to olaparib with a false discovery rate of 0.01%. These loci contain multiple genes that are reported to impact DNA repair and could potentially have such a co-operative role. The chromosome 3q locus includes *LRRC31* which when overexpressed is reported to inhibit DNA repair and sensitize to cell death following radiation-induced double strand DNA breaks (31). The chromosome 10 locus includes the genes *SIRT1*, *DNA2*, *TET1* which have been implicated in DNA repair regulation and the ubiquitin ligase *HERC4* which has been previously identified as impacting PARP inhibitor sensitivity in a reported genome wide screen (32). The chromosome 15 locus includes *PARP6*; *ARIH1* which is implicated in DNA damage-induced translation arrest (33);

C15orf60 (REC114 Meiotic Recombination Protein) which is reportedly involved in regulating DSB formation (34); and *CD276* (*B7-H3*). The chromosome 19 locus also includes several genes implicated in the DNA damage response including *AURKC*, *KMT5C* and *POLD1*. Overall, however, in light of the significant risk of false positivity in these genome-wide associations we recommend that validation studies from other PARP inhibitor clinical trials are necessary to confirm these findings and the pursuit of wet laboratory studies of the impact of genes at these loci both as altered single, or multiple, genes.

We acknowledge that these analyses have limitations. First, the number of patients on this trial of the individual subsets was small, therefore the analyses performed are largely exploratory and hypothesis-generating with any findings needing validation in larger cohorts. Secondly, patients were recruited to the trial based on a targeted tumor-only NGS assay, and in some cases, there was insufficient material to pursue the extended deeper genomic analyses and IHC and IF assays. Despite this, however, these analyses remain the largest such deep analyses of advanced prostate cancer samples on a prospective clinical trial of a PARP inhibitor. Lastly, TOPARP-B was a phase II trial without a control treatment arm, so some of these findings may be confounded should any of these biomarkers have prognostic, rather than predictive, value; hence, validation in randomized trials is necessary.

In conclusion, our data have identified a group of exceptional responders to PARP inhibition characterized by *BRCA2* homozygous deletions, and shown that *BRCA* altered tumors unlike *PALB2* and *ATM* aberrant tumors usually have

biallelic loss. These data may help refine stratification strategies in clinical practice for identifying prostate cancer patients benefiting from this now approved class of molecularly stratified treatment. Our study also suggests that functional assays assessing HRR, such as RAD51 foci IF, may have clinical utility for patient stratification in prostate cancer, although these now require prospective validation in larger cohorts.

Methods

Study design, patients and outcomes

The results of the TOPARP-B trial have been previously published (11). Briefly, we conducted an open-label, randomized phase 2 trial where patients with tumors known to have deleterious DDRm that may sensitize to PARP inhibition were randomized to receive olaparib at either 300 mg BID or 400 mg BID tablets. After providing written informed consent, archival primary tumor or fresh metastatic biopsies were tested using an investigational amplicon-based targeted NGS panel. Patients had to have been treated with at least one line of taxane-based chemotherapy; they received olaparib until radiographic progression, unacceptable toxicities or withdrawal of consent. Patients treated with 300 mg BID were offered dose escalation to 400 mg BID on confirmation of radiographic progression, if clinically indicated but no significant difference in antitumor activity was seen between the two dose levels.

The primary endpoint was confirmed tumor response, defined as a composite of: objective response by RECIST 1.1 (with PCWG2 caveats) and/or PSA decline of $\geq 50\%$ from baseline and/or conversion of CTC count from ≥ 5 cells/7.5 ml blood at baseline to < 5 cells/7.5 ml. Secondary endpoints included radiographic progression-free survival (rPFS), defined as time from randomization to first evidence of radiographic progression (by RECIST 1.1 or bone scan as per PCWG2 criteria) or death and overall survival (OS), defined as time from randomization to death by any cause.

The study was approved by the London, Surrey Borders, Research Ethics Committee (REC reference 11/LO/2019), and co-sponsored by The Royal Marsden Hospital and The Institute of Cancer Research (ICR), London, UK.

Sequencing and bioinformatics.

DNA was extracted from the same formalin-fixed and paraffin-embedded (FFPE) tumor samples tested for study inclusion using the FFPE Tissue DNA kit (Qiagen) and quantified with the Quant-iT high-sensitivity PicoGreen doublestranded DNA Assay Kit (Invitrogen). DNA quality control was performed by quantitative PCR (qPCR) using the Illumina FFPE QC kit (WG-321-1001) according to the manufacturer's protocol as previously described (11,14).

Libraries for whole exome sequencing (WES) were performed using Kapa Hyper Plus Library Prep Kits and the Agilent SureSelectXT V6 target enrichment kit. Paired-end sequencing was performed using the NovaSeq 6000 S2 flow cell (2x100 cycles; Illumina) at the Centre for Molecular Pathology Translational Genomics Lab (RMH). FASTQ files were generated from the sequencer's output using Illumina bcl2fastq2 software (v.2.17.1.14, Illumina) with the default settings. All sequencing reads were aligned to the human genome reference sequence (GRCh37-hg19) using the BWA-MEM algorithm (v. 0.7.12). Picard tools (v.2.1.0) were used to remove PCR duplicates and to calculate sequencing metrics for quality control check. The Genome Analysis Toolkit (GATK, v. 3.5-0) was applied to realign local indels, recalibrate base scores, and identify genetic variants. Somatic point mutations and small indels

were called using paired tumor-normal design using MuTect2 with stand_call_conf 30 and stand_emit_conf 30. Somatic variant was further filtered by quality PASS, does not appear in normal sample, coverage depth > 10 and allele frequency >5%. By comparing tumor DNA to its matched germline DNA control, copy number estimation was obtained through modified ASCAT2 package using 1) BAF data matrix derived from GATK variants calling and 2) LogR data matrix of sequencing coverage at GATK variant location from Picard CalculateHsMetrics.

Low-pass whole genome sequencing (lp-WGS) was performed with libraries constructed using the NEBNext Ultra FS II DNA kit (New England Biolabs) according to the manufacturer's protocol. Samples were pooled and run on the NextSeq (Illumina) at $\times 0.5$ mean coverage, using the 300 cycles High Output v2.5 kit (Illumina). BCL files were converted to FASTQ files using the bcl2fastq2 software (v.2.17.1.14, Illumina). Sequence alignments were performed using the BWA-MEM algorithm (v. 0.7.12) to the human genome reference sequence (GRCh37-hg19). Copy number analysis was performed using IchorCNA (35). In short, hg19 genomes (filtered centromeres) were divided into 500-kb nonoverlapping bins, and the abundance of the mapped reads was counted by HMMcopy Suite in each bin and predicted segments of CNAs. GC and mappability bias were corrected by loess regression and based on a panel of germline DNA sequencing from healthy donors. The maximum CNA detection was set to 20 copies.

Raw sequencing data have been deposited at the European Nucleotide Archive (ENA) with accession number: PRJEB45010.

For the purpose of this analyses, we considered “biallelic” events those cases with either: 1) two pathogenic mutations; 2) a pathogenic mutation and a shallow deletion; 3) a pathogenic mutation and loss-of-heterozygosity; 4) or cases with homozygous deletions in the genes of interest; after analyzing data from the targeted, whole exome, and low pass whole genome sequencing and reviewing manually the cases when discordance was detected. Those cases where there was no evidence for any of those conditions were declared “not confirmed biallelic” loss (**Supplementary data file1**).

ATM Immunohistochemistry

ATM protein expression in the ATM group was determined by immunohistochemistry (IHC) staining on 3 to 4 μ M-thick FFPE sections using the rabbit monoclonal anti-ATM antibody Y170 at 1:400 (catalog no. ab32420; Abcam plc, Cambridge, UK) as previously described (28). IHC slides were assessed by a pathologist, blinded to the patients’ clinical characteristics, sequencing findings, and outcome data. Nuclear staining was semi-quantitatively assessed using an H-score formula: 3 times percentage of strongly staining cells and 2 times percentage of moderately staining cells and percentage of weakly staining cells, giving a H-score range of 0–300. ATM negative was considered if there was a complete absence of ATM staining or weak intensity staining in 10% or less of cancer cells (H-score \leq 10).

RAD51 Immunofluorescence

Immunofluorescence for RAD51, geminin (GMN) and phospho-histone H2AX (γ H2AX) was performed as described previously (19,20), using sections of paraffin-embedded formalin-fixed (FFPE) tumor biopsies. RAD51 was used as a biomarker of HRR function; GMN and γ H2AX were used as quality checks to confirm that RAD51 foci were quantified in cells in the S/G2 cell cycle phase when HRR takes place and in the presence of DNA double-strand breaks respectively. For target antigen retrieval, sections were microwaved in DAKO Antigen Retrieval Buffer pH 9.0. Sections were permeabilized with DAKO Wash Buffer for 5 minutes, followed by 5 minutes incubation in blocking buffer (DAKO Wash Buffer with 1% bovine serum albumin). Primary antibodies were diluted in DAKO Antibody Diluent and incubated at room temperature for 1 hour. Sections were washed and blocked again. Secondary antibodies were diluted in blocking buffer and incubated for 30 minutes at room temperature. Finally, sections were dehydrated with increasing concentrations of ethanol and mounted with DAPI ProLong Gold antifade reagent.

The following primary antibodies were used: rabbit anti-RAD51 (Abcam ab133534, 1:1000), mouse anti-GMN (NovoCastra NCL-L, 1:60), rabbit anti-GMN (ProteinTech 10802-1-AP, 1:400) and mouse anti- γ H2AX (Millipore #05636, 1:200). Goat anti-rabbit Alexa fluor 568 (Invitrogen; 1:500), goat anti-mouse Alexa fluor 488 (Invitrogen; 1:500), donkey anti-mouse Alexa fluor 568 (Invitrogen; 1:500), and goat anti-rabbit Alexa fluor 488 (Invitrogen; 1:500) were used as secondary antibodies. Scoring was performed independently by two readers independently and blinded to clinical outcome and genomics data.

Scores were assessed on life images using a 60x immersion oil objective with a Nikon Eclipse Ti-E microscope. RAD51 was quantified in tumor areas by scoring the percentage of GMN-positive cells with five or more RAD51 nuclear foci. The mean of the scores obtained by the two observers was used. RAD51 scores were classified as “high” or “low” by applying a predefined cut-off of 10% (20,21). All samples included in the analysis fulfilled the QC criteria of having at least 40 GMN positive cells and more than 25% of γ H2AX/GMN positive tumor cells. Immunofluorescence images were acquired with a 60x objective using a Nikon DS-Qi2 digital camera and generated using NIS-Elements-AR (version 4.40) software.

Statistical considerations

All randomized patients (n=98) were considered for this analysis, regardless of the dose group (300 mg and 400 mg) or evaluability status. Time-to-event endpoints were summarized across different gene subgroups by Kaplan-Meier curves, and median times estimated with 95% confidence intervals. Local radiological response assessment was used for all radiological endpoints. For rPFS, patients alive and without radiological progression were censored at the last scheduled disease assessment on study, at time of treatment discontinuation (in case of clinical progression not leading to death), or at time of starting a new treatment for mCRPC. Patients alive at the end of follow-up were censored for the analysis of OS. Within each of the gene-subgroups, the proportion of homozygous deletions vs somatic mutations vs germline mutations were described, as well as the proportion of mutations with a demonstrated biallelic event vs mutations without confirmation of biallelic loss.

Response, time on treatment, rPFS and OS were estimated by type and origin of mutation, with time to event endpoints compared by log-rank tests. In the ATM gene subgroup, outcome of patients with ATM loss vs no loss as per IHC were also compared. The levels of RAD51 foci assessed by IF were graphically described by gene subgroup. The association of RAD51 score categories with outcomes was described graphically as above and analyzed by Chi-Square and log-rank tests, respectively.

Statistical analyses were conducted with the use of Stata software (version 15), on a snapshot of the data taken on September 21, 2020.

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TABLE 1. Responses to olaparib (composite trial endpoint and per each of the components) based on the origin and type of the qualifying genomic alterations.

	All patients, by type of response										Composite overall response by gene subgroup							
	Composite Overall response		RECIST 1.1 Objective Response		PSA fall ≥50%		CTC conversion		RECIST 1.1 or PSA response		BRCA1/2		PALB2		ATM		CDK12	
	resp/n	RR	resp/n	RR	resp/n	RR	resp/n	RR	resp/n	RR	resp/n	RR	resp/n	RR	resp/n	RR	resp/n	RR
All randomized patients	45/98	45.9	14/75	18.7	31/95	32.6	29/57	50.9	33/98	33.7	26/32	81.3	4/7	57.1	8/21	38.1	5/21	23.8
By origin/type alteration																		
Germline mutation	19/30	63.3	6/22	27.3	12/28	42.9	11/19	57.9	13/30	43.3	10/13	76.9	4/6	66.7	4/5	80	1/1	100
Somatic homozygous deletion	10/16	62.5	4/11	36.4	10/16	62.5	7/11	63.6	10/16	62.5	9/11	81.8	0/0	0	1/1	100	0	0
Somatic mutation	16/52	30.8	4/42	9.5	9/51	17.6	11/27	40.7	10/52	19.2	7/8	87.5	0/1	0	3/15	20	4/20	20
Based on the evidence of biallelic loss																		
Bi-allelic hit detected	33/64	51.6	10/47	21.3	24/64	37.5	22/39	56.4	25/64	39.1	19/24	79.2	4/4	100	5/12	41.7	4/18	22.2
Mono-allelic hit detected	12/34	35.3	4/28	14.3	7/31	22.6	7/18	38.9	8/34	23.5	7/8	87.5	0/3	0	3/9	33.3	1/3	33.3

Resp=number of responses, n=patients available, RR=response rate (%)

FIGURE LEGENDS

Figure 1: Consort diagram showing the sample disposition in this study.

Figure 2: Outcomes with olaparib in the BRCA1/2 and PALB2 cohorts. **A.** Kaplan-Meier curves showing rPFS and OS in BRCA1/2 cohort depicting homozygous deletions, germline and somatic mutations: Tumors with homozygous BRCA deletions have the best outcomes. **B.** Kaplan-Meier curves rPFS and OS in BRCA1/2 cohort depicting outcomes in homozygous deletions and mutated genes with or without a detectable second hit. **C.** Swimmer plots depicting time on treatment per origin/type alterations in BRCA1/2 patients. **D.** Swimmer plots depicting time on treatment per origin/type alterations in PALB2 patients.

Figure 3: Outcomes with olaparib in ATM-altered prostate cancer indicating that complete ATM loss associates with better outcome on PARP inhibition. **A.** Kaplan-Meier curves depicting rPFS and OS outcomes in the prostate cancer cohort treated with olaparib and germline and somatic mutations. **B.** Kaplan-Meier curves depicting rPFS and OS outcomes with olaparib treatment in prostate cancers with and without second detectable genomic hits on the ATM gene. **C.** Kaplan-Meier

curves depicting rPFS and OS outcomes in prostate cancer patients treated with olaparib in the ATM cohort with and without ATM loss by IHC. **D.** Swimmer plots depicting time on treatment per origin/type alterations in the ATM gene.

Figure 4: Genomic landscape of the TOPARP-B cohort. A. Oncoprint of the prostate cancer biopsies of the patients treated with olaparib on the TOPARP-B trial, separating those cases where a treatment-naïve vs castration-resistant biopsy was used in the trial for NGS. **B.** CNV Frequency plots of the advanced prostate cancers in the TOPARP-B patients and significant differences in the genomic copy number profile between responders and non-responders. **C.** Cooccurrence and mutually exclusive alterations plot for prostate cancer associated genes.

Figure 5: Loss of RAD51 as a functional marker of HRR deficiency in prostate cancer and PARPi sensitivity. A. Percentage of GMN-positive cells positive for RAD51 and γ H2AX foci per patient, sorted based on the pre-defined subgroups per genes of interest; 10% of GMN-positive cells positive for RAD51 foci was used as the threshold to classify samples as RAD51 low vs high. **B.** Kaplan-Meier curves depicting rPFS and OS depending on the RAD51 assay. **C.** Swimmer plots depicting time on treatment in patients with high (grey) vs low (blue) RAD51 scores.

Figure 1: Consort diagram of sample disposition

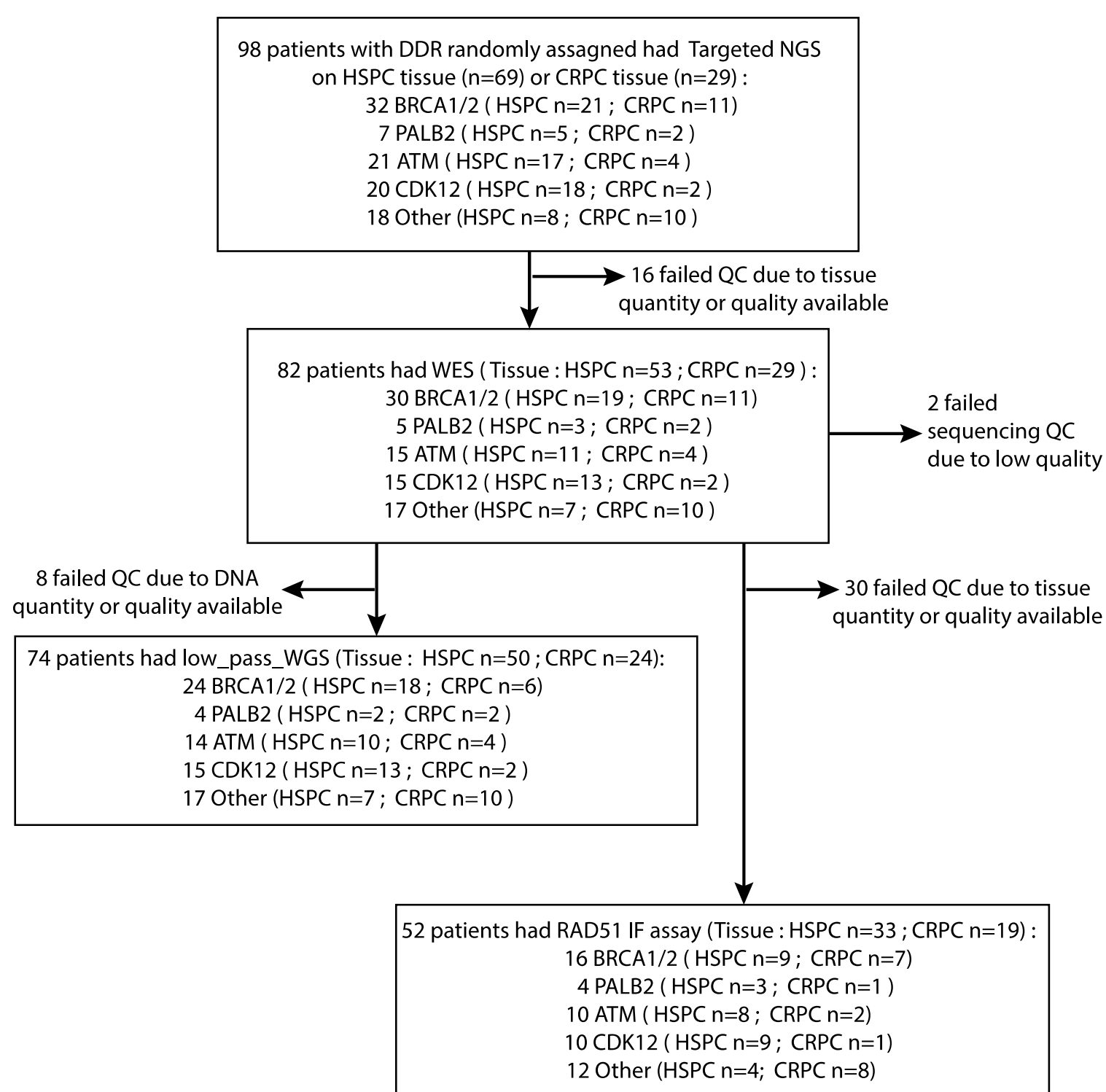
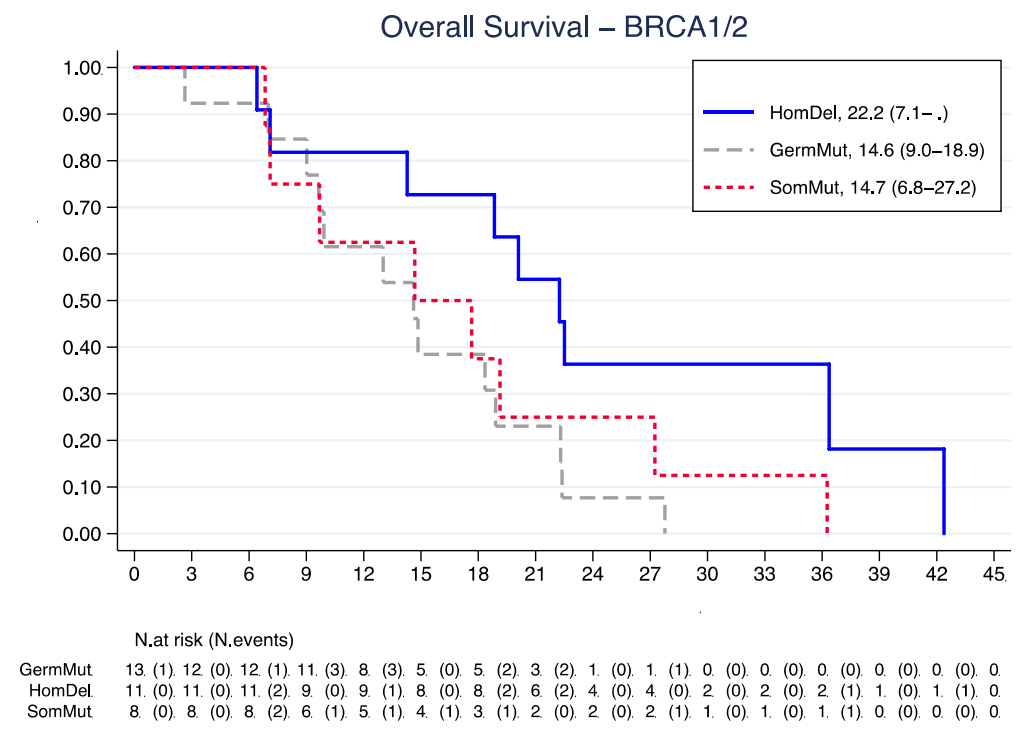
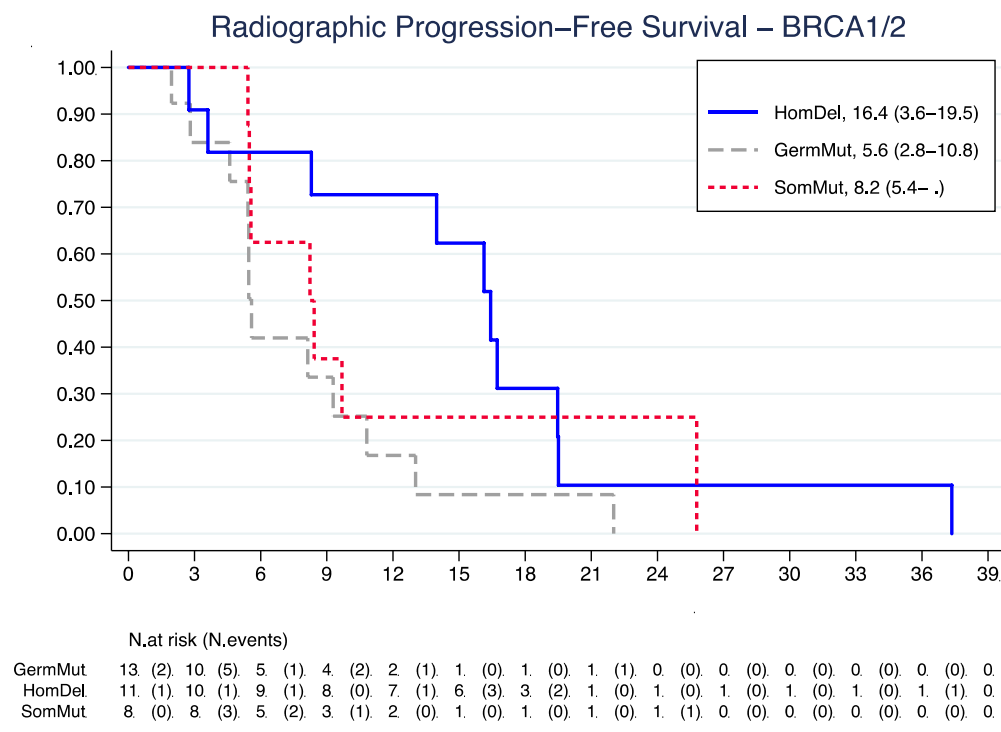
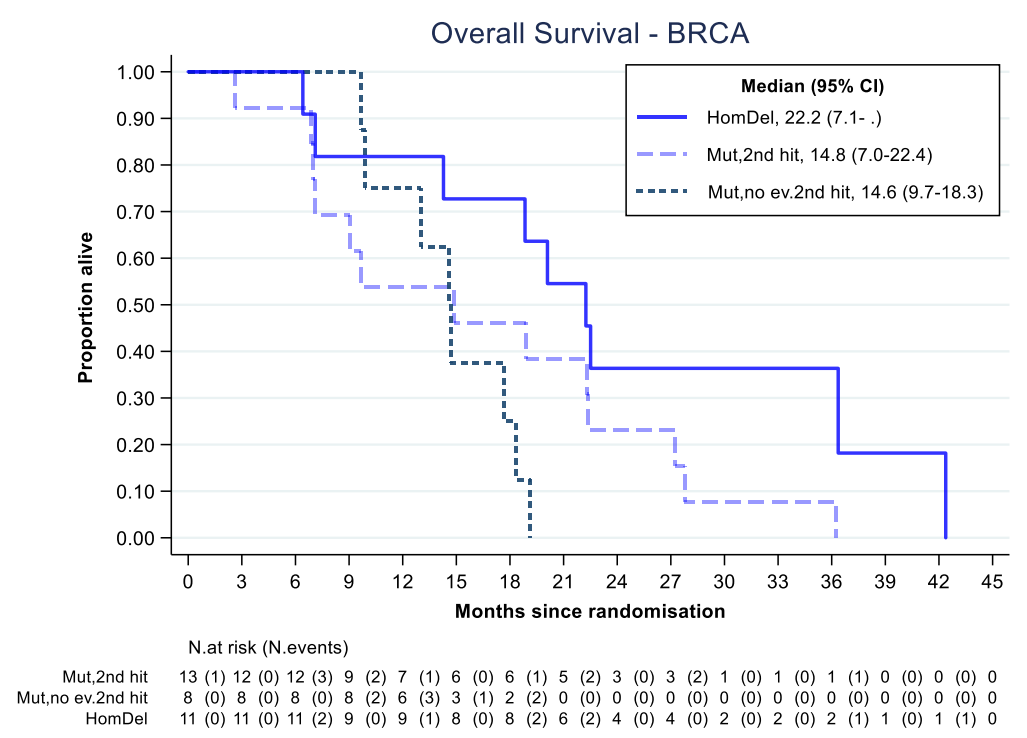
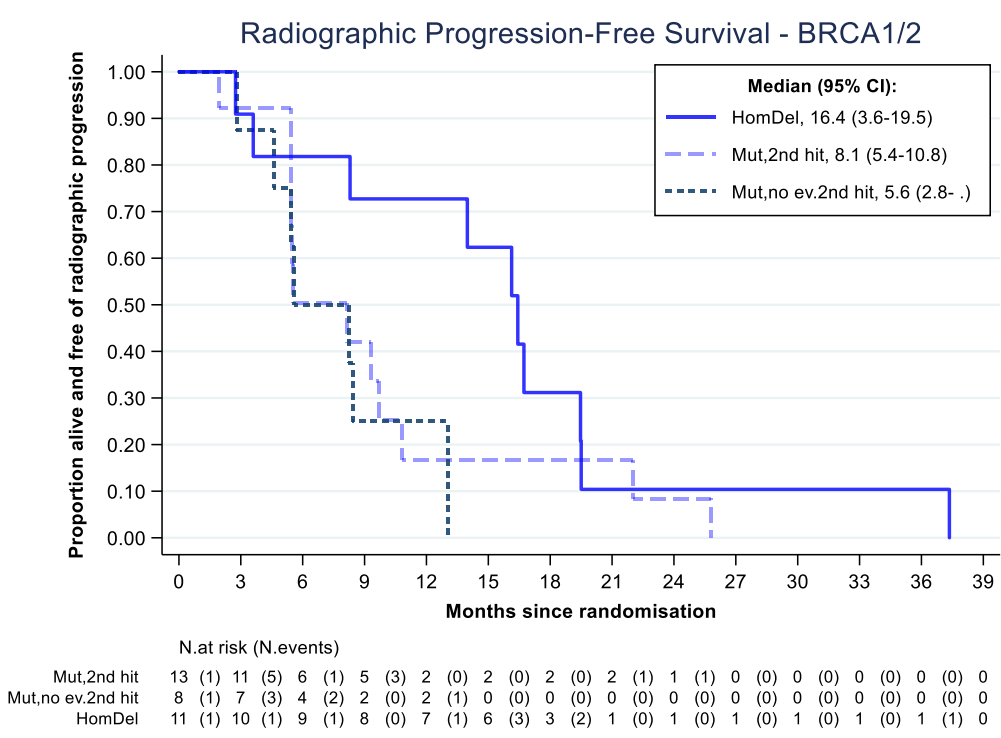


Figure 2 : Outcomes with olaparib in the BRCA1/2 and PALB2 cohorts

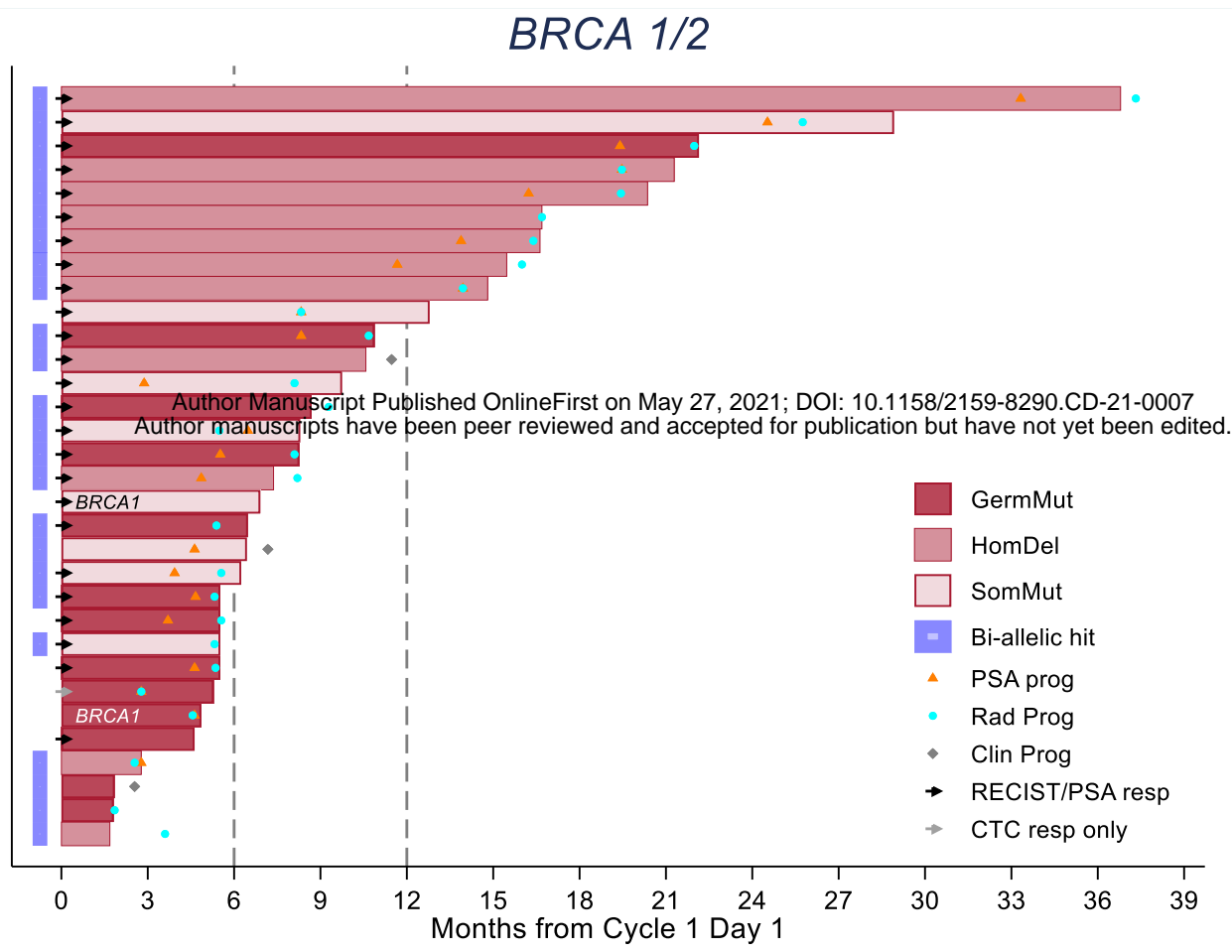
A : rPFS and OS in BRCA1/2 cohort depicting homozygous deletions, germline and somatic mutations: Tumours with homozygous BRCA deletions have the best outcomes



B : rPFS and OS in BRCA1/2 cohort depicting outcomes in homozygous deletions and mutated genes with or without a detectable second hit



C : Swimmer plots depicting time on treatment per origin/type of alterations in BRCA1/2 patients



D : Swimmer plots depicting time on treatment per origin/type of alterations in PALB2 patients

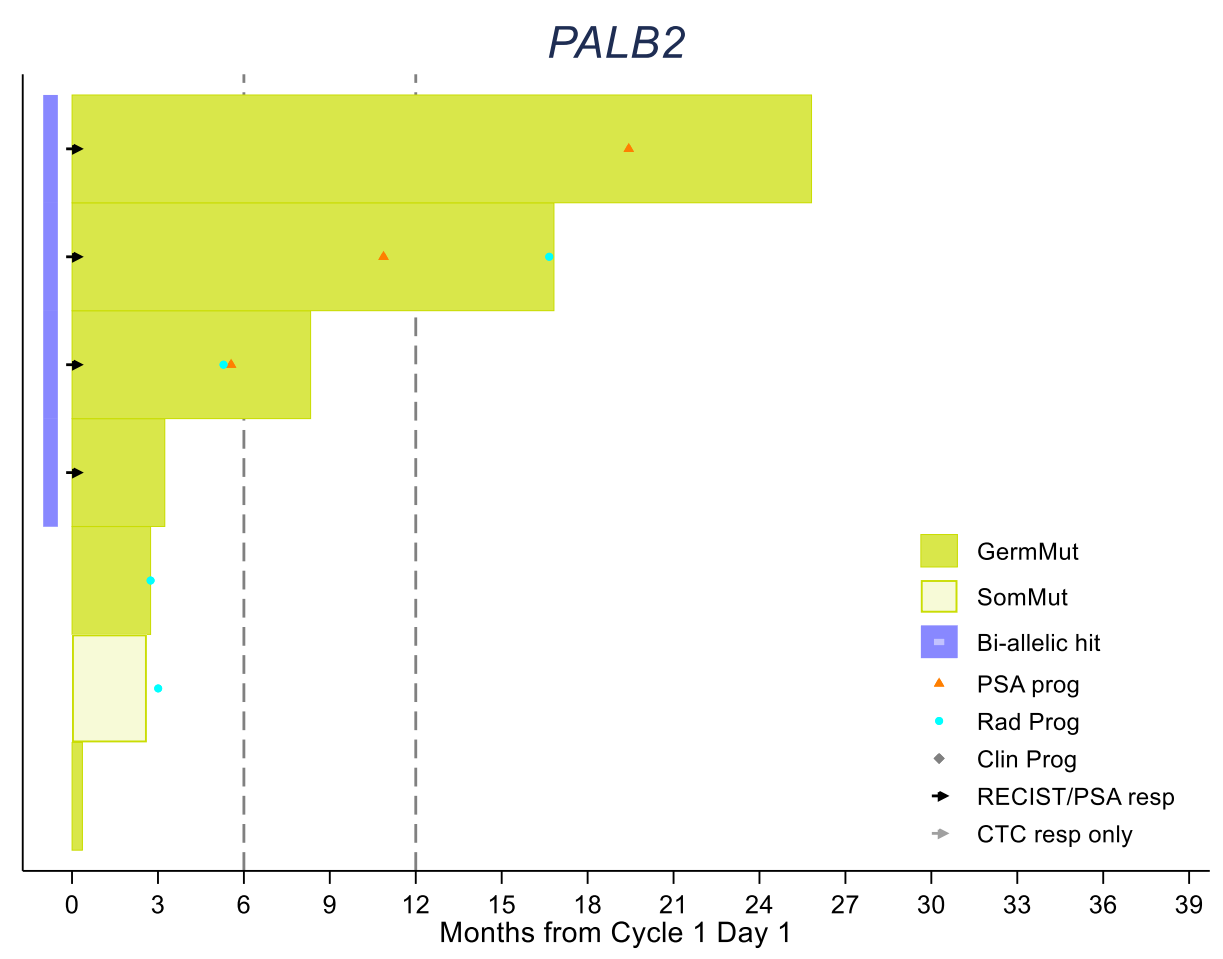
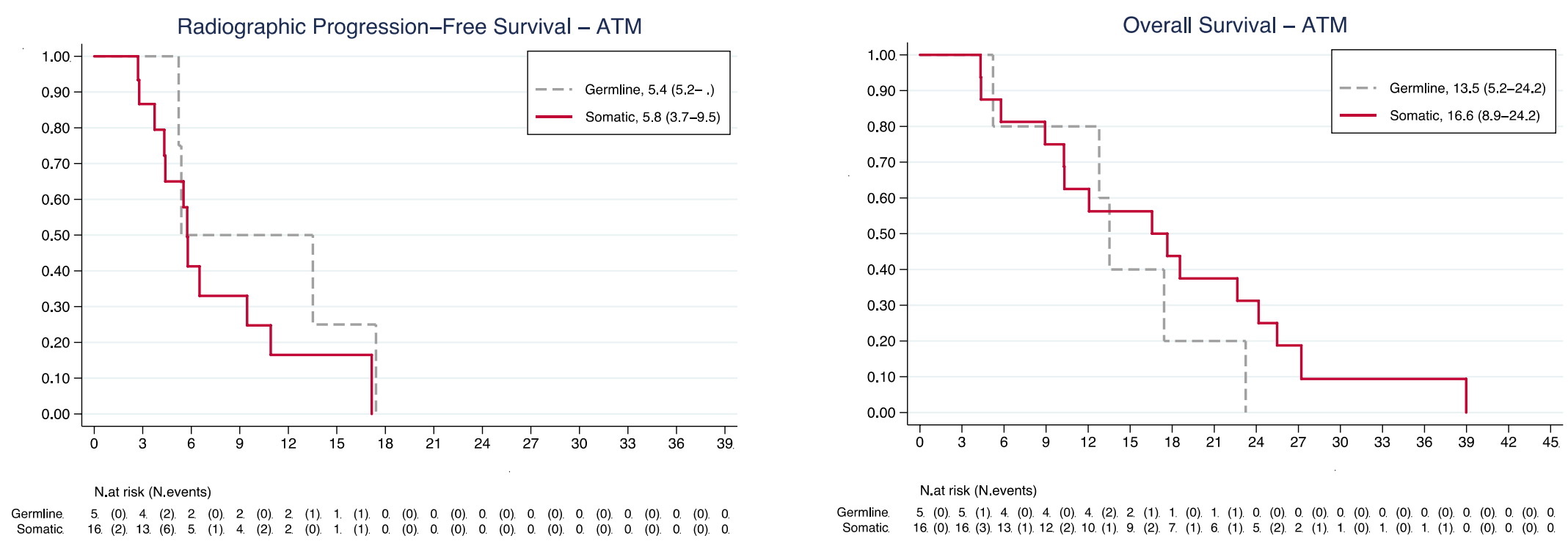
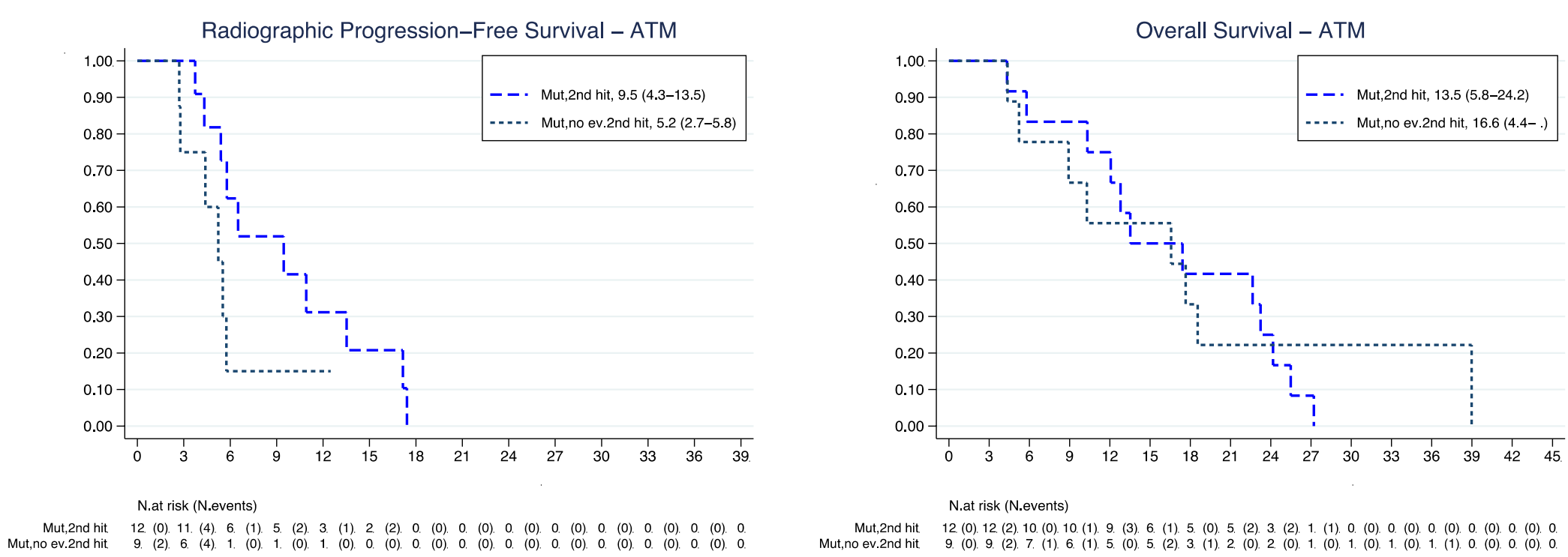


Figure 3: Outcomes with olaparib in ATM-altered prostate cancer indicating that complete ATM loss associates with better outcome on PARP inhibition.

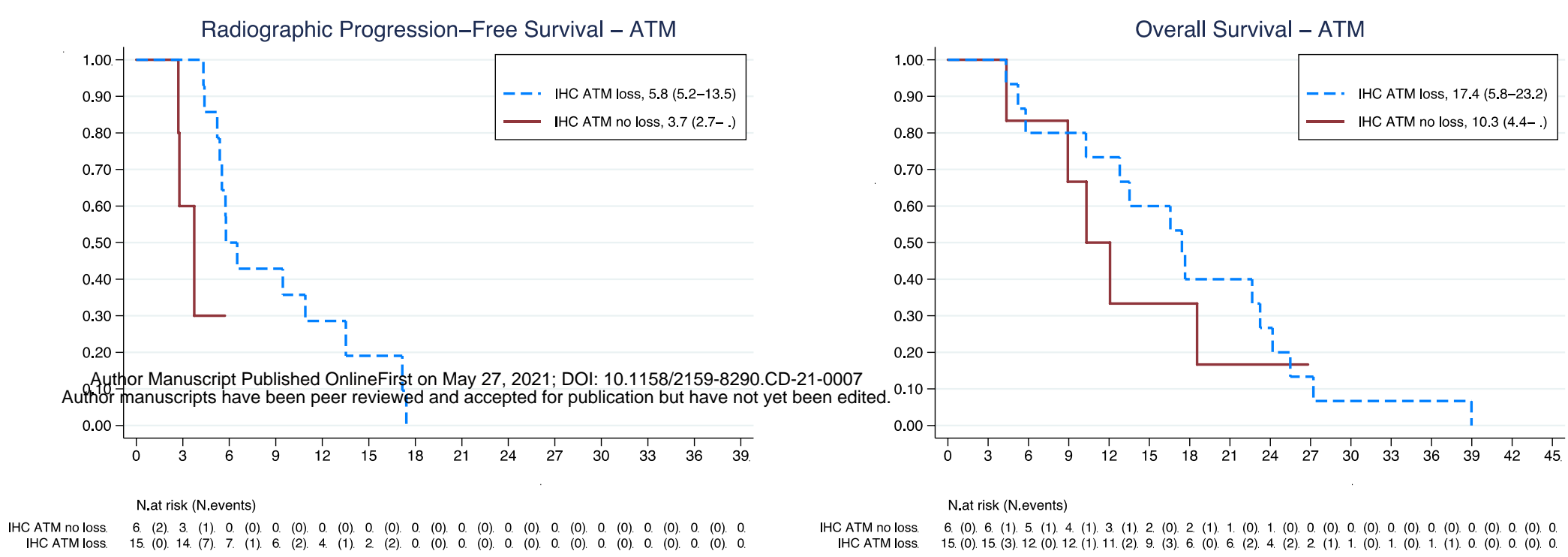
A : rPFS and OS outcomes in the prostate cancer hit cohort treated with olaparib and germline and somatic mutations.



B : rPFS and OS outcomes with olaparib treatment in prostate cancers with and without second detectable genomic hits on the ATM gene.



C : rPFS and OS outcomes in prostate cancer patients treated with olaparib in the ATM cohort with and without ATM loss by IHC.



D : Swimmer plots depicting time on treatment per origin/type of alterations in the ATM gene

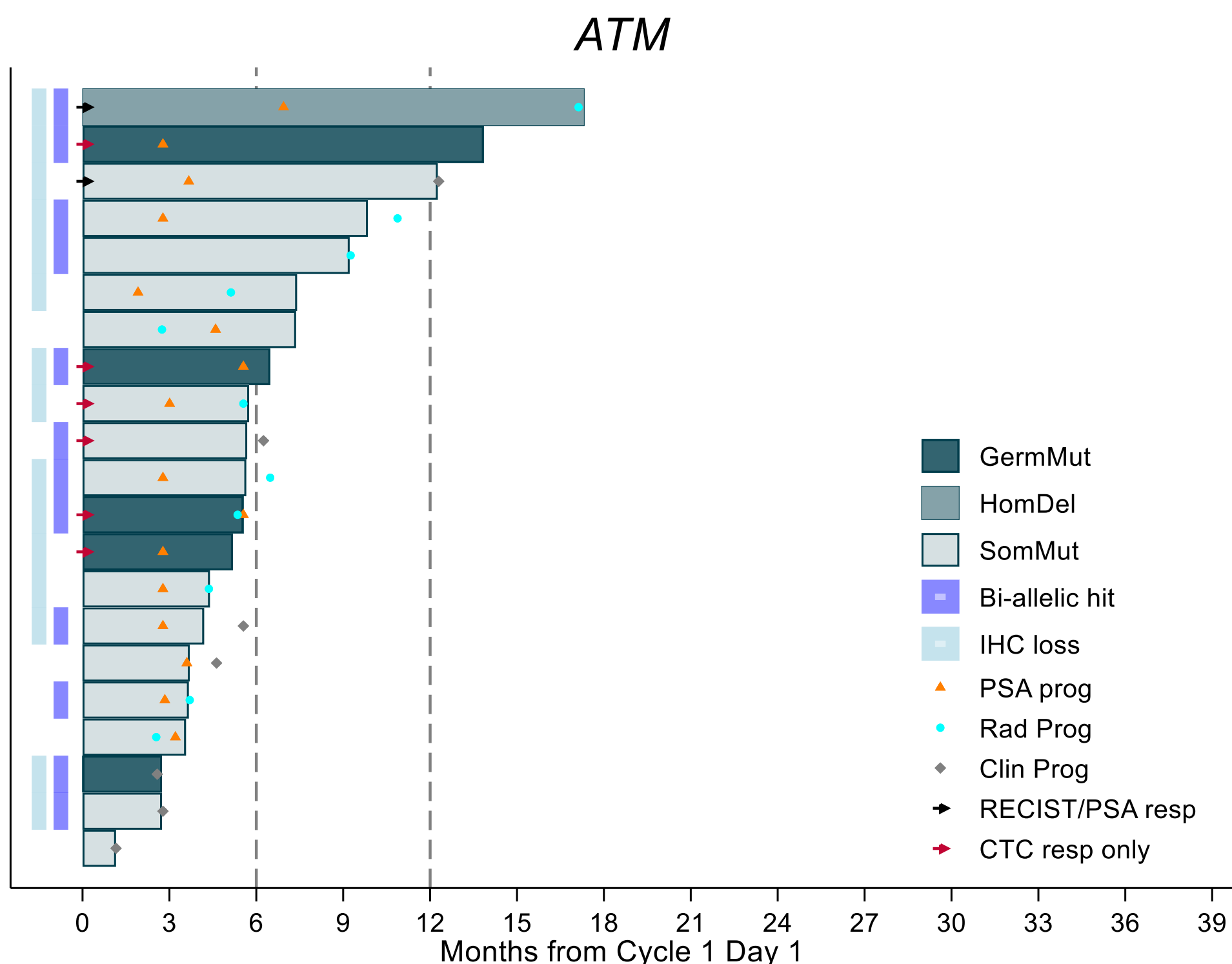
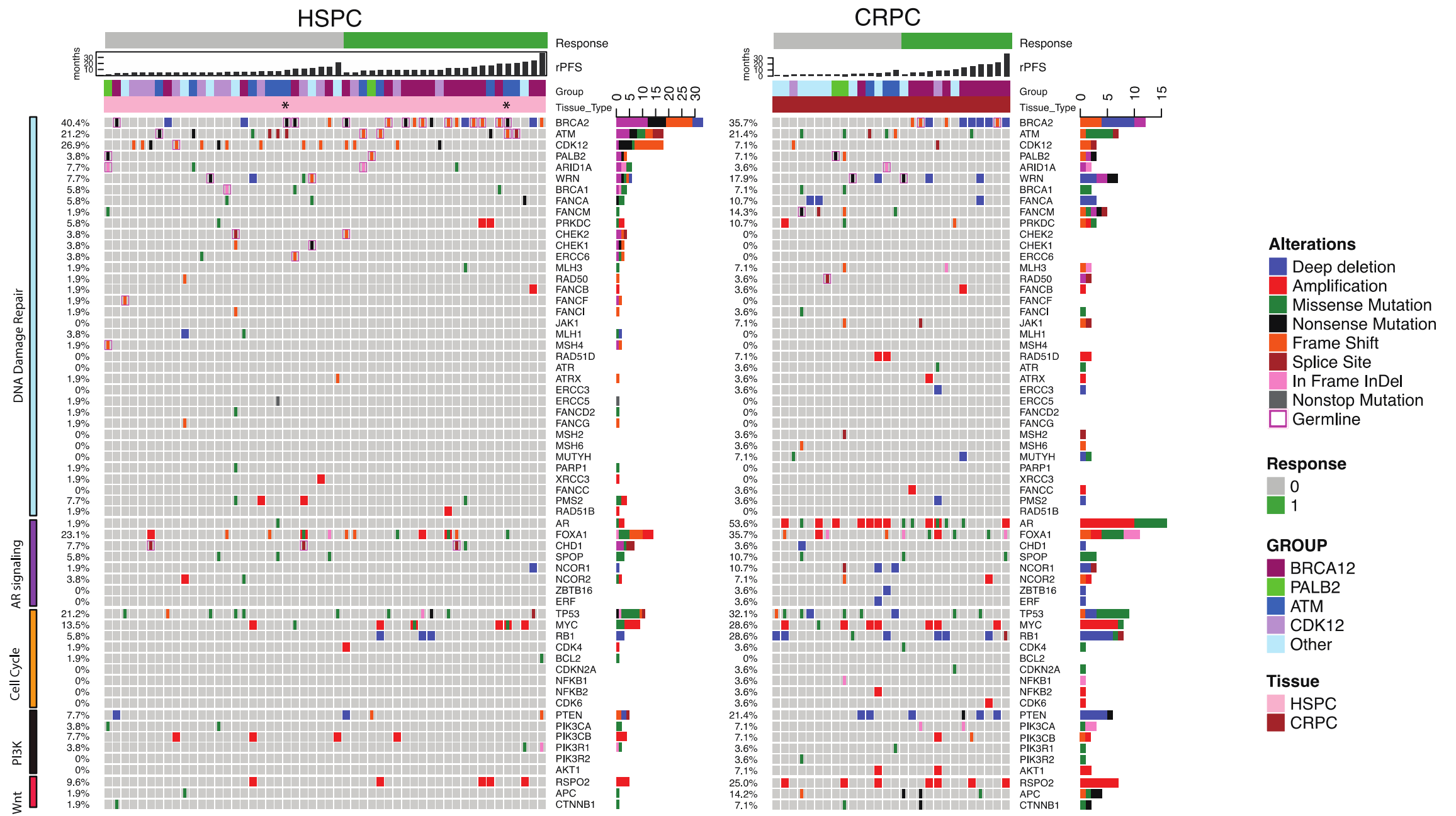
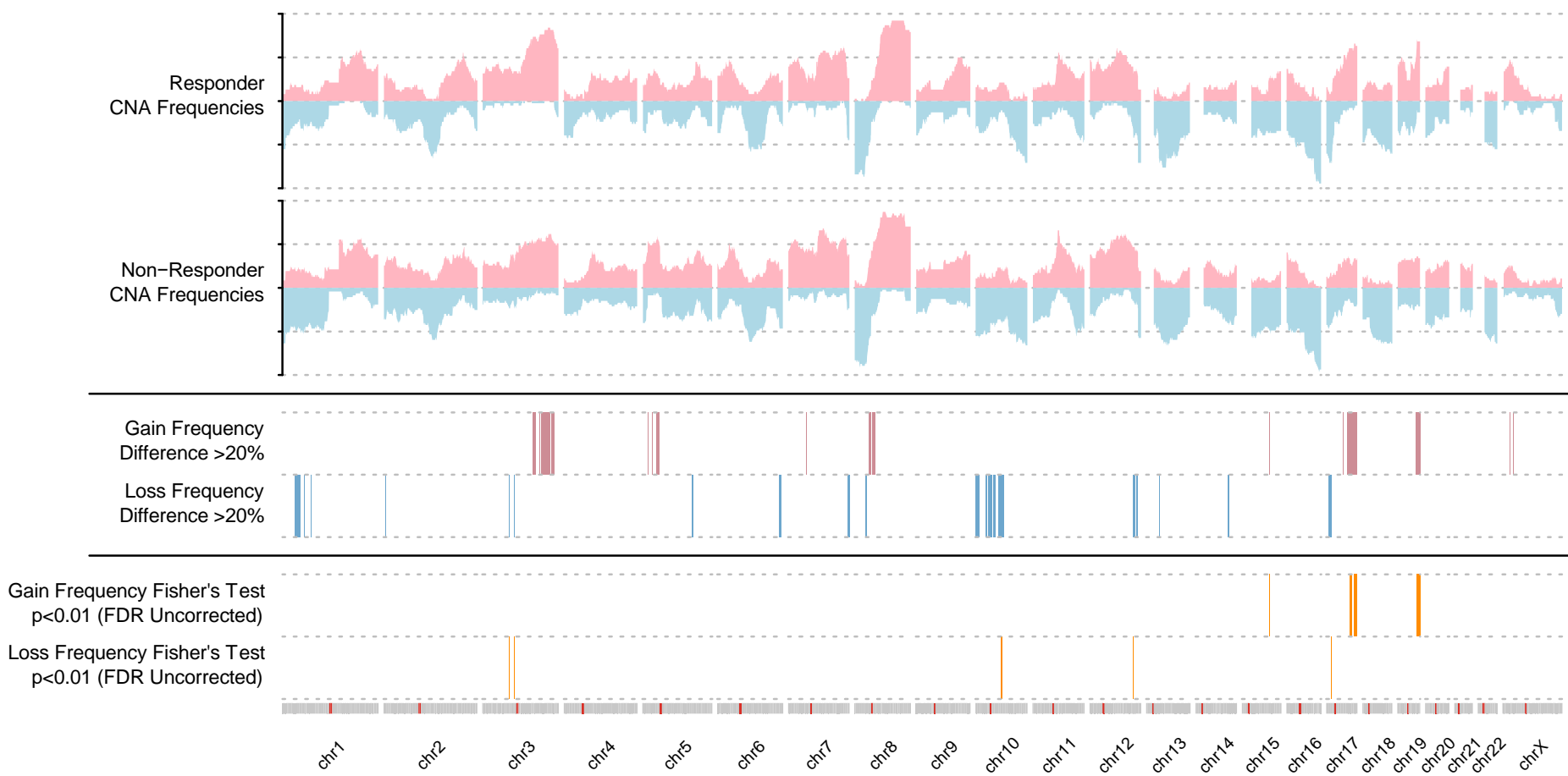


Figure 4 : Genomic landscape of the TOPARP-B cohort

A : Oncoprint of the prostate cancer biopsies of the patients treated with olaparib on TOPARP-B



B : CNV Frequency plots of the advanced prostate cancers in the TOPARP-B Responders vs Non Responders and significant genomic copy number differences between the two groups.



C : Co-occurrence and mutually exclusive alterations

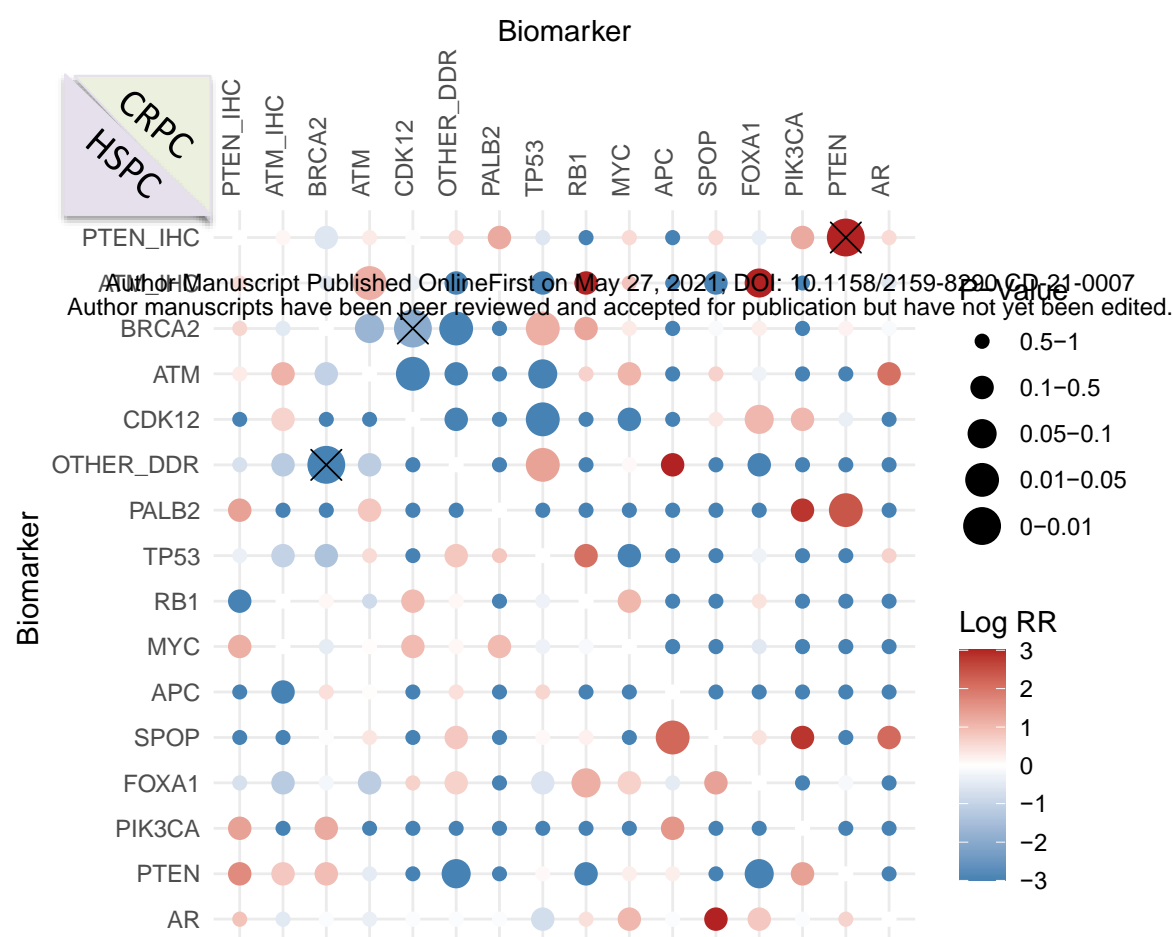
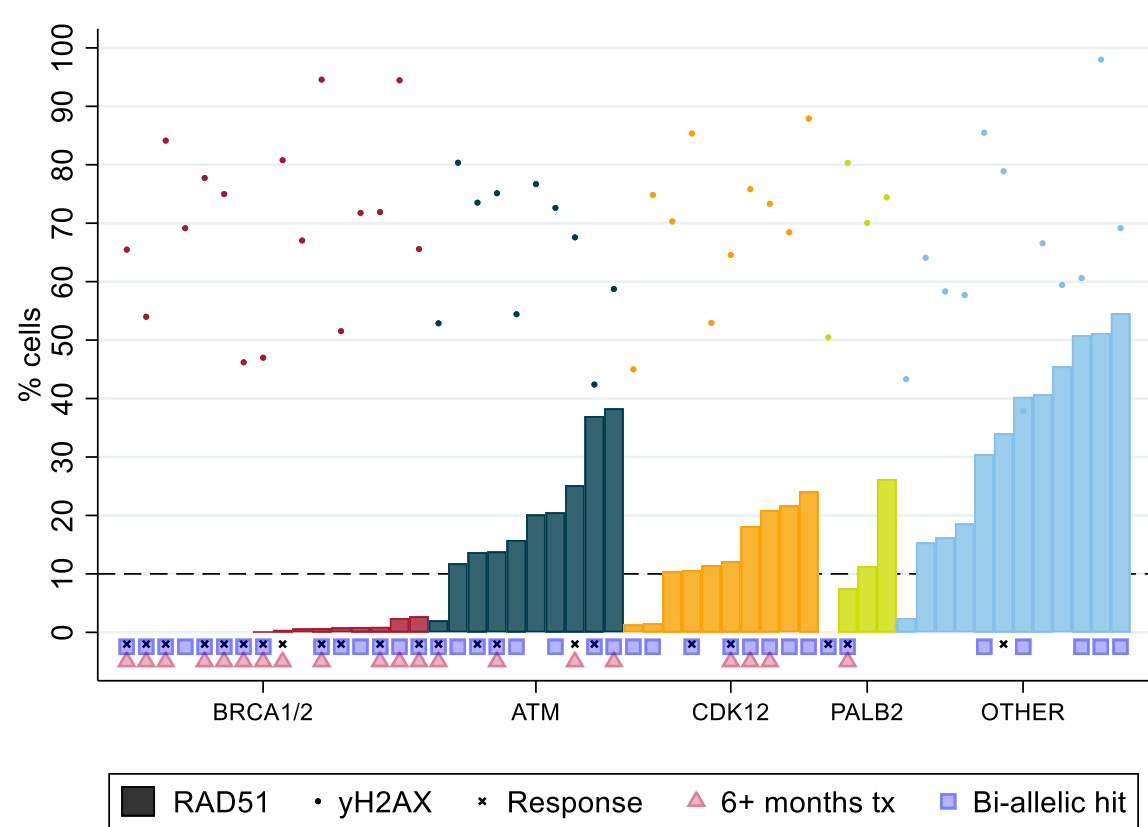
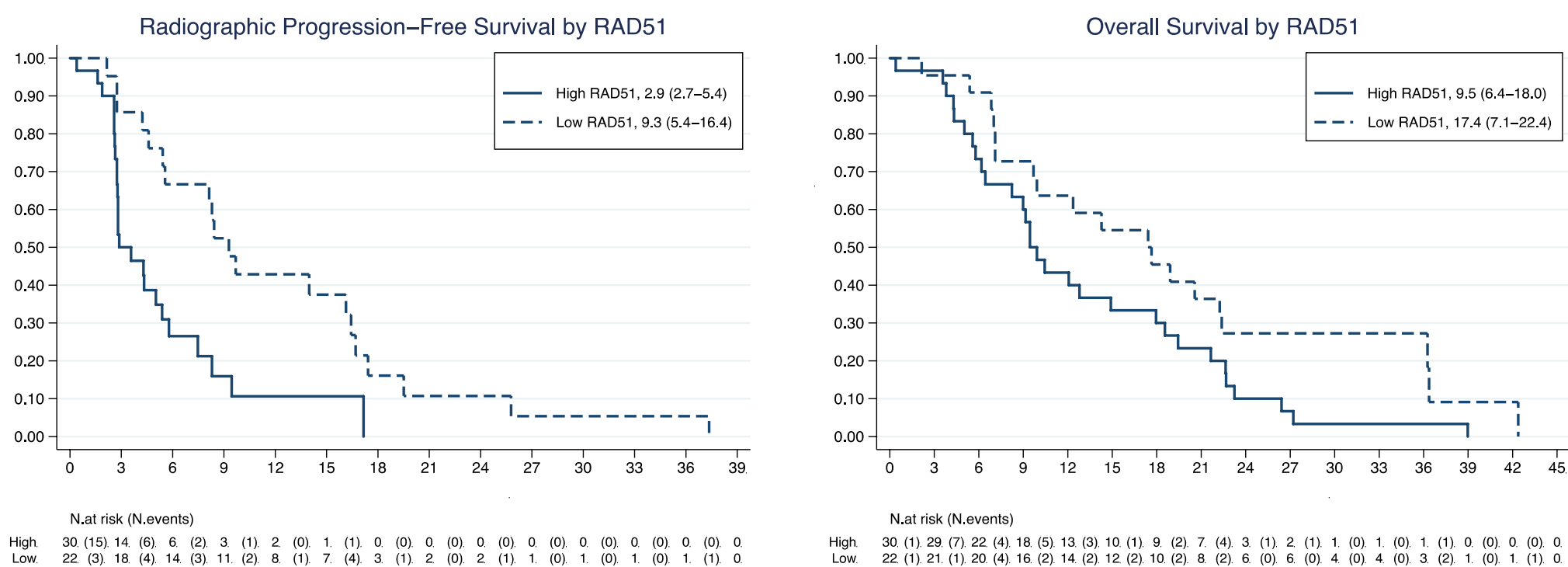


Figure 5 : Loss of RAD51 foci as a functional marker of HRR deficiency in prostate cancer and PARPi sensitivity

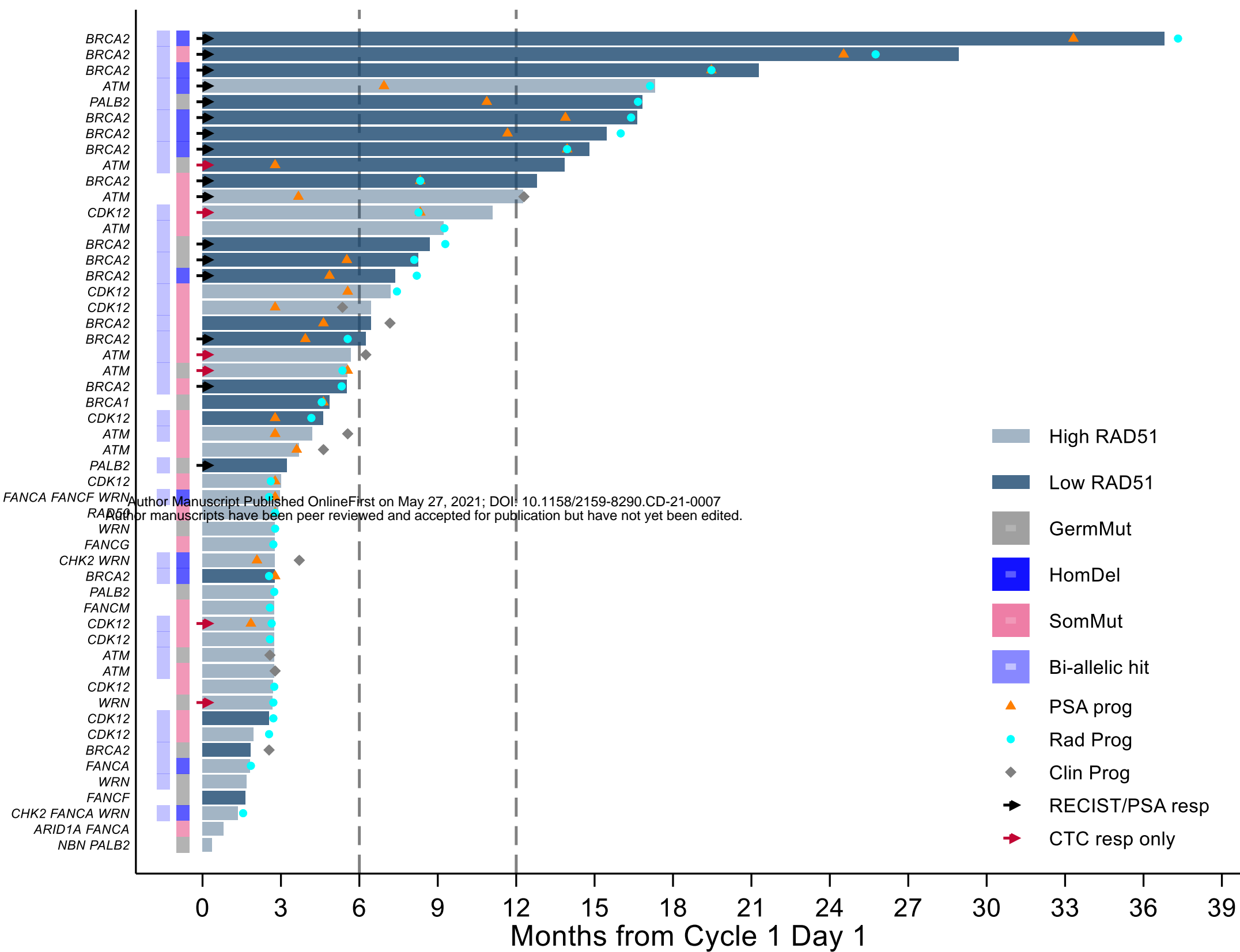
A. RAD51 and γ H2AX assay positivity in TOPARP-B DDR groups



B. rPFS and OS outcomes in prostate cancer patients depending on their RAD51 assay positivity.



C. Swimmer plots depicting time on treatment with olaparib per RAD51 assay positivity



CANCER DISCOVERY

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