TP53 outperforms other androgen receptor biomarkers to predict abiraterone or enzalutamide outcome in metastatic castration-resistant prostate cancer

Running title: Comprehensive AR and TP53 profiling in mCRPC liquid biopsies

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Declaration of interest

BDL, SVL, and LD have a patent “WO2017207702: ANDROGEN RECEPTOR SPLICE VARIANTS AND ANDROGEN DEPRIVATION THERAPY” pending. SS has a patent “WO2016133387: USE OF CABAZITAXEL IN THE TREATMENT OF PROSTATE CANCER” pending. All other authors declare no potential conflicts of interest.

Statement of translational relevance

Although single *AR* biomarkers and *TP53* gene perturbations have shown to be of prognostic value, no large-scale studies have simultaneously investigated multiple *AR* and *TP53* biomarkers. Synchronous profiling of all outcome-associated somatic alterations in *AR* and *TP53* in liquid biopsies of mCRPC patients (n=168) prior to abiraterone and enzalutamide treatment demonstrates that *TP53*, but not *AR,* is an independently-associated negative response biomarker. We present and validate a three-stratum risk stratification system using clinical variables and *TP53* alterations to assist treatment decisions in mCRPC. Hence, efficient prognostication of mCRPC patients, before starting abiraterone or enzalutamide treatment, is achievable by combining *TP53* liquid biopsy profiling and clinical variables. Further comprehensive *AR* profiling studies are required to determine which patients have a relevant *AR* biomarker output.

Abstract

Purpose: To infer the prognostic value of simultaneous androgen receptor (*AR*) and *TP53* profiling in liquid biopsies from metastatic castration-resistant prostate cancer (mCRPC) patients starting a new line of *AR* signalling inhibitors (ARSi).

Experimental design: Between March 2014 and April 2017, we recruited mCRPC patients (n=168) prior to ARSi in a cohort study encompassing 10 European centres. Blood samples were collected for comprehensive profiling of CellSearch-enriched circulating tumour cells (CTCs) and circulating tumour DNA (ctDNA). Targeted CTC RNA-seq allowed the detection of eight *AR* splice variants (ARVs). Low-pass whole-genome and targeted gene-body sequencing of *AR* and *TP53* was applied to identify amplifications, loss-of-heterozygosity, mutations and structural rearrangements in ctDNA. Clinical or radiological progression-free survival (PFS) was estimated by Kaplan-Meier analysis, and independent associations were determined using multivariable Cox-regression models.

Results: Overall, no single *AR* perturbation remained associated with adverse prognosis after multivariable analysis. Instead, tumour burden estimates (CTC counts, ctDNA fraction, and visceral metastases) were significantly associated with PFS. *TP53* inactivation harbored independent prognostic value (HR 1.88, 95%CI 1.18-3.00, p = 0.008), and outperformed ARV expression and detection of genomic *AR* alterations. Using Cox coefficient analysis of clinical parameters and *TP53* status, we identified three prognostic groups with differing PFS estimates (median, 14.7 vs 7.51 vs 2.62 months, p < 0.0001), which was validated in an independent mCRPC cohort (n=202) starting first-line ARSi (median, 14.3 vs 6.39 vs 2.23 months, p < 0.0001).

Conclusions: In an all-comer cohort, tumour burden estimates and *TP53* outperform any *AR* perturbation to infer prognosis.

Key words

mCRPC, circulating tumour cells, cfDNA, androgen receptor, abiraterone, enzalutamide, AR, TP53

Introduction

The androgen receptor (*AR*) remains the central target in the treatment of metastatic prostate cancer (mPC), which eventually develops lethal castration-resistance (mCRPC), for which current standard-of-care therapies lack prognostic biomarkers. Although second-generation *AR* signalling inhibitors (ARSi) are effective in both chemotherapy-naïve and -pretreated mCRPC, *a priori* resistance is observed in up to 40% of patients (1). Genomic analyses revealed pivotal roles for *AR*, phosphoinositide-3-kinase (PI3K), DNA-repair and cell cycle pathways in mPC (2). *AR* alterations encompass copy numbers variants (CNVs), mutations and the expression of *AR* splice variants (ARVs), which are associated with poor outcome on ARSi treatment (3-6). Additionally, intra-*AR* genomic structural rearrangements (GSRs) have been described in (pre-)clinical mCRPC samples (7-9). DNA-repair or PI3K pathway aberrations have been proposed as ARSi biomarkers, but the results are currently discordant (10-13). However, *TP53* inactivation has consistently been associated with poor prognosis (11,12,14). To date, information on the simultaneous detection of multiple *AR* perturbations and other genomic events, and their association with outcome is lacking (9). Here, we investigated the prognostic value of a combined *AR*- and *TP53*-focussed circulating tumor cell (CTC) and circulating tumor DNA (ctDNA) liquid biopsy to identify prognostic biomarkers for ARSi.

Methods

A detailed description of materials and methods is provided in Supplementary Materials and Methods. In brief, we recruited mCRPC patients with histologically confirmed prostate adenocarcinoma, starting a new line of second-generation ARSi, i.e. abiraterone or enzalutamide, for biochemically-defined progressive disease (PD) according to EAU guidelines (1). At baseline, 10-12 weeks follow-up and PD, a blood sample was collected for CellSearch CTC enumeration, CTC-ARV targeted RNA-seq and low-pass whole genome and targeted sequencing of plasma cell-free DNA (cfDNA) for *AR* and *TP53* to infer amplifications, loss-of-heterozygosity, mutations and structural rearrangements, as previously described (9). Treating physicians were blinded to the CTC/ctDNA results during clinical practice. Primary outcome measure was progression-free survival (PFS), according to Prostate Cancer Clinical Trials Working Group 3 criteria (15). Secondary outcomes encompassed PSA waterfall plots and confirmed ≥50%PSA response rates at 10-12 weeks (16), and overall survival (OS). The association between somatic variations and time-to-event outcomes were evaluated by Kaplan-Meier (KM) analysis with logrank test and assessment of effect by uni-(UV-Cox) and multivariable Cox (MV-Cox) regression models, including the following covariates: PSA level, CTC count and ctDNA fraction at baseline,

prior chemotherapy, prior exposure to abiraterone or enzalutamide, and presence of visceral metastases (5,17,18). Co-occurrence was tested using Chi-squared or Fisher's Exact tests. Correlations and comparisons by Pearson's, Spearman's and Mann-Whitney tests, respectively. Statistical analysis was performed in R (v3.3.2), with two-sided p values < 0.05 considered as statistically significant.

Results

Patient cohort and sample collection

Between March 2014 and April 2017, 168 mCRPC patients were recruited, starting ARSi (Supplementary Figure 1A; Table 1). In total, 148/168 (88.1%) patients had not received prior ARSi for CRPC. We profiled 249 CTC and 252 cfDNA samples, with a baseline ARV and *AR/TP53* gene profile in 131 and 145 evaluable samples, respectively, and matching datasets in 108 cases (Supplementary Figure 1B). The median PFS in the studied cohort was 6.8 months (IQR: 3.4-13.2), with 129/168 (76.8%) patients progressed at the time of analysis. The median follow-up time was 12.4 months (IQR: 7-17.3), with 65/168 (38.7%) patients deceased at the time of analysis.

CTC and ctDNA profiling

CTC-ARV sequencing at baseline (n=131), follow-up (n=61) and PD (n=57) demonstrated dominance of the full-length *AR* isoform, with ARV fractions ranging from 0.5±1.6%, 0.06±0.1% to 1.6±4.9%, respectively (Supplementary Figure 2A). ARV expression demonstrated inter- and intra-patient heterogeneity and was more prevalent in samples harvested at the time of PD. At baseline, ARVs were frequently co-expressed with AR-V3 (53/131, 40.4%) and AR-V7 (34/131, 25.9%) being the most prevalent constitutively active ARVs (Supplementary Table 1). AR45 and AR-V3 were most abundantly expressed (Supplementary Figure 2B&C and Supplementary Figure 3).

Plasma *AR* sequencing revealed genomic alterations in 63/145 (43.4%), 14/45 (31.1%) and 33/62 (53.2%) patients at baseline, follow-up and PD, respectively (Supplementary Figure 4A). *AR* was amplified in 54/145 (37.2%), 9/45 (20%) and 26/62 (41.9%) patients at baseline, follow-up and progression, respectively. Hotspot mutations were detected in 13/145 (8.9%), 3/45 (6.7%) and 7/62 (11.3%) patients at baseline, follow-up and PD, respectively, with p.L702H and p.H875Y as most frequently detected. Tiled *AR* sequencing revealed GSRs in

26/145 (17.9%), 7/45 (15.6%) and 16/62 (25.8%) patients at baseline, follow-up and PD, respectively. Excluding structural variants of unknown significance (SVUS) and focussing on rearrangements affecting coding or cryptic exon (CE) regions, an increased prevalence was observed at the time of PD compared to baseline (12/62 (19.4%) vs 12/145 (8.3%) patients, χ^2 test: $p = 0.04$). Also, the number of events in GSR-positive patients increased at progression (Mann-Whitney U test: $p = 0.014$), accompanied with more rearrangement complexity (Supplementary Figure 4B&C). GSRs typically co-occurred with *AR* amplifications, with 43/49 (87.8%) GSR-positive samples having gained copy numbers (χ^2 test: $p < 0.0001$).

Plasma *TP53* sequencing revealed genomic alterations in 36/145 (24.8%), 12/45 (26.7%) and 27/62 (42.9%) patients at baseline, follow-up and PD, respectively, with bi-allelic inactivation in 24/36 (66.7%), 6/12 (50.0%) and 17/26 (65.4%) of *TP53*-perturbed patients, respectively.

Integrating ARV data with genomic alterations in the *AR* gene

Comprehensive CTC and ctDNA profiles were available for 108, 31 and 49 patients at baseline, follow-up and PD, respectively (Figure 1). Of note, we observed that CTC-negative enumeration samples were occasionally positive for ctDNA and/or ARV expression in their temporally-matched plasma and/or blood samples, respectively (Supplementary Figure 5). For *AR*, when combining CNVs, GSRs, mutations and ARVs (excluding AR-V1/2, which were expressed in nearly all patients), we detected perturbations in 77/108 (71.3%), 23/31 (74.2%) and 48/49 (97.9%) patients at baseline, follow-up and PD, respectively. ARV expression (excluding AR-V1/2) occurred in patients with and without *AR* amplifications, which at baseline suggested a higher prevalence in *AR*-amplified disease (65.9% vs 45.3%, χ^2 test: $p = 0.05$). However, ARV abundance was higher in *AR*-amplified ($p = 0.027$) or -rearranged ($p = 0.002$) samples obtained at PD. Interestingly, when focusing on exon1-deleting GSRs (i.e. ARv45), we observed increased expression levels of the exon 1b-2 junction, corresponding to the AR45 isoform (Mann-Whitney U test: $p = 0.002$) (Supplementary Figure 6).

CTC-ARV profiling and clinical outcome

A shorter PFS was observed in patients expressing AR45, AR-V3, AR-V4, AR-V5 and AR-V7 at baseline (all $p < 0.05$) (Supplementary Figure 7). However, in MV-Cox analysis, the individual ARVs were not prognostic, whereas CTC count and prior chemotherapy exposure were independently associated with poor outcome

(Supplementary Table 2). Logrank testing identified a a shorter OS in patients expressing AR45, AR-V3, AR-V5, AR-V7 and AR-V9 (all $p < 0.01$) (Supplementary Figure 7).

When combining PFS-associated ARVs from univariable analysis, we observed that 69/131 (52.6%) patients were expressing at least one of these ARVs, demonstrating a shorter PFS (median, 4.00 vs 11.0 months, $p = 0.00014$) (Figure 2A). However, in MV-Cox analysis, combined ARV expression was not prognostic, and only CTC counts were independently associated with poor outcome (hazard ratio (HR) 1.33, 95% confidence interval (CI) 1.14-1.55, $p < 0.001$) (Supplementary Table 2). For 116/131 (88.5%) cases PSA follow-up data at 10-12 weeks (or before in case of early PD) were available (Supplementary Figure 8) which demonstrated fewer confirmed ≥50% PSA responses in ARV-expressing patients (20% vs 48%, χ^2 test: $p = 0.006$) (Figure 2A).

Plasma-*AR* genomic profiling and clinical outcome

AR-amplified patients had a shorter PFS compared to patients who were copy-number neutral (median, 3.9 vs 9.5 months, $p < 0.0001$). Patients with intra-*AR* GSRs (with or without SVUS) had a shorter PFS compared to patients with a wild-type *AR* (median, 3.6 vs 7.8 months, $p < 0.001$) (Supplementary Figure 9). No association between *AR* mutations and outcome was observed (Supplementary Table 2). For 132/145 (91%) cases PSA follow-up data were available, which demonstrated no association between genomic alterations and PSA response at 10-12 weeks (Supplementary Figure 9). In MV-Cox analysis, *AR* amplification and GSRs lost significance, whereas the ctDNA fraction, baseline PSA level and presence of visceral metastases were independently associated with poor outcome (Supplementary Table 2). Logrank testing identified a shorter OS in *AR*-amplified (median, 11.2 vs 29.0 months, $p < 0.0001$) and GSR-positive patients, regardless if SVUS were in- or excluded (median, 7.7 vs 26.7 or 7.3 vs 25.6 months, both $p < 0.001$) (Supplementary Figure 9). The twelve patients harbouring GSRs within coding or CE regions (of whom 11/12 (91.7%) patients were *AR*-amplified) represented a unique subpopulation with worse PFS (median, 3.3 vs 4.8 vs 10.0 months, $p < 0.0001$) and OS (median, 7.3 vs 11.2 vs 29.7 months, $p < 0.0001$), compared to GSR-negative/*AR*-amplified and wild-type patients (Supplementary Figure 10).

When combining PFS-associated genomic AR alterations from univariable analysis, we observed that 55/145 (37.9%) patients had a shorter PFS (median, 3.9 vs 10.0 months, $p < 0.0001$) (Figure 2B). In MV-Cox analysis, the combined plasma-*AR* status lost significance, whereas ctDNA fraction (HR 1.02, 95%CI 1.01-1.04, p <

0.0001), baseline PSA levels (HR 1.12, 95%CI 1.00-1.26, p = 0.047) and presence of visceral metastases (HR 1.82, 95%CI 1.11-3.00, p = 0.02) remained independently associated with poor outcome (Supplementary Table 2). No associations between the combined plasma-*AR* status and PSA response were observed (Figure 2B).

Plasma *TP53* genomic profiling and clinical outcome

Patients with a *TP53* perturbation had a shorter PFS compared to patients who were wild-type (median, 3.0 vs 8.7 months, p < 0.0001) (Figure 2C). The poorest PFS was observed for patients harbouring a bi-allelic inactivation, compared to patients with a mono-allelic perturbation or wild-type genotype (median, 2.7 vs 5.3 vs 8.7 months, p < 0.0001). However, the PFS difference between mono- and bi-allelic inactivation was not significant (p = 0.4) (Supplementary Figure 11A). PSA follow-up data at 10-12 weeks demonstrated fewer confirmed ≥50% PSA responses in *TP53*-perturbed patients (15.4% vs 46.8%, χ^2 test: p = 0.008) (Figure 2C). In MV-Cox analysis, a perturbed *TP53* status was independently associated with poor outcome (HR 1.88, 95%CI 1.18-3.00, p = 0.008), together with ctDNA fraction (HR 1.02, 95%CI 1.01-1.03, p = 0.0005) and presence of visceral metastases (HR 1.72, 95%CI 1.05-2.84, p = 0.032) (Supplementary Table 2). Logrank testing identified a shorter OS in *TP53*-perturbed disease (median, 7.8 vs 26.7 months, p < 0.0001) (Supplementary Figure 11B).

Benchmarking outcomes of ARV, genomic *AR*, *and TP53* profiling

In the light of previously-published data (3,5,18,19), we were surprised by our findings of lack of association between ARV expression, combined plasma *AR*-status, and outcome in our MV-Cox analysis. Even considering different AR-V7 expression level thresholds for positivity failed to identify independent associations with outcome (Supplementary Figure 12). We tested the associative power of *TP53* alterations against *AR*-derived biomarkers in a MV-Cox analysis, by including ARV, *AR* and *TP53* genomic data (Supplementary Figure 13A). Perturbed *TP53* status was the only molecular biomarker independently associated with poor outcome (HR 1.97, 95%CI 1.14-3.40, p = 0.015), together with baseline PSA levels (HR 1.24, 95%CI 1.07-1.44, p = 0.005) and presence of visceral metastases (HR 2.11, 95%CI 1.21-3.66, p = 0.008). Even against the well-established *AR* amplification and AR-V7 biomarkers, *TP53* remained independently associated with poor outcome (HR 1.89, 95%CI 1.08-3.32, p = 0.026) (Supplementary Figure 13B).

Inferring prognosis using clinical features and a *TP53*-driven liquid biopsy

To facilitate prognostication of patients initiating ARSi, we developed a scoring algorithm using the *TP53* MV-Cox regression coefficients (Supplementary Table 2; Figure 3A). We generated a PFS-score by summation of the individual variables multiplied by their corresponding Cox regression coefficient (Figure 3B). Quartile index stratification of the PFS-scores (<Q1, Q1-Q3 and ≥Q3) identified three prognostic groups (good, intermediate and poor) with different KM PFS estimates (median, 14.7 vs 7.51 vs 2.62 months, $p < 0.0001$). Next, we validated the developed classifier in an independent cohort of 201 evaluable treatment-naïve mCRPC patients, initiating abiraterone or enzalutamide (14). Stratification based on the PFS-score quartiles partitioned the independent cohort into three prognostic groups with 81 (40.3%), 89 (44.3%) and 31 (15.4%) patients with similar median PFS estimates of 14.3, 6.39 and 2.23 months, respectively (Figure 3C and D).

Discussion

This is the first large-scale prospective multicentre study to perform simultaneous profiling of CTC and ctDNA liquid biopsies from all-comer mCRPC patients before, during and at progression on ARSi. By accounting for both ARVs and *AR* genomic alterations, we observed that 71.3% of mCRPC patients carry at least one relevant *AR* perturbation at baseline. Interestingly, other ARVs, besides AR-V7, are also associated with outcome in univariable analyses. In addition, 18% of mCRPC patients demonstrate intra-*AR* rearrangements, which typically co-occur with *AR* amplifications, and have a poor prognosis. However, our key finding is that *TP53* inactivation outperforms any *AR*-derived biomarker as negative prognosticator for second-generation ARSi. Using a clinical feature and *TP53*-driven liquid biopsy-derived classifier, we observe that 50-55% of mCRPC patients starting ARSi can be reliably stratified into good (median PFS ≥ 14.0 months) or poor (median PFS ≤ 2.5 months) prognosis groups.

The present study demonstrates how *AR* perturbations, such as AR-V7 and *AR* amplifications, can be detected in the majority of mCRPC patients, however, none of the *AR* biomarkers were independently associated with treatment outcomes in MV-Cox analyses. Although the initial discovery by Antonarakis et al (20) suggested that AR-V7 could act as a negative response biomarker for ARSi, subsets of patients expressing AR-V7 still demonstrate clinical benefit (21). Hence the clinical utility of AR-V7 is currently controversial (22), and a

recent consensus concluded that there is insufficient evidence to support the implementation of AR-V7 testing in clinical practice (23).

Intra-*AR* rearrangements have been described as a potential endocrine resistance mechanism, and could be detected in up to 50% of heavily pre-treated mCRPC patients using tumor tissue or plasma ctDNA (8,9). Most recently, structural rearrangements were detected in 19/50 (38%) preselected patients with known high ctDNA fractions prior to ARSi and typically demonstrated inferior outcome (14). Here, we demonstrate for the first time how patients with intra-*AR* rearrangements encompass a unique subpopulation with poorest prognosis. However, in MV-Cox we observed that none of the *AR*-derived biomarkers were independently associated with outcome, thereby confirming the recent report investigating the association between *AR* amplification and response to ARSi (14). Since both ctDNA fraction and CTC enumeration were independently associated with outcome in our MV-Cox analysis, our study exemplifies the importance adjusting for tumor burden estimates when performing biomarker discovery studies. Tumor burden may be correlated to the number of pre-existing resistant cells harbouring subclonal mutations before the start of therapy, which may prevent molecularly-targeted trials to reach their primary endpoints (24,25).

Despite not reaching statistical significance when associating with outcome, we believe that *AR* perturbations may still play a key role in the disease. However, there are inherent challenges with using *AR* as a baseline biomarker. *AR* biomarkers were detectable in the vast majority of patients at baseline and almost all at progression in our study. If at least one *AR* biomarker is detectable in the majority of men, comprehensive profiling needs to be undertaken to determine which patients express a relevant biomarker-output from the *AR* locus in relation to the upcoming therapy. In addition, as the chemo-hormonal therapy landscape for mPC evolves (26-28), the somatic evolutionary trajectory of the *AR*-locus is likely to be altered and needs to be explored as guidelines are updated.

However, until the molecular heterogeneity of *AR* has been completely resolved, *TP53* profiling can be applied to identify poor prognosis patients. Beyond circulating and clinical disease burden estimates, *TP53* status remained significantly associated with outcome in our MV-Cox analysis. This emphasises the importance of looking into other pathways or transdifferentiation processes, which have been implicated in endocrine resistance and *AR*-independent tumour growth (2,29,30). Recent clinical studies have demonstrated an

association between *TP53* inactivation and poor response to next generation ARSi (11,12,14). Our study provides confirmatory evidence for the molecular characterization of *TP53,* reproducing its independent prognostic value, together with ctDNA fraction and presence of visceral metastasis, in an all-comer cohort of men with mCRPC.

Additionally, we developed a robust and reliable three-stratum risk stratification system, using both clinical features and a *TP53*-driven liquid biopsy to identify patients with good and poor prognosis in the context of ARSi. Our PFS classifier was tested in a large mCRPC cohort (n=201), recruited in a randomised clinical trial (RCT) (14), and identified 31/201 (15.4%) patients in this independent cohort with poorest prognosis despite ARSi, who may be better served with other treatment modalities.

Limitations of the present study include the absence or incomplete collection of data on patient performance status and routine clinical parameters. For example baseline alkaline phosphatase and lactate dehydrogenase concentrations were missing in approximately 30% of the studied cohort, and hence not included in MV-Cox analysis. Additionally the number of metastastatic lesions was not collected. Formal performance status scores, which are associated with OS in mCRPC patients starting first-line chemotherapy (31) but not with time to progression in context of ARSi (14), are not collected as standard practice in the recruiting centres. We validated our prognostic classifier in an independent cohort of patients with *a priori* knowledge that *TP53*, ctDNA fraction, and visceral metastases were independently associated with outcome. However, and importantly, we demonstrate that our stratification method, which was generated on an all-comer cohort of men with mCRPC, gave similar PFS estimates and HR in a completely different cohort from an RCT. Although our study was prospectively designed to test the hypothesis that a combined ARV profiling strategy is prognostic in the context of ARSi, our exploratory plasma-derived biomarker analyses were undertaken retrospectively. Furthermore, our study of a heterogeneous cohort may be underpowered to identify PFS differences in specific subgroups of patients expressing ARVs.

Conclusion

The present study is the first large-scaled prospective multicentre study to perform comprehensive *AR* and *TP53* profiling in CTCs and cfDNA in an all-comer cohort of men with mCRPC starting abiraterone or enzalutamide outside the context of a RCT. Besides emphasizing the importance of comprehensive *AR* profiling, a major strength of our study is the identification of a single molecular *TP53* biomarker and tumor burden-driven stratification system for all comer patients commencing ARSi. The activity and efficacy of treatment selection driven by *TP53*, *AR* and other molecular biomarkers will need to be tested in a future prospective interventional RCT.

Contributors

BDL, PR, HG, JL and LD conceived of and designed the study. HG, SVL, PR, JL and LD acquired financial support from the funding organisations. BDL, SO, ABr and VvD provided administrative support. PVO, CG, JA, PO, WD, LH, DS, BB, WL, EE, DDM, MS, ABo, KF, NB, IdK, AR, SS, AU, JY, HG and LD recruited study patients, collected blood samples and performed clinical analyses. BDL, JL, SO, MM, TW, PJvD, DG, LH, GVdE and JDF collected and assembled the data. BDL, MM, TW, PJvD and JL performed bioinformatics analysis and data preprocessing. BDL, MM, TW, PJvD, MR, PR and JL performed the data analysis and interpretation. BDL, MR, PR and JL performed the statistical analysis. All authors contributed in the manuscript preparation. All authors provided the final approval of the manuscript.

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Figure and Table legends

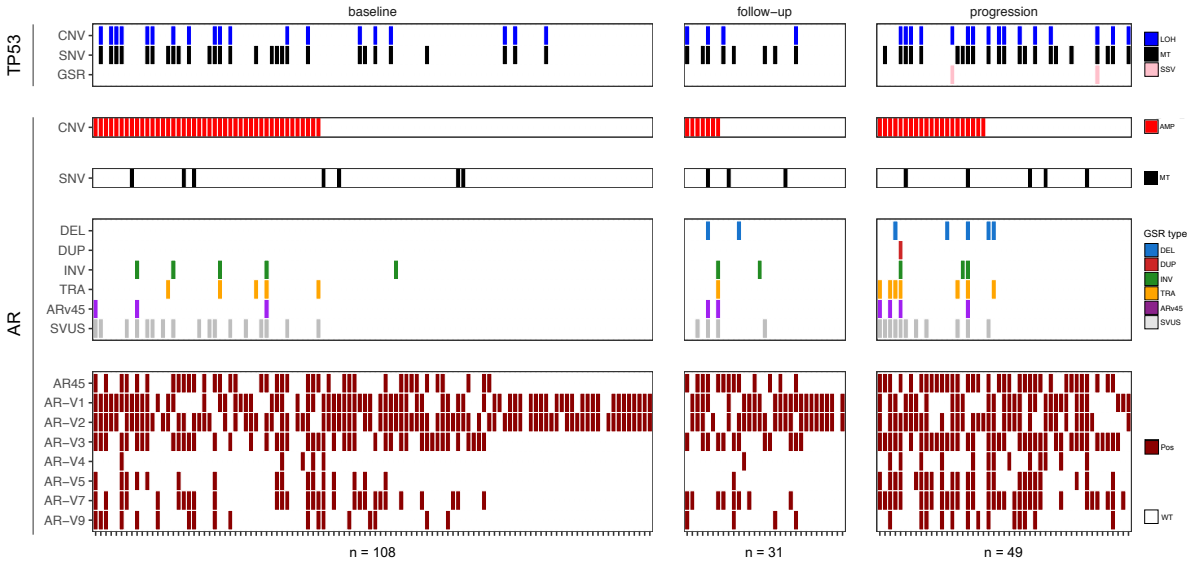


Figure 1 – Comprehensive landscape of somatic *AR* and *TP53* perturbations in liquid biopsies from patients with mCRPC at baseline (n=108), follow-up (n=31) or at progression (n=49) on abiraterone or enzalutamide. Samples are grouped according to sample type. Upper: *TP53* panel with copy-number, mutation and structural rearrangement status. Lower: *AR* panel, encompassing a CNV panel: *AR* copy number stratified according to amplification status. SNV panel: hotspot mutations within the ligand-binding domain of *AR*. GSR panel: genomic structural rearrangements across the *AR* gene. ARV panel: Presence of absence of *AR* splice variant expression. CNV denotes copy number variation. SNV denotes single nucleotide variation. GSR denotes genomic structural rearrangements. ARV denotes *AR* splice variants. LOH denotes loss-of-heterozygosity. MT denotes mutant. SSV denotes significant structural variant. AMP denotes amplified. MT denotes mutant. DEL denotes deletion. DUP denotes duplication. INV denotes inversion. TRA denotes translocation. ARv45 denotes structural variant deletion *AR* exon 1, which may result in AR45 expression. SVUS denotes structural variant of unknown significance. SSV denotes significant structural variant. Pos denotes positive. WT denotes wild-type.

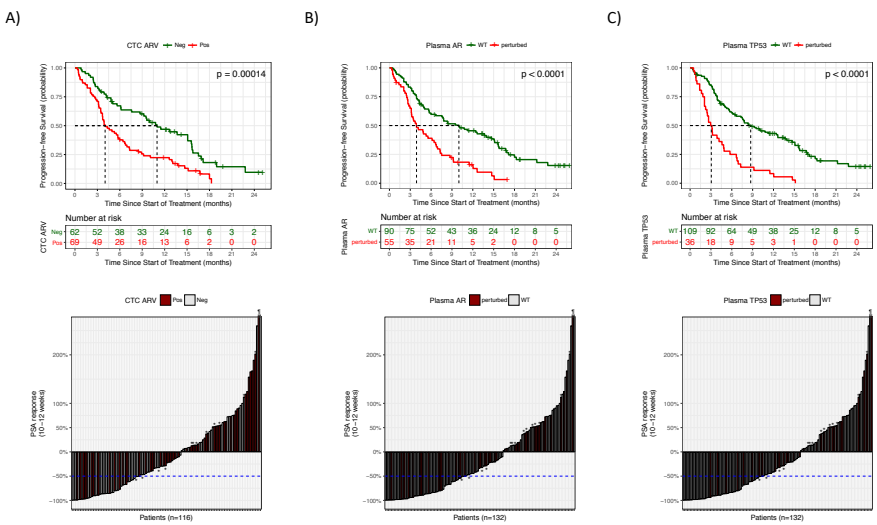
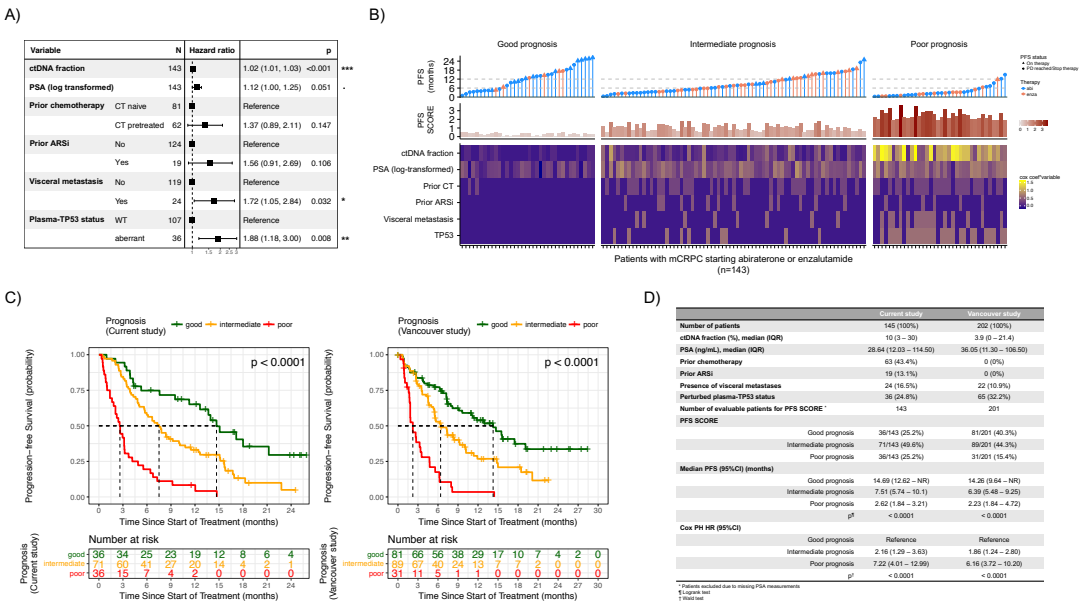


Figure 2 – AR splice variant expression in circulating tumour cells, detection of genomic *AR* and *TP53* perturbations in plasma cell-free DNA, progression-free survival and PSA response on abiraterone or enzalutamide. Kaplan-Meier (KM) analysis of progression-free survival (upper) and waterfall plots (WF) of prostate-specific antigen (PSA) responses after 10-12 weeks (or before in case of early disease progression) on therapy (lower), stratified according to outcome-associated ARV expression in CTCs (**A**), genomic *AR* (**B**) or *TP53* (**C**) perturbations in plasma cfDNA at baseline. *p*-value in KM plot is calculated via logrank test. In WF plots: * denotes PFS < 10-12 weeks, ¶ denotes PSA increase > 200% and dashed blue horizontal lines represent 50% decrease in PSA.



Variable		N	Hazard ratio	p	
ctDNA fraction		143	1.02 (1.01, 1.03)	<0.001	***
PSA (log transformed)		143	1.12 (1.00, 1.25)	0.051	.
Prior chemotherapy	CT naive	81	Reference		
	CT pretreated	62	1.37 (0.89, 2.11)	0.147	
Prior ARSi	No	124	Reference		
	Yes	19	1.56 (0.91, 2.69)	0.106	
Visceral metastasis	No	119	Reference		
	Yes	24	1.72 (1.05, 2.84)	0.032	*
Plasma-TP53 status	WT	107	Reference		
	aberrant	36	1.88 (1.18, 3.00)	0.008	**

	Current study	Vancouver study
Number of patients	145 (100%)	202 (100%)
ctDNA fraction (%), median (IQR)	10 (3 – 30)	3.9 (0 – 21.4)
PSA (ng/mL), median (IQR)	28.64 (12.03 – 114.50)	36.05 (11.30 – 106.50)
Prior chemotherapy	63 (43.4%)	0 (0%)
Prior ARSi	19 (13.1%)	0 (0%)
Presence of visceral metastases	24 (16.5%)	22 (10.9%)
Perturbed plasma-TP53 status	36 (24.8%)	65 (32.2%)
Number of evaluable patients for PFS SCORE *	143	201
PFS SCORE		
Good prognosis	36/143 (25.2%)	81/201 (40.3%)
Intermediate prognosis	71/143 (49.6%)	89/201 (44.3%)
Poor prognosis	36/143 (25.2%)	31/201 (15.4%)
Median PFS (95%CI) (months)		
Good prognosis	14.69 (12.82 – NR)	14.26 (9.64 – NR)
Intermediate prognosis	7.51 (5.74 – 10.1)	6.39 (5.48 – 9.25)
Poor prognosis	2.62 (1.84 – 3.21)	2.23 (1.84 – 4.72)
p¶	< 0.0001	< 0.0001
Cox PH HR (95%CI)		
Good prognosis	Reference	Reference
Intermediate prognosis	2.16 (1.29 – 3.63)	1.86 (1.24 – 2.80)
Poor prognosis	7.22 (4.01 – 12.99)	6.16 (3.72 – 10.20)
p†	< 0.0001	< 0.0001

* Patients excluded due to missing PSA measurements
¶ Logrank test
† Wald test

Figure 3 – Development and validation of a three stratum risk stratification system using clinical features and molecular profiling. A) Multivariable Cox regression analysis (hazard ratio (confidence interval)) of progression-free survival using baseline clinical characteristics, ctDNA fraction estimate and *TP53* status in patients with mCRPC. **B)** Multi-level landscape of cox coefficient-adjusted variable values (bottom), calculated clinical progression (i.e. PFS) score (middle) and progression-free survival (top). Patients are grouped according to the PFS score category (i.e. < Q1, Q1-Q3 and > Q3 level) and ordered according to increasing progression-free survival. Horizontal dashed lines represent 12- and 6-month landmarks. **C)** Kaplan-Meier analysis of progression-free survival, stratified according to clinical progression score category at baseline for the current study (i.e. training cohort, n=143) and Vancouver Prostate Centre study (i.e. testing cohort, n=201). *p*-value is calculated via logrank test. **D)** Performance characteristics of the three stratum risk stratification system, comparing risk group prevalences, median PFS times and Cox hazard ratios.

Table 1 – Patient characteristics

	All Patients	
	n	%
Patients	168	100%
Age at registration, yr, mean ± SD	76 ± 7.7	
Tumor stage at diagnosis		
T1/2	45	26,79%
T3/4	41	24,40%
M1	45	26,79%
node-positive	12	7,14%
Not specified	25	14,88%
Gleason score at diagnosis		
≤ 7	63	37,50%
8 - 10	83	49,40%
Not specified	22	13,10%
Primary treatment		
ADT (± RT)	76	45,24%
Radical Px (± RT)	61	36,31%
Radical Px + ADT	5	2,98%
Other	15	8,93%
Not specified	11	6,55%
Previous Chemotherapy		
Chemotherapy naïve	100	59,52%
Chemotherapy pretreated	68	40,48%
Previous ARS inhibitor for CRPC		
no	148	88,10%
yes	20	11,90%
Initiating Therapy		
Abiraterone Acetate	111	66,07%
Enzalutamide	57	33,93%
Metastatic burden at start Therapy		
LN only	20	11,90%
Bone only	73	43,45%
Bone and LN	45	26,79%
Visceral and bone and/or LN	26	15,48%
Not specified	4	2,38%
Baseline blood chemistry	median	IQR
LDH, U/L (n=119)	335	217 - 655.5
AP, U/L (n=123)	102	73 - 160.5
PSA, µg/L (n=164)	36.92	13.5 - 144.9
Baseline circulating tumor cells	median	IQR
CTC, #/7.5mL (n=164)	2	0 - 17.5

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