Increased Pathway Complexity Is a Prognostic Biomarker in Metastatic Castration-Resistant Prostate Cancer

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Abstract: Metastatic castration-resistant prostate cancer (mCRPC) is a heterogeneous disease, characterized by common and rare driver gene alterations that provide a selective growth advantage for progressing tumour cells. We hypothesized that the number of distinct gene driver alteration-affected pathways or gene classes was associated with poor prognosis in patients initiating androgen receptor signalling inhibitors (ARSi). We performed a post hoc analysis of an amalgamated baseline circulating tumour DNA (ctDNA) mutational landscape dataset of ARSi-treated men with mCRPC (n = 342). We associated the detected hotspot, pathogenic, and/or high impact protein function-altered pathways or gene classes were affected (PFS HR (95%CI): 1.7 (1.02–2.84), p = 0.04, and OS HR (95%CI): 2.5 (1.06–5.71), p = 0.04). Additionally, grouping genomic alterations with other events within the same cellular pathways would offer increased precision for biomarker discovery and utilization.

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visceral disease and baseline PSA and plasma ctDNA levels were independently associated with poor prognosis. Elevated PCI is associated with poor ARSi outcome and supports comprehensive genomic profiling to better infer mCRPC prognosis.

**Keywords:** mCRPC; biomarker; ctDNA

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1. **Introduction**

Comprehensive genomic profiling of metastatic castration-resistant prostate cancer (mCRPC) has demonstrated extensive inter-patient heterogeneity, with the identification of relatively few commonly perturbed genes and a large number of infrequently mutated cancer drivers in the so-called “long tail” [1]. Consequently, any precision medicine feasible trial will be underpowered for the stratified evaluation of the majority of reported driver genes. The mCRPC genomic landscape in metastatic tissue is faithfully mirrored in plasma-derived circulating tumour DNA (ctDNA) [2] or circulating tumour cells [3]. Our prior work demonstrated that ctDNA profiling can identify microsatellite instability (MSI), genomic structural rearrangements (GSRs), and that TP53 alterations can stratify mCRPC patients on androgen receptor signalling inhibitors (ARSi) into clinically relevant prognostic groups [4,5]. Thus, the potential clinical utility of minimally-invasive liquid biopsies to identify driver DNA alterations has been demonstrated and has set the stage for precision urologic oncology trials, aiming towards improving patient prognostication and clinical management.

A prerequisite for identifying an association between TP53 perturbations and ARSi prognosis was the high (24.8%) frequency of TP53-altered patients [5]. Although TP53 is the most frequently mutated gene in cancer, alterations in TP53 have the same basic consequence as uncommonly altered oncogenic genes; namely to provide a selective growth advantage. Most cancers harbour alterations in a handful of driver genes, but heterogeneity exists. The known drivers of cancer, commonly or uncommonly altered, are all associated with a limited number of cellular signalling pathways (e.g., AR pathway, PI3K pathway, cell cycle signalling, DNA damage repair, etc.) which in turn affect core cellular processes, resulting in increased cancer cell fitness [6].

Although the sizes of currently existing datasets prevent clinical evaluation of genes in the long tail, we hypothesized that an increase in the number of perturbed distinct pathways in an individual cancer, contributed to by common and uncommon driver genes, will lead to an increase in biological versatility. Therefore, we investigated if the number of altered pathways, coined the pathway complexity index (PCI), was associated with prognosis in men with mCRPC starting ARSi.

2. **Results**

2.1. **Study Patient Populations**

A baseline ctDNA mutational landscape dataset from abiraterone- or enzalutamide-treated men with MSI- and/or hypermutator-negative mCRPC (n = 342), recruited between 2014 and 2017, was assembled using publicly available datasets (i.e., our CORE/PROBIO cohort study (n = 142) [4,5] and the prospective NCT02125357 trial (n = 200) [7]) (Figure 1). Whilst the Vancouver Prostate Centre (defined as VANC) encompassed 200 mCRPC patients starting ARSi as first-line therapy, the CORE/PROBIO-enrolled patients also consisted of 61/142 (43%) and 19/142 (13.4%) patients previously treated with chemotherapy and ARSi, respectively (Table S1). We observed a higher median ctDNA fraction (9.15% vs. 3.90%, p < 0.001) in the CORE/PROBIO cohort compared with the VANC cohort. However, when comparing clinical features within the subgroup of treatment-naïve patients (n = 277) from both cohorts, no differences were observed (Table S2). Overall, we did not observe significant differences between progression-free (PFS) (median, 6.67 vs. 7.40 months, p = 0.23) and overall survival (OS) (median, 24.8 vs. 20.3 months, p = 0.09) estimates.
between the CORE/PROBIO and VANC cohorts respectively (Figure S1). Nonetheless, due to the observed variability between both cohorts in terms of prior systemic therapy exposure and ctDNA fractions, cox-regression models are stratified by cohort to allow for differences in cohort-specific hazards.

Figure 1. CONSORT diagram of patients with metastatic castration-resistant prostate cancer (mCRPC) treated with androgen receptor signalling inhibitors (ARSi). A total of 342 patients with mCRPC who underwent cell-free DNA profiling received ARSi (i.e., abiraterone acetate or enzalutamide). Five patients were excluded from downstream analysis as they were positive for the microsatellite instability (MSI) or hypermutator genotype. VANC, Vancouver Prostate Centre cohort; CORE/PROBIO, Centre for Oncological Research and Prostate Biomarker cohort; PCI, pathway complexity index.

2.2. The ctDNA Landscape of Driver and Pathway Alterations in mCRPC

Driver gene perturbations were detected in 192/342 (56.1%) evaluable patients at baseline (Figure S2). Overall, AR (135/342, 39.5%), TP53 (93/342, 27.2%), PTEN (43/342, 12.6%), FOXA1 (20/342, 5.8%), and SPOP (20/342, 5.8%) were the most commonly perturbed genes (Figure 2A, Table S3). For these top 5 perturbed genes, the prevalence in both cohorts was similar except for PTEN perturbations, which were higher in the CORE/PROBIO cohort in comparison to the VANC cohort (19.7% vs. 7.5%, respectively, \( p = 0.001 \)) (Table S4). When these genes were grouped into their defined pathways and/or gene classes, the AR pathway (139/342, 40.6%), TP53 gene class (93/342, 27.2%), the PI3K pathway (54/342, 15.8%), the cell cycle signalling (37/342, 10.8%), and DNA repair (32/342, 9.4%) were the most commonly perturbed pathways or gene classes (Figure 2B, Table S3). No driver alterations were detected in MSH2, CDK4, NFE2L2, IDH1, FANCG, GNAS, FBXW7, and IDH2.
2.3. Individual Pathways or Gene Classes and Outcome

In univariate analysis, the median PFS and OS estimates were significantly shortened in patients carrying AR pathway, TP53, PI3K pathway, cell cycle signalling, and DNA repair driver perturbations (all \( p < 0.01 \)) (Tables S5 and S6). For OS, we additionally observed WNT/\( \beta \)-catenin pathway and transcription factor perturbations to be associated with inferior prognosis (all \( p < 0.05 \)) (Table S6). The majority of these observations, except for DNA repair, were recapitulated in both cohorts (Figure S3). Multivariate (MV) analysis of an individual pathway or gene class, corrected for PSA and ctDNA levels, prior chemotherapy and ARSi exposure, and presence of visceral metastases, revealed that the TP53 and DNA repair gene classes maintained independent prognostic value for both PFS and OS (Tables S5 and S6). This was preserved when the MV analysis for PFS and OS incorporated all pathway or gene classes (Figure S4). In both models, prior ARSi exposure, presence of visceral metastases and elevated ctDNA and PSA levels were also associated with inferior prognosis. Interestingly, when putting the TP53 and DNA repair gene classes head to head, and allowing for interaction with the underlying cohorts, we observed that the PFS prognostic value of the TP53 gene class could be recapitulated in both cohorts, whereas the association of the DNA repair category with PFS and OS was only observed in the VANC cohort (Figure S4B).

2.4. Pathway Complexity Index (PCI) and Outcome

When associating the number of the pathway or gene class hits with outcomes we observed a decremental gradient in PFS and OS as the number of affected pathways or gene classes accumulated (Figure S5). We observed how outcome estimates did not differ between patients with 1–2 and \( \geq 3 \) affected pathways or gene classes. Grouping these patients resulted in 123/342 (36%) and 69/342 (20.2%) patients with a pathway complexity index (PCI) of 1–2 and \( \geq 3 \), respectively, with decremental PFS (median, 12.5 vs. 5.8 vs. 3.2 months, \( p < 0.0001 \)) and OS (median, not reached vs. 18.2 vs. 9.7 months, \( p < 0.0001 \)) survival curves in comparison to patients without driver pathway hits (Figure 3A,C).
Figure 3. Increasing pathway complexity is associated with poor outcomes to abiraterone or enzalutamide in metastatic castration-resistant prostate cancer. Kaplan–Meier analysis of progression-free (A) and overall survival (C), stratified according to the pathway complexity index (PCI) at baseline. p-value is calculated via the log-rank test. Multivariate Cox regression analysis (hazard ratio (confidence interval)) of progression-free (B) and overall (D) survival using baseline characteristics and pathway complexity index (using a PCI = 0 as reference). Within the multivariate cox regression model, serum PSA and plasma ctDNA levels were incorporated and modelled as continuous variables using restricted cubic spline models. The correlation between increasing levels of serum PSA or plasma ctDNA and time-to-event hazard ratio’s (for progression-free (PFS) and overall survival (OS)) is graphically presented in Panel B and D (right), using the median PSA (36 ng/mL) and ctDNA fraction (6%) as reference points (i.e., HR = 1). The observed positive correlation was tested for significance using the Wald test (both \( p < 0.001 \)). Abbreviations: ARSi, androgen receptor signalling inhibitors; CT, chemotherapy; ctDNA, circulating tumour DNA fraction; PSA, prostate-specific antigen.

No frequency differences were observed between both cohorts (Chi-square: \( p = 0.10 \)) (Table S1). In multivariate analysis the PCI, using patients without driver pathway hits (PCI = 0) as a reference, carried independent PFS (HR 1.7, 95%CI 1.02–2.84, \( p = 0.04 \)) (Figure 3B) and OS (HR 2.5, 95%CI 1.06–5.71, \( p = 0.04 \)) (Figure 3D) prognostic value once \( \geq 3 \) pathway or gene classes were affected. Additionally, we observed how, besides a high PCI the presence of visceral metastases, serum PSA and plasma ctDNA levels (all \( p < 0.001 \)) were independently associated with inferior outcomes. Importantly, incorporating and modelling the PSA and ctDNA fraction as continuous variables within the Cox regression model (Figure 3C,D, right) revealed how increasing levels of serum PSA or plasma ctDNA resulted in a strong positive correlation with the time-to-event hazard ratio’s, i.e., the higher...
the PSA or ctDNA level at baseline, the higher the chance of an event (progression or death) occurring.

The decremental PFS and OS with increasing PCI were preserved in the prior treatment-naive cohort-stratified subgroup analysis (Figure S6). In an exploratory analysis, we looked into recurrent combinations in poor prognosis patients with ≥3 affected pathways or gene classes (n = 69), which revealed that 44/69 (63.8%) patients harboured combined AR pathway and TP53 gene class perturbations, of whom 24/44 (54.5%) carried an additional perturbation within the PI3K pathway (Figure S7). Surprisingly, alterations in genes of the same pathway (thought to be evolutionary redundant [6]), were not mutually exclusive in all patients. This warrants future investigation of the potential prognostic value of the number of altered driver genes that overlap in the same signalling pathway (Figure S7).

3. Discussion

We demonstrate how plasma ctDNA profiling can identify subpopulations of patients with mCRPC that have accumulated multiple hits in cancer driver pathways, which is independently associated with poor ARSi outcome and survival. Our study has some limitations. Firstly, this retrospective analysis is hypothesis-generating and was not designed to assess the effect of (sub)clonal mutations; which likely harbour prognostic information in the context of the disease trajectory and therapeutic selection pressure [8], nor to benchmark the prognostic value of PCI relative to tumour mutational burden (TMB). The relatively small sizes of our panels cannot provide a robust TMB estimate [9], unless in the context of microsatellite instability or hypermutation [4,7]. Additionally, the CORE/PROBIO study had incomplete data collection on clinical features and blood-based parameters (e.g., Gleason score, number of metastatic lesions, comorbidities, ECOG, LDH, etc.), which are associated with prognosis [10]. Furthermore, the merged variant dataset was generated from overlapping regions of two hybridisation-based capture assays with different capture designs, which could be exemplified by the rate of PTEN perturbations. This was markedly higher in the CORE/PROBIO cohort as a result of the intronic sequencing performed for the CORE/PROBIO patients, enabling the detection of GSRs [11]. Finally, these data were derived from different patient populations. Although similar outcome estimates were observed, we took the inherent differences into account in our cohort-stratified time-to-event analyses. Higher ctDNA fractions were detected in the CORE/PROBIO cohort in comparison to the treatment-naive Vancouver (VANC) patients, which can be in part explained by the all-comer nature of the former cohort, where a group of patients initiated ARSi at a later line of therapy. In the context of genomic biomarkers, this underlying demographic difference was most clearly demonstrated for the association between the DNA repair deficiency status and ARSi outcome. Although a significant association in multivariate analysis was observed in the total population, this was driven by the Vancouver patients. Genes with different roles in the DNA repair process (e.g., sensing, signalling or repair) can cause distinct mutational signatures [12] and responses to, e.g., PARP inhibition [13]. It is, therefore, likely that the interplay with endocrine therapy may differ between individual genes. The CORE/PROBIO-cohort has a significantly lower proportion of men with pathogenic BRCA2 germline alterations and instead demonstrates increased levels in ATM and CHEK2 [4]. The differences in clinically relevant germline DNA repair alterations may have contributed to the differences observed here and warrants further investigation in future biomarker-driven studies. Other tissue biopsy-based studies have suggested SPOP mutations [14] and biallelic inactivation of RB1 [15] to be independently associated with good and poor outcomes of ARSi, respectively. Our ctDNA data revealed no association for SPOP and demonstrated how perturbed cell cycle signalling, in general, was associated with poor ARSi outcomes in univariate, but not in multivariate, analysis. We did not study RB1 alone due to its low prevalence (i.e., 10/342 (2.9%) cases). Importantly, RB1 intronic sequencing was not carried out in the plasma samples from the Vancouver cohort, which may have led to a misclassification of a subset of patients as not carrying a driver RB1 event, due to an inability of detecting inactivating GSRs. Additionally, it is unclear if
Abida et al. [15] incorporated correction for tumour burden estimates in the multivariate analysis, which questions the current generalizability of RB1’s independent association with ARSi outcomes, especially since successful biopsy profiling is correlated with high tumour burden [16]. Although a different OS definition and patient population were used in comparison to Chen et al. [17], we were able to recapitulate the association between RB1 bi-allelic inactivation and poor overall survival using the CORE/PROBIO-cohort data (Figure S8). To the best of our knowledge, these data represent the first demonstration of the clinical validity of the pathway complexity index, which dictates the prognosis of mCRPC patients on an upcoming ARSi therapy. In patients with a high PCI, we observed how AR and TP53 perturbations most commonly co-occurred, with >50% of those patients having a PI3K pathway perturbation as well. Targeting both the AR and PI3K pathway was tested in a randomised controlled trial, and demonstrated durable responses in PTEN-null tumours when treated with abiraterone and the Akt-inhibitor ipatasertib [18]. The reported data warrants the continued investigation of new combination therapy modalities or biomarker signature-therapy matches in large liquid biopsy-driven multi-arm prospective clinical studies, such as PC-BETS (ClinicalTrials.gov Identifier: NCT03385655) [19] and the randomized clinical trial ProBio (ClinicalTrials.gov Identifier: NCT03903835) [20], where longitudinal monitoring will make it possible to study the PCI dynamics throughout the course of multiple therapeutic interventions. Additionally, with the decreasing cost of DNA sequencing and increasing DNA capture panel sizes we anticipate that the novel PCI metric will be subject to continued evaluation and fine-tuning in future biomarker-driven research.

4. Materials and Methods

4.1. Patient Cohort and ctDNA Dataset Generation

A baseline ctDNA mutational landscape dataset from abiraterone- or enzalutamide-treated men was assembled using publicly available datasets (i.e., our CORE/PROBIO cohort study (n = 142) [4,5] and the prospective ClinicalTrials.gov Identifier: NCT02125357 trial (n = 200) [7]) (Figure 1). All reported genomic alterations, encompassing copy-number aberrations, mutations and GSRs, within 39 genes that overlapped between both datasets were merged. The following filters were applied to the annotated variants to generate a list of driver genomic alterations: (1) For indel and missense variants: only known hotspots, pathogenic, and/or annotated as high-impact variants by the Variant Effect Predictor (PMID: 27268795) were kept; (2) Deleterious copy-number aberrations need to encompass deep and/or homozygous deletions (log ratio < -1.0); (3) Only high-level copy-number amplifications (log ratio > 0.6) were retained, except for AR, where any gain was annotated as a driver hit in the AR gene; and (4) Only significant genomic structural rearrangements (GSRs), resulting in loss of- or gain of protein function were kept.

The driver genomic alterations were grouped into pathways except for TP53, SPOP, IDH1, and CDK12 who were kept as individual gene classes. TP53 is involved in multiple pathways and hard to assign to a specific pathway [21], SPOP and IDH1 represent distinct prostate cancer subtypes [22], as does CDK12 [23]. The pathway and driver complexity index were defined as the number of detectable driver genomic alterations in the selected 39 genes, which in turn were associated with 13 pathways or gene classes: AR pathway (AR, FOXA1, ZBTB16), TP53 class (TP53), PI3K pathway (AKT1, PIK3CA, PIK3CB, PIK3R1, PTEN), cell cycle signalling (RB1, CCND1, CDKN1B, CDKN2A, CDK4, MYC), DNA repair deficiency (BRCA1, BRCA2, ATM, FANCA, MLH1, MSH2, MSH6), WNT/b-catenin pathway (APC, CTNNB1), SPOP class (SPOP), chromatin modifiers (CHD1, KDM6A, ARID1A), CDK12 class (CDK12), transcription factors (MED12, NFE2L2, NKKX3.1), MAPK pathway (KRAS, BRAF), IDH1 subtype (IDH1), and other (FBXW7, GNAS, IDH2, FANCG). The ctDNA fraction was estimated as described previously [4,7].
4.2. Statistical Analysis

The current post hoc analysis evaluated progression-free (PFS) and overall (OS) survival as clinical endpoints. PFS was defined as time to first confirmed PSA progression, clinical and/or radiologic progression, or death from disease. Overall survival (OS) outcome was defined as the time from baseline blood sampling to death from any cause. Survival curves for the individual and the number of affected pathways or gene classes (i.e., PCI) were estimated by Kaplan–Meier (KM) analysis. Survival differences were determined using the log-rank test. The effects of pathway/gene class alterations and PCI were quantified by hazard ratios (HRs) in cohort-stratified multivariate Cox regression models, adjusted for the following covariates: baseline PSA and ctDNA levels, prior chemotherapy, prior ARSi exposure, and presence of visceral metastases. The baseline ctDNA fraction and PSA concentration were incorporated in the multivariate model as continuous variables, and were modeled in the Cox regression model using restricted cubic splines. The association of baseline ctDNA fraction and PSA concentration as continuous variables with the HRs for time-to-event outcomes (PFS and OS) were graphically presented using their median values as reference points. Differences between the two cohorts were assessed using the Mann–Whitney U (continuous variable) and Chi-square tests (categorical variables). All tests were performed in R (v.1.1.463), with a two-sided \( p \)-value < 0.05 as being considered as statistically significant.

5. Conclusions

We demonstrate for the first time that the elevated pathway mutational complexity is independently associated with poor prognosis, which warrants comprehensive genomic profiling for the prognostication of patients with advanced prostate cancer.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/cancers13071588/s1, Figure S1: Kaplan–Meier analysis of progression-free survival (A) and overall (B) survival, stratified according to the study cohort, i.e., CORE/PROBIO (\( n = 142 \)) and VANC (\( n = 200 \)), Figure S2: The ctDNA landscape of driver gene perturbations in baseline liquid biopsies from patients with metastatic castration-resistant prostate cancer (\( n = 342 \)) initiating abiraterone or enzalutamide, Figure S3: Cohort-stratified Kaplan–Meier analyses of progression-free and overall survival, stratified according to a wild-type or mutant/perturbed pathway or gene class status, Figure S4: Multivariate Cox regression analysis of progression-free and overall survival, Figure S5: Kaplan–Meier analysis of progression-free survival and overall survival, stratified according to the number of perturbed pathways or gene classes, Figure S6: Cohort-stratified Kaplan–Meier analysis of progression-free and overall survival in treatment-naive patients, Figure S7: Pathway or gene class characteristics in patients with an elevated pathway complexity index, Figure S8: Overall survival between patients with and without biallelic \( RB1 \) inactivation from the date of ARSi initiation, Table S1: All-comer patient characteristics for the total and separate cohorts, Table S2: Treatment-naive patient characteristics for the total and separate cohorts, Table S3: Gene- and Pathway/Gene class-level prevalences of genomic alterations in baseline plasma ctDNA samples from patients with metastatic castration-resistant prostate cancer initiating abiraterone or enzalutamide (\( n = 342 \)), Table S4: Comparative analysis of the prevalence of the top 5 most commonly perturbed genes, Table S5: Kaplan–Meier analyses, and uni- and multivariate Cox regression of progression-free survival, stratified according to a wild-type or perturbed pathway/gene class status. Table S6: Kaplan–Meier analyses, and uni- and multivariate Cox regression of overall survival, stratified according to a wild-type or perturbed pathway/gene class status.

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Institutional Review Board Statement: The data reported in this study encompasses a retrospective analysis. The data were gathered from publicly available datasets from clinical studies that were conducted according to the guidelines of the Declaration of Helsinki, and approved by the Institutional Review Boards in Belgium (Antwerp University Hospital, registration number: B300201524217; 16 March 2015), Sweden (Karolinska University Hospital, registration number: 2016/101-32; 27 January 2016), and Canada (University of British Columbia Research Ethics Board; Registration Number H14–00738; 4 September 2014).

Informed Consent Statement: Written informed consent was obtained from all subjects involved in the study.

Data Availability Statement: All data relevant for the interpretation of our findings is provided in the current and main manuscripts (i.e., De Laere et al. (PMID: 30209161) and Annala et al. (PMID: 29367197); or the Supplementary Materials and methods for the manuscript, except for the raw sequence data. Any data providing genotype information is considered to be a personal registry by the Swedish law (Personal Data Act), thereby prohibiting the submission to a public repository. The raw sequence data is instead available upon request from the authors (For Belgium/Sweden: contact johan.lindberg@ki.se; For Canada: contact awyatt@prostatecentre.com) if approval has been obtained from a Regional Ethical Vetting Board.

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