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TITLE: Noninvasive Detection of AR-FL/AR-V7 as a Predictive Biomarker for Therapeutic Resistance in Men with Metastatic Castration-Resistant Prostate Cancer

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14. ABSTRACT

Understanding primary and acquired resistance to abiraterone and enzalutamide, and developing analytically validated and clinically qualified predictive biomarkers, remains a critically important unmet medical need. We propose non-invasive detection of full-length androgen receptor (AR-FL) and the androgen receptor splice variant 7 (AR-V7) (AR-FL/AR-V7) as a predictive biomarker for therapeutic resistance in men with metastatic castration-resistant prostate cancer. Using a laboratory-developed, RNA-based assay modified from a commercially available circulating tumor cell (CTC) detection platform, we have developed standard operating procedures and performed extensive internal validation and quality control studies to determine its feasibility for detection of AR-FL/AR-V7 in blood samples. Although our recent studies show data supporting this predictive biomarker, analytical validation is required prior to clinical use, and a large-scale, multi-institutional study is needed to further establish clinical utility. The overall objective of the project is to enable precision therapy of metastatic castration-resistant prostate cancer by developing non-invasive tests for the AR-FL/AR-V7.

15. SUBJECT TERMS

None listed

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1. **INTRODUCTION:** Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.

Understanding primary and acquired resistance to abiraterone and enzalutamide, and developing analytically validated and clinically qualified predictive biomarkers, remains a critically important unmet medical need. We propose non-invasive detection of full-length androgen receptor (AR-FL) and the androgen receptor splice variant 7 (AR-V7) (AR-FL/AR-V7) as a predictive biomarker for therapeutic resistance in men with metastatic castration-resistant prostate cancer. Using a laboratory-developed, RNA-based assay modified from a commercially available circulating tumor cell (CTC) detection platform, we have developed standard operating procedures and performed extensive internal validation and quality control studies to determine its feasibility for detection of AR-FL/AR-V7 in blood samples. Although our recent studies show data supporting this predictive biomarker, analytical validation is required prior to clinical use, and a large-scale, multi-institutional study is needed to further establish clinical utility. The overall objective of the project is to enable precision therapy of metastatic castration-resistant prostate cancer by developing non-invasive tests for the AR-FL/AR-V7.

2. **KEYWORDS:** Provide a brief list of keywords (limit to 20 words).

Prostate cancer, CRPC, AR-V7, liquid biopsy, resistance, abiraterone, enzalutamide

3. **ACCOMPLISHMENTS:** The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction.

What were the major goals of the project

Major Task 1: Development of robust and standardized SOPs pertaining to the accurate and reliable detection of AR-FL/ARV7.

Subtask 1: To conduct essential study planning activities including IRB and HRPO approval, ordering of a common set of reagents, equipment readiness, protocol review, distribution of SOPs, personnel assignment, and review of documentation requirements (Months 1-6).

Subtask 2: Testing SOPs pertaining to the accurate and reliable detection of AR-FL/AR-V7.

Subtask 3: Development of robust SOPs for sample collection, processing, and transfer (Months 7-12).

Major Task 2: Correlation between CTC AR expression with contemporaneously acquired fresh CRPC biopsy expression, and with expression detected in cell-free exosome RNA.

Subtask 1: Correlation between CTC AR expression with contemporaneously acquired fresh CRPC biopsy expression. (Months 7-24).

Subtask 2: Correlation between CTC AR expression with expression detected in cell-free exosome RNA. (Months 7-24).

Major Task 3: Development of new CTC selection and molecular detection platforms

Subtask 1: Evaluation of new CTC selection platform for the purpose of detection of AR-FL/AR-V7 (Months 12-24).

Subtask 2: Evaluation of new molecular detection platforms (Months 12-24).

Major Task 4: Clinical validation of the AR-FL/AR-V7 test

Subtask 1: Prospective recruitment of 300 patients with mCRPC initiating standard-of-care treatment with abiraterone, enzalutamide, or chemotherapy consenting for blood draw (baseline, 2nd at the time of response if any, and 3rd time at the time of progression), and optional biopsy (~n=50) (Months 12-30) Subtask 2: Biomarker implementation in certified labs (Months 12-30).

Subtask 3: Data analysis (Months 30-36).

Major Task 5: Biomarker-embedded trial of enzalutamide and AKT inhibitor

Subtask 1:

ï Recruit, consent, and enroll 140 patients/human subjects to Phase I/II trial.

ï Evaluation of the association between CTC counts, ARFL/ AR-V7 expression, and PTEN status, and all these parameters to response to treatment (Months 6-30).

Subtask 2: Collection and documentation of 20 pre and post-treatment biopsies from men enrolled in the trial for collaborative studies with Dr. Luo (Months 6-12).

Major Task 6: Alternative approaches

Subtask 1: Formulation of additional biomarker-driven clinical trials (Months 24-36).

Subtask 2: Additional studies according to FDA/EMA guidance (Months 24-36).

What was accomplished under these goals?

Task 1: We have completed this task. All regulatory documents were in place and all required collaborative agreements were signed during Year 1 of the project period. In addition, we have distributed SOPs and compared the data across different institutions during Year 1 and Year 2 of the project period. The test was analytically validated at Johns Hopkins University during Year 1 of the project period, leading to a publication focusing on analytical performance of the test (1).

Task 2: We have completed this task. An article focusing on evaluating the correlation between CRPC tissue biopsy and CTC marker status was published (2).

Task 3: Subtask 1 will be reported by one of the principle PIs, Dr. Stephen Plymate. Subtask 2 has been completed by Drs. Luo, de Bono, and Plymate, leading to multiple joint publications (3, 4).

Task 4: We have completed this task. A manuscript on clinical utility of the test has been published (5). We also published the results from the expanded Johns Hopkins cohort (6). We conducted tests on blood samples shipped from the ICR partnering site (PI: de Bono). The results from this collaborative study was recently published (2).

Task 5: Will be mainly reported by the de Bono group from the ICR partnering site.

Task 6: We have completed this task. We participated in two biomarker selection trials. Trial results have been published (7, 8). Collaborative clinical trials beyond the scope of this study are ongoing.

What opportunities for training and professional development has the project provided?

The laboratory of Dr. Luo hosted a Scientific Officer from the Prof. de Bono group to train in the Adnatest to ensure good technical practice. A postdoc research fellow from Dr. Luo group (Dr. Yezi Zhu) and a clinical fellow from Dr. de Bono group (Dr. Adam Sharp) have collaborated and co-authored a manuscript published in European Urology. Both Dr. Zhu and Sharp presented their most recent work at the annual Prostate Cancer Foundation retreat.

How were the results disseminated to communities of interest?

Results from this project were disseminated to communities of interest through peer-reviewed publications as well as poster and podium presentations at national meetings.

What do you plan to do during the next reporting period to accomplish the goals?

Nothing to report.

4. IMPACT: Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:

What was the impact on the development of the principal discipline(s) of the project?

Following analytical validation, we have realized patient benefit by making a clinical grade test available to patients at the Johns Hopkins University. Since the implementation of the test, more than 800 patients have been tested. As a result of this project, the AR-V7 test was validated and implemented for clinical use. Our published studies motivated other collaborative studies and commercial interest, and may have indirectly contributed to the development and commercialization of the EPIC AR-V7 test (9).

What was the impact on other disciplines?

Nothing to Report.

What was the impact on technology transfer?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Nothing to Report.

What was the impact on society beyond science and technology?

We believe men with metastatic CRPC will benefit from the availability of the test. A manuscript evaluating how the test results are utilized by providers and patients and whether the availability of the test resulted in better patient outcome has been published (5). This information will provide guidance to providers, patients, and insurers.

5. CHANGES/PROBLEMS: The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:

Nothing to Report.

Actual or anticipated problems or delays and actions or plans to resolve them

Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

Nothing to Report.

Changes that had a significant impact on expenditures

Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

Nothing to Report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Significant changes in use or care of human subjects

Nothing to Report.

Significant changes in use or care of vertebrate animals

Nothing to Report.

Significant changes in use of biohazards and/or select agents

Nothing to Report.

6. **PRODUCTS:** List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."

Publications, conference papers, and presentations

Report only the major publication(s) resulting from the work under this award.

Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

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Books or other non-periodical, on(10)e-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series.*

Nothing to Report.

Other publications, conference papers and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

Nothing to Report.

ï **Website(s) or other Internet site(s)**
List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

Nothing to Report.

ï **Technologies or techniques**
Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.

Nothing to Report.

ï **Inventions, patent applications, and/or licenses**
Identify inventions, patent applications with date, and/or licenses that have resulted from the research. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

Nothing to Report.

ï **Other Products**

Nothing to Report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name	Role	Percent Effort
Sokoll , Lori	Logistical and regulatory consult, Co-Investigator	5
Luo , Jun	Principle Investigator, overall management	30
Demarzo , Angelo	Tissue-based studies, Co-Investigator	3.99
Eshleman , James	CLIA lab activities, Co-Investigator	4.02
Paller , Channing	Oncology planning, Co-Investigator	3.67
Isaacs , William	Scientific guidance, Co-Investigator	7.83
Antonarakis , Emmanuel	Oncology lead, Co-Investigator	8.40
Wang , Hao	Statistician, Co-Investigator	15
Lu , Changxue	quality control, protocol development	50
Zhu, Yezi	technological development	60
Riel, Stacy	CLIA coordination, lab management	50

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

Nothing to Report.

What other organizations were involved as partners?

Nothing to report

6. **8. PECIAL REPORTING REQUIREMENTS**

COLLABORATIVE AWARDS: For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to **https://ers.amedd.army.mil** for each unique award.

QUAD CHARTS: If applicable, the Quad Chart (available on https://www.usamraa.army.mil) should be updated and submitted with attachments.

9. APPENDICES: Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.

Published articles supported by the Award are attached.

available at www.sciencedirect.com
journal homepage: www.europeanurology.com



European Association of Urology

Platinum Priority – Prostate Cancer
Editorial by XXX on pp. x–y of this issue

Novel Junction-specific and Quantifiable In Situ Detection of AR-V7 and its Clinical Correlates in Metastatic Castration-resistant Prostate Cancer

Yezi Zhu [a,1], Adam Sharp [b,c,1], Courtney M. Anderson [d], John L. Silberstein [a], Maritza Taylor [a], Changxue Lu [a], Pei Zhao [a], Angelo M. De Marzo [a,e,f], Emmanuel S. Antonarakis [f], Mindy Wang [d], Xingyong Wu [d], Yuling Luo [d], Nan Su [d], Daniel Nava Rodrigues [b], Ines Figueiredo [b], Jonathan Welti [b], Emily Park [d], Xiao-Jun Ma [d], Ilsa Coleman [g], Colm Morrissey [h], Stephen R. Plymate [h], Peter S. Nelson [g,h], Johann S. de Bono [b,c,], Jun Luo [a,*]*

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Abstract

Background: Androgen receptor splice variant 7 (AR-V7) has been implicated in resistance to abiraterone and enzalutamide treatment in men with metastatic castration-resistant prostate cancer (mCRPC). Tissue- or cell-based in situ detection of AR-V7, however, has been limited by lack of specificity.
Objective: To address current limitations in precision measurement of AR-V7 by developing a novel junction-specific AR-V7 RNA in situ hybridization (RISH) assay compatible with automated quantification.
Design, setting, and participants: We designed a RISH method to visualize single splice junctions in cells and tissue. Using the validated assay for junction-specific detection of the full-length AR (AR-FL) and AR-V7, we generated quantitative data, blinded to clinical data, for 63 prostate tumor biopsies.
Outcome measurements and statistical analysis: We evaluated clinical correlates of AR-FL/AR-V7 measurements, including association with prostate-specific antigen progression-free survival (PSA-PFS) and clinical and radiographic progression-free survival (PFS), in a subset of patients starting treatment with abiraterone or enzalutamide following biopsy.
Results and limitations: Quantitative AR-FL/AR-V7 data were generated from 56 of the 63 (88.9%) biopsy specimens examined, of which 44 were mCRPC biopsies. Positive AR-V7 signals were detected in 34.1% (15/44) mCRPC specimens, all of which also co-expressed AR-FL. The median AR-V7/AR-FL ratio was 11.9% (range 2.7–30.3%). Positive detection of AR-V7 was correlated with indicators of high disease burden at baseline. Among the 25 CRPC biopsies collected before treatment with abiraterone or enzalutamide, positive AR-V7 detection, but not higher AR-FL, was significantly associated with shorter PSA-PFS (hazard ratio 2.789, 95% confidence interval 1.12–6.95; $p = 0.0081$).

[1] These authors contributed equally to this work.
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Conclusions: We report for the first time a RISH method for highly specific and quantifiable detection of splice junctions, allowing further characterization of AR-V7 and its clinical significance.
Patient summary: Higher AR-V7 levels detected and quantified using a novel method were associated with poorer response to abiraterone or enzalutamide in prostate cancer.
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1. Introduction

Androgen receptor splice variant 7 (AR-V7) is one of the AR aberrations implicated in the development of castration-resistant prostate cancer (CRPC) [1,2]. AR-V7 originates from contiguous splicing of AR exons 1, 2, and 3 and the cryptic exon 3 (CE3) within the canonical intron 3 of the *AR* gene [1]. Specific detection of AR-V7 can be achieved by targeting the exon 3/CE3 splice junction via reverse transcription polymerase chain reaction (RT-PCR) [3]. A number of previous studies have demonstrated the prognostic value of AR-V7 detection by RT-PCR in men with metastatic CRPC (mCRPC) treated with abiraterone and/or enzalutamide. These studies used biological substrates such as prostate cancer tissues [4–8] and liquid biopsy samples, including circulating tumor cells (CTCs) [9–11], plasma exosomes [12], peripheral blood mononuclear cells (PBMCs) [13], and even whole blood samples [14,15]. While these approaches generally allow sensitive and specific detection of AR-V7, they are limited by a number of analytical and preanalytical challenges mainly attributable to low amounts of AR-V7 mRNA in liquid biopsy samples [16]. Critically, determination of AR-V7 status and its quantification were not possible in a significant proportion of mCRPC patients who were CTC-negative, even though the CTC-based AR-V7 test has been analytically validated and implemented in a clinical laboratory [17].

An alternative and potentially complementary approach to RT-PCR–based detection is RNA in situ hybridization (RISH). In contrast to the RT-PCR approach, RISH allows visualization of gene expression with spatial and morphological context [18]. Traditional RISH methods have been hampered by low sensitivity and a low signal-to-noise ratio, as well as the time-consuming effort required to develop experimental protocols for each detection target [19]. The RNAscope method is a recently developed RISH technique that uses an integrated probe design and signal amplification strategy to amplify target-specific signals by thousands fold without amplifying the background noise [20]. Importantly, this technique is compatible with routine formalin-fixed paraffin-embedded (FFPE) tissues. Following an initial report on AR-V7 RISH by RNAscope [10], two recent reports showed that AR-V7 detected in FFPE tissue specimens by two different RISH methods was associated with CRPC and prognostic in those treated with AR-targeting therapies [21,22]. However, these RISH methods, while revolutionary in RNA detection, require multiple tiling probes covering a target sequence of ~1 kb, and therefore lack the resolution for detecting a variant-specific splice junction. For AR-V7 detection, the published methods [10,21,22] targeted the 1.3-kb CE3 sequence. Because the CE3 sequence is also present in AR genomic DNA and AR pre-mRNA that are retained in the nucleus before being spliced and exported to the cytoplasm, detection of the CE3 sequence described in these previous studies should not be equated to detection of AR-V7. Indeed, detection of pre-mRNA was reported in a previous study [21] and detection of AR genomic DNA cannot be ruled out, particularly in mCRPC specimens with AR amplification. In addition, specificity for AR-V7 detection that targets the CE3 sequence may be further compromised by simultaneous detection of AR-V9, another androgen receptor variant that shares the same 3′ CE3 sequence [23]. Therefore, accurate detection and quantification of AR-V7 mRNA in intact cells would not be possible given the lack of resolution and detection specificity of existing RISH methods.

In the present study, we developed a novel RISH detection method targeting a single splice junction using probes straddling the targeted junction. We applied this novel method to detect and quantify AR-V7, by targeting the exon 3/CE3 junction, and full-length AR (AR-FL), by targeting the exon 7/exon 8 junction. Following validation of junction-specific detection of the AR transcripts in cell lines and in FFPE specimens from mCRPC patients, we applied the prototype technology and quantified AR-V7/AR-FL levels in biopsies from mCRPC patients. We then conducted exploratory clinical correlative analysis for men treated with abiraterone or enzalutamide. We present the first example of visualization of splice junctions in morphologically intact cells, and demonstrate for the first time a highly specific and quantifiable AR-V7 RISH test for detection of clinically significant levels of AR-V7 mRNA in mCRPC patients.

2. Patients and methods

2.1. Patients

Two biopsy cohorts, one from the Johns Hopkins University School of Medicine (JHU cohort) and one from the Institute of Cancer Research and Royal Marsden NHS Foundation Trust (UK cohort), were used in this study. For the JHU cohort, 35 patients with metastatic prostate cancer gave informed consent to undergo the biopsy procedure under a study protocol approved by the institutional review board. Within this unselected and diverse cohort (Supplementary Table 1), nine patients with mCRPC underwent treatment with abiraterone or enzalutamide immediately following the biopsy procedure. For the UK cohort, 28 retrospective biopsies, including mainly bone marrow and prostate biopsies (Supplementary Table 1) were selected from patients treated with first-line abiraterone or enzalutamide (mainly abiraterone) following the biopsies. All study participants had given written informed

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consent and were enrolled in institutional protocols approved by a multicenter research ethics committee (Chelsea Research Ethics Committee, reference 04/Q0801/60). There were no other sample selection criteria; all samples tested are included in Supplementary Table 1. All experimental processes were performed while blinded to the sample type and related data.

2.2. RISH by BaseScope

The BaseScope assays (Advanced Cell Diagnostics, Inc., Hayward, CA) for AR-FL/AR-V7 were developed to achieve junction-specific detection of the AR transcripts. The BaseScope assay is based on the RNAscope technology [20] but uses an additional signal amplification step and requires only one "double Z" (1 ZZ) probe pair for single-molecule detection. The 1-ZZ probe for AR-V7 was designed to target the AR-V7–specific junction of exon 3 and CE3 (AR-E3/CE3) (ZZ probe target sequence GAC TCT GGG AGA AAA ATT CCG GGT TGG CAA TTG CAA GCA TCT C), and the 1-ZZ probe for AR-FL was designed to target the splice junction of exon 7 and exon 8 (AR-E7/E8) (ZZ probe target sequence GCT CAC CAA GCT CCT GGA CTC CGT GCA GCC TAT TGC GAG A), as illustrated schematically in Figure 1A. For each sample, four probes were used in four adjacent sections: AR-E7/E8, AR-E3/CE3, 1-ZZ Hs-POLR2A as a positive control, and 1-ZZ DapB as a negative control. Slides with negative POLR2A staining ($n = 4$ in the JHU cohort and $n = 3$ in the UK cohort), indicative of poor tissue quality, were excluded from analysis. Automated quantification of AR transcripts was performed using RNAscope Spot Studio software (Supplementary material).

2.3. Statistical analysis

The baseline clinical characteristics in the JHU cohort ($n = 28$, excluding 4 disqualified samples and 3 samples diagnosed with small cell carcinoma/neuroendocrine [SC/NE]), and UK cohort ($n = 16$, including all those collected before abiraterone or enzalutamide treatment) were separately compared according to AR-V7 status (positive vs negative). Categorical and continuous variables were compared using Fisher's exact test and a Mann-Whitney test, respectively.

Exploratory evaluations of an association between AR status and treatment outcome were conducted among the combined cohort of all patients treated with abiraterone or enzalutamide ($n = 25$) following the biopsy procedure. Outcome measures included

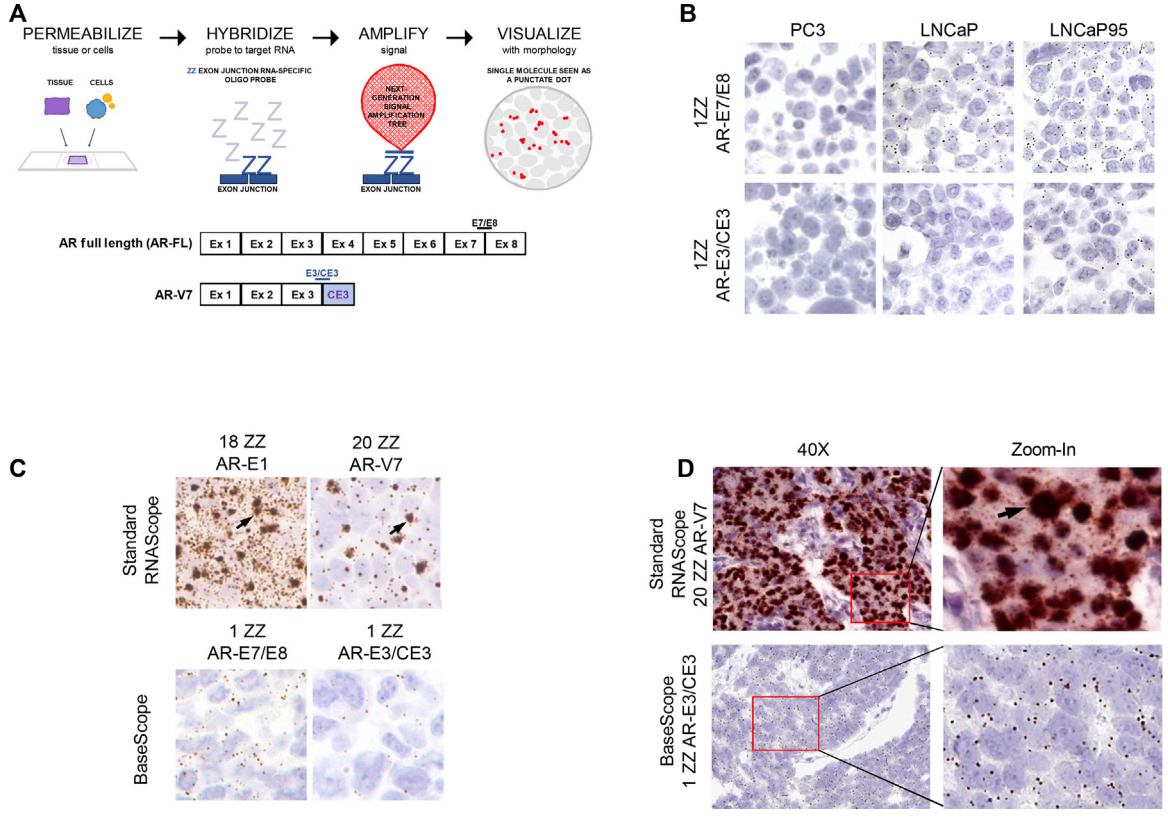


Fig. 1 – Development of the BaseScope RNA in situ hybridization assay for detection of splice junctions specific to AR-FL and AR-V7. (A) Schematic illustration of the BaseScope assay and the splice junctions targeted for probe design. Top: Overview of the BaseScope assay workflow. Sections containing fixed tissues or cells were permeabilized, and exposed mRNA were hybridized with a single pair of BaseScope probes (ZZ pair) that straddle the exon/exon junction of interest. Following amplification by an advanced, next-generation signal amplification system, junction-specific signals can be visualized as punctate dots under a standard bright-field microscope. Bottom: AR splice junctions targeted for BaseScope probe design. The splice junction between AR exons 7 and 8 (E7/E8) was targeted for specific detection of the full-length AR (AR-FL), while the splice junction between exon 3 and cryptic exon 3 (CE3) (E3/CE3) was targeted for specific detection of AR-V7. (B) Specificity of the BaseScope AR probes as demonstrated by signals detected in prostate cancer cell lines with known AR-FL/AR-V7 profiles. The cell lines PC3 (AR-FL–negative, AR-V7–negative), LNCaP (AR-FL–positive, AR-V7–negative), and LNCaP95 (AR-FL–positive, AR-V7–positive) were stained using the following 1 ZZ BaseScope probes: AR-E7/E8 for AR-FL and AR-E3/CE3 for AR-V7. (C) The BaseScope assay detects mature mRNA exclusively in cytoplasm. LNCaP95 cells (AR-FL–positive, AR-V7–positive) were stained with standard RNAscope (top) and BaseScope (bottom) assays. Both cytoplasmic and intranuclear (arrows) signals were detected with 18 ZZ AR-E1 and 20 ZZ AR-V7 probes used in the standard RNAscope (top) assay, while the 1 ZZ probes used in the BaseScope (bottom) assays detected punctate signals representing mature mRNA exclusively in the cytoplasm. (D) Comparison of AR-V7 signals detected by the standard RNAscope and BaseScope assays in a metastatic castration-resistant prostate cancer (mCRPC) biopsy specimen. The same mCRPC biopsy core was processed and stained for AR-V7 using 20 ZZ AR-V7 probes in the standard RNAscope assay (top) and the BaseScope assay (bottom) using the 1 ZZ AR-E3/CE3 probe. Note the intense intranuclear AR-V7 signal (arrow) with the RNAscope assay.

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prostate-specific antigen progression-free survival (PSA-PFS) and clinical/radiographic progression-free survival (PFS). Survival time differences were analyzed using a log-rank test. In all tests, $p \leq 0.05$ was considered statistically significant. Statistical analyses were performed using GraphPad Prism version 7.0 (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Junction specific AR RISH assay development

The splice junction between AR exon 3 and CE3 (E3/CE3) is specific to AR-V7 mRNA. Detection of this junction (ie, specific detection of AR-V7) has not been possible in morphologically intact cells and the native tissue environment because of technical constraints of the existing RNAscope RISH assay requiring 20 ZZ probes targeting the 1.3-kb CE3 sequence [10,21,22]. We designed and optimized a novel AR-V7 RISH probe consisting of a 1-ZZ pair of oligonucleotide sequences straddling the AR E3/CE3 junction, in parallel with a novel 1-ZZ probe for the AR-FL that straddles the splice junction between AR exon 7 and exon 8 (E7/E8; Fig. 1A). To validate the specificity of these novel junction-specific AR probes, we first performed RISH in human prostate cancer cell lines with known AR-FL/AR-V7 expression profiles. As shown in Figure 1B, probes each consisting of 1-ZZ pairs (termed BaseScope probes) detected punctate cytoplasmic signals consistent with the known AR-FL/AR-V7 status of the cell lines. The improvement in specificity of the BaseScope assay over the RNAscope assay was shown by comparison of two RISH assays in LNCaP95 cells (positive for both AR-FL and AR-V7). Consistent with previous findings [21], the RNAscope probes (20 ZZ over ~1 kb) designed to target the entire CE3 sequence detected both cytoplasmic dots from mature AR-V7 mRNA and nonspecific intranuclear signals from AR-V7 pre-mRNA (Fig. 1C), precluding accurate quantification. By contrast, the junction-specific AR-V7 probe (<50 bases) detected signals for mature AR-V7 mRNA exclusively in the cytoplasm (Fig. 1C). Parallel comparison of these two AR-V7 RISH assays in a metastatic CRPC biopsy specimen further confirmed this distinction (Fig. 1D).

Although the novel prototype AR-V7 RISH assay appeared to detect fewer transcripts than the RNAscope assay owing to significantly fewer ZZ pairs for detection (Fig. 1C,D), the junction-specific detection made it possible to conduct automated quantification of AR-V7–specific signals (Supplementary Fig. 1). As shown in Supplementary Figure 2, quantitative measurements of AR-V7, AR-FL, and AR-V7/AR-FL ratios from the novel assay were consistent with values derived from RT-PCR in a set of metastatic biopsies from CRPC patients ($n = 13$) with matching FFPE and frozen specimens. AR-V7 can also be detected in a tissue microarray containing autopsy specimens from CRPC patients (Supplementary Fig. 3), although no statistically significant correlation between RISH and RNA-Seq was found ($n = 7$; Supplementary Fig. 3). Therefore, we have demonstrated the validity and feasibility of AR-V7 quantification by the novel RISH assay.

3.2. AR-V7/AR-FL quantification in biopsy specimens and correlation with baseline clinical characteristics

Having established the novel junction-specific AR RISH method, we generated quantitative AR-V7 and AR-FL RISH data from two independent biopsy cohorts while blinded to the sample identity. The first cohort consisted of 35 biopsies from patients with metastatic prostate cancer collected at JHU (Supplementary Table 1). After excluding four samples that did not meet the quality control criteria (no signal with the POLR2A-positive control probe), samples were grouped into SC/NE ($n = 3$), castration-sensitive prostate cancer (CSPC; $n = 3$), and CRPC ($n = 25$) on the basis of pathology reports and clinical notes. Representative images showing AR-V7/AR-FL measurements were shown in Figure 2A, and quantitative values for all 31 samples were shown in Figure 2B. Notably, samples with AR-V7 signals were always concurrently positive for AR-FL and, without exception, AR-FL measurement values were higher than those for AR-V7 (Fig. 2B and Supplementary Table 1).

Because AR-V7 values exhibited a continuous range (Supplementary Table 1), it was necessary to define AR-V7 "positivity" before clinical correlative analysis. We used a cutoff value of 0.4 to define AR-V7 "positivity" (Supplementary material). Using this cutoff, six of the 12 samples (50%) that had an AR-V7 RISH value above zero were AR-V7–positive (Fig. 2B and Supplementary Table 1). AR-V7 positivity was associated with prior treatment with ketoconazole, abiraterone, or enzalutamide, but not with any other baseline variable in this set of 28 biopsies (Supplementary Table 2). After defining the cutoff, a second cohort of 28 biopsies (UK cohort) was evaluated (Supplementary Table 1) using the same RISH method, among which nine biopsies were AR-V7–positive according to the predefined cutoff (Fig. 2C). In this cohort, 16 samples had baseline data available at the sampling time before treatment with abiraterone or enzalutamide (Supplementary Table 2). AR-V7 positivity was associated with serum PSA, but not with any other baseline variables in this cohort (Supplementary Table 2). Quantitative AR-V7/AR-FL RISH values from the combined 56 biopsy samples are presented in Supplementary Figure 4. Notably, all CSPC specimens ($n = 9$) and SC/NE samples ($n = 3$) were negative for AR-V7 according to this novel detection method (Supplementary Fig. 4). Among the CRPC specimens ($n = 44$), the AR-V7–positive rate was 34.1% (15/44), and the median AR-V7/AR-FL ratio was ~11.9% among AR-V7–positive samples (Supplementary Fig. 4).

3.3. Comparison of AR-V7 RISH and AR-V7 immunohistochemistry (IHC)

Detection of clinically significant AR-V7 can also be achieved by IHC using antibodies raised against the AR-V7–specific peptide [8,24]. However, detection of nonspecific, unidentified protein targets in AR/AR-V7–negative cells has been reported [8]. To allow comparison of AR-V7 RISH and IHC results, we developed an optimized AR-V7 IHC method (Supplementary material) that uses a new AR-V7

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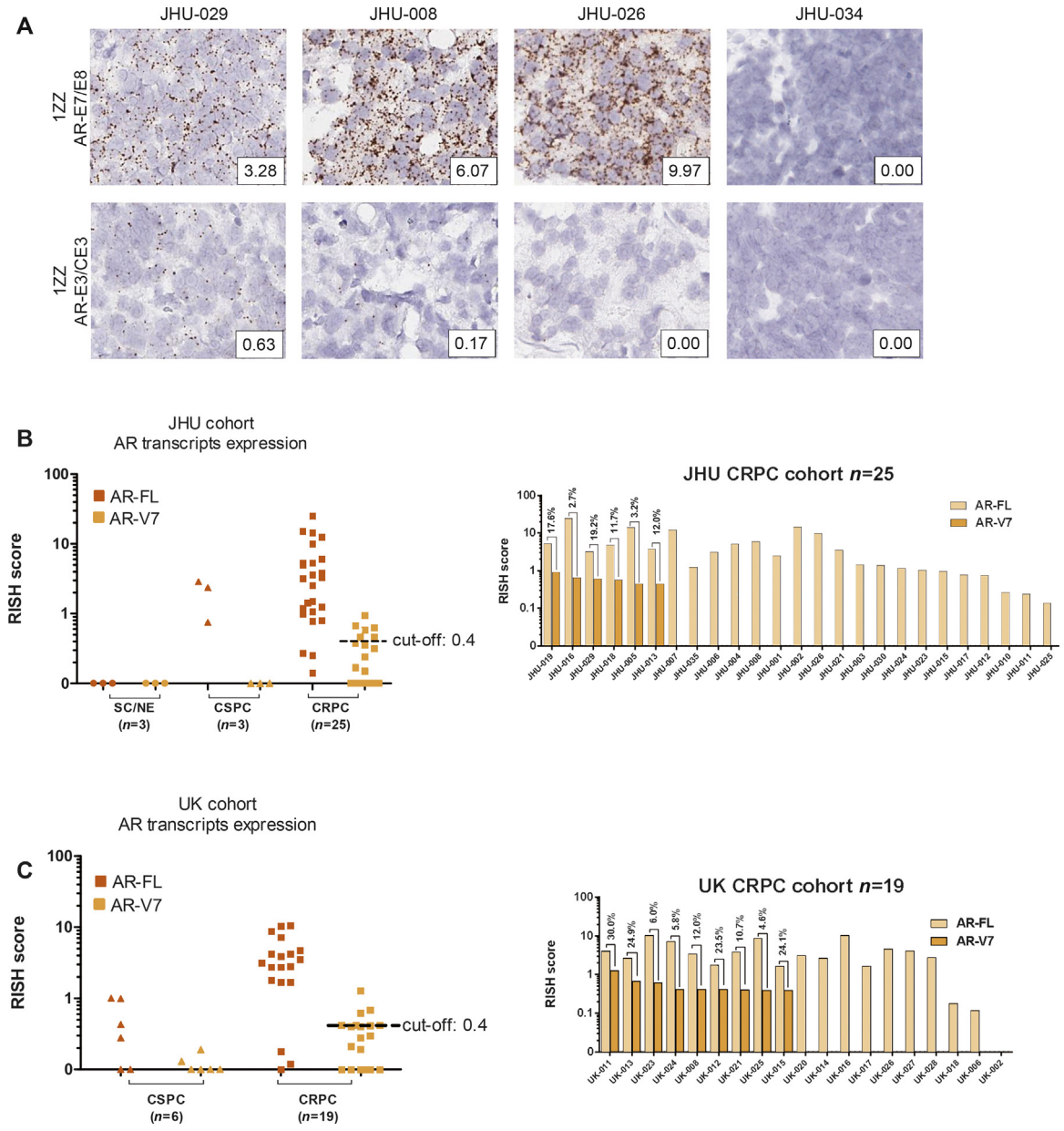


Fig. 2 – Detection and quantification of AR-FL and AR-V7 in two independent biopsy cohorts. (A) Representative images and quantified RNA in situ hybridization (RISH) scores for AR-FL (probe AR-E7/E8, top) and AR-V7 (probe AR-E3/CE3, bottom) mRNA detection in tissue biopsies from patients with metastatic prostate cancer. (B) AR quantification by junction-specific RISH in the JHU cohort. Left panel: Quantified AR-FL and AR-V7 mRNA expression in three small cell carcinoma/neuroendocrine (SC/NE) biopsies, three castration-sensitive prostate cancer (CSPC) biopsies, and 25 castration-resistant prostate cancer (CRPC) biopsies from the JHU cohort. The line indicates the value for the AR-V7 cutoff (0.4). Right panel: Relative AR-V7/AR-FL values and ratios (for those that were AR-V7–positive defined by the cutoff) in each of the 25 CRPC specimens. (C) AR quantification by junction-specific RISH in the UK cohort. Left panel: Quantified AR-FL and AR-V7 mRNA expression in six CSPC biopsies and 19 CRPC biopsies from the UK cohort. The line denoted the AR-V7 cutoff (0.4). Right panel: Relative AR-V7/AR-FL values and ratios (for those that were AR-V7–positive defined by the cutoff) in each of the 19 CRPC specimens.

antibody that specifically detected AR-V7 protein in cells with known AR-V7 status (Fig. 3A). In addition, areas of positive IHC staining corresponded to positive RISH staining in a sample with mixed SC/NE and adenocarcinoma histology (Fig. 3B). To further characterize the novel AR-V7 RISH test, we compared AR-V7 measurements obtained with RISH and IHC methods (Supplementary material) in matched sections from 36 mCRPC biopsies (mainly from the UK cohort). The IHC results robustly correlated with the RISH results (Fig. 3C,D, Supplementary Table 3).

3.4. *Association with treatment outcome*

We conducted exploratory treatment outcome analyses after combining biopsies collected from patients treated with abiraterone or enzalutamide in the two cohorts. A total of 25 patients (n = 9 in the JHU cohort and n = 16 in the UK cohort) were biopsied before treatment with abiraterone or enzalutamide. PSA response rates were not significantly different by AR-V7 status, although a numerically better PSA response rate was observed in subjects with AR-V7 scores

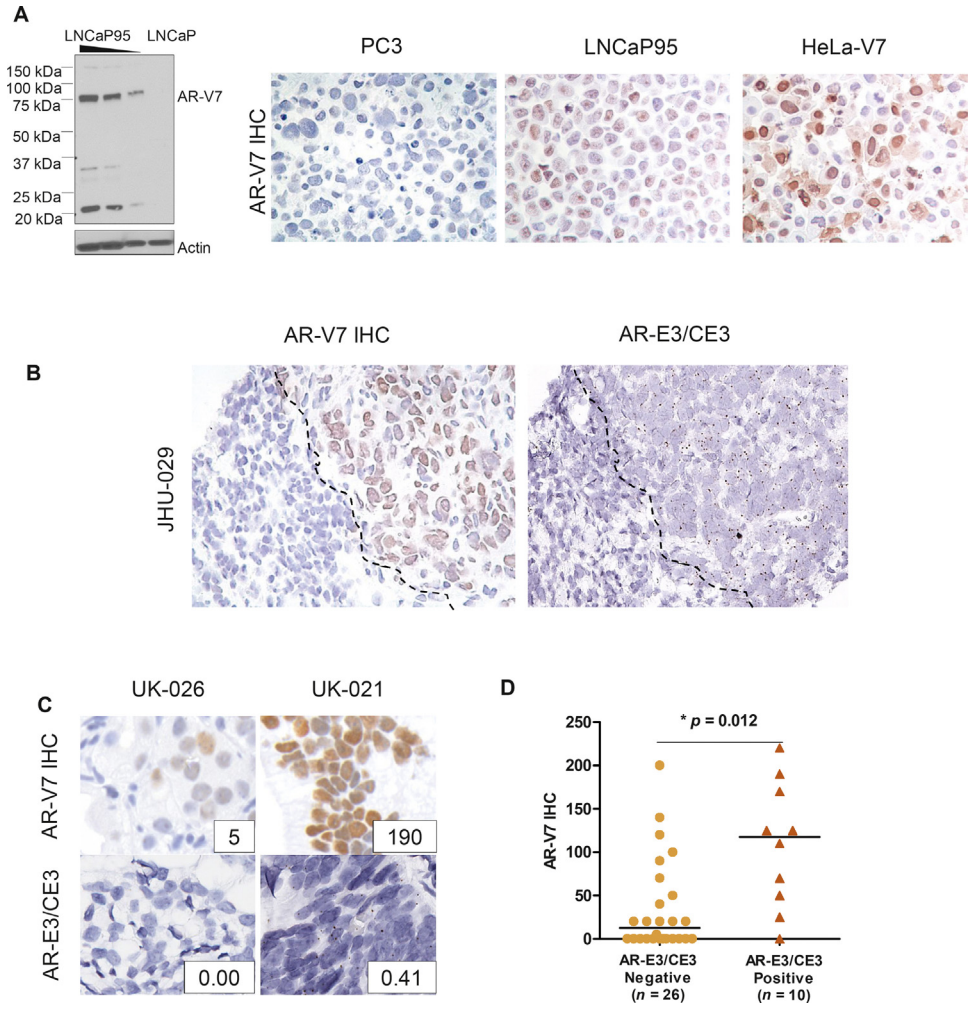


Fig. 3 – Comparison of AR-FL/AR-V7 levels quantified by RNA in situ hybridization (RISH) and immunohistochemistry (IHC). (A) Western blot and IHC using the RevMab-RM7 AR-V7 antibody in prostate cancer cells with known AR profiles. Western blot showed the ~80-kDa AR-V7 band consistent with known AR-V7 status in LNCaP (AR-V7–negative) and LNCaP95 (AR-V7–positive) cells. Different doses of LNCaP95 protein lysates were loaded. Non-specific staining was shown at approximately 30 and 23 kDa. β-Actin was blotted as a loading control. In IHC experiments, PC3 cells showed negative AR-V7 IHC staining, LNCaP95 cells showed moderate AR-V7 staining, and HeLa cells transiently transfected with AR-V7 showed the highest level of AR-V7 IHC staining (heterogeneity reflected the transfection efficiency). (B) AR-V7 IHC staining was compared with the AR-E3/CE3 BaseScope assay in a metastatic castration-resistant prostate cancer CRPC biopsy with mixed SC/NE and adenocarcinoma histology. (C) Representative images and quantified scores comparing IHC and RISH results in biopsies from the UK cohort. (D) Comparison of AR-V7 IHC values in AR-V7–positive (n = 10) and AR-V7–negative biopsies (n = 26) defined by junction-specific RISH. The p value was determined using an unpaired t test.

below the cutoff (Supplementary Fig. 5). AR-V7 status was significantly associated with shorter PSA-PFS (p = 0.0081; Fig. 4A) and showed a trend towards an association with PFS (p = 0.054; Fig. 4B). However, AR-FL status was not associated with either PSA-PFS or PFS in this combined cohort (Fig. 4C,D).

4. Discussion

Here we present the first example of visualization of splice junctions in morphologically intact cells using a novel RISH assay, and quantitative analysis of AR-FL/AR-V7 mRNA levels in FFPE biopsies obtained from mCRPC patients. Although the study was limited by cohort size, AR-V7 status was correlated with clinical characteristics and clinical outcomes after treatment with abiraterone or enzalutamide. This novel AR-V7 RISH test may help to address some of the limitations of the RT-PCR–based test, for which clinical development may be limited by preanalytical and analytical challenges because of reliance on detection of CTCs and low levels of the analytes in liquid biopsy samples [16]. For example, the CTC-based test requires relatively fresh blood samples delivered and processed within 24 h of collection. In addition, reporting of AR-V7 status would not be possible for patients with no detectable CTCs, although they usually present with lower disease burden and favorable treatment outcome [25]. For AR-V7 tests using biological substrates other than CTCs (exosomes, PBMCs, and whole blood), full analytical performance data have not been reported [12–15]. Although tissue-based tests require an invasive sampling procedure and may be further compromised by tissue heterogeneity, the role of molecular aberrations detected in tissue biopsies

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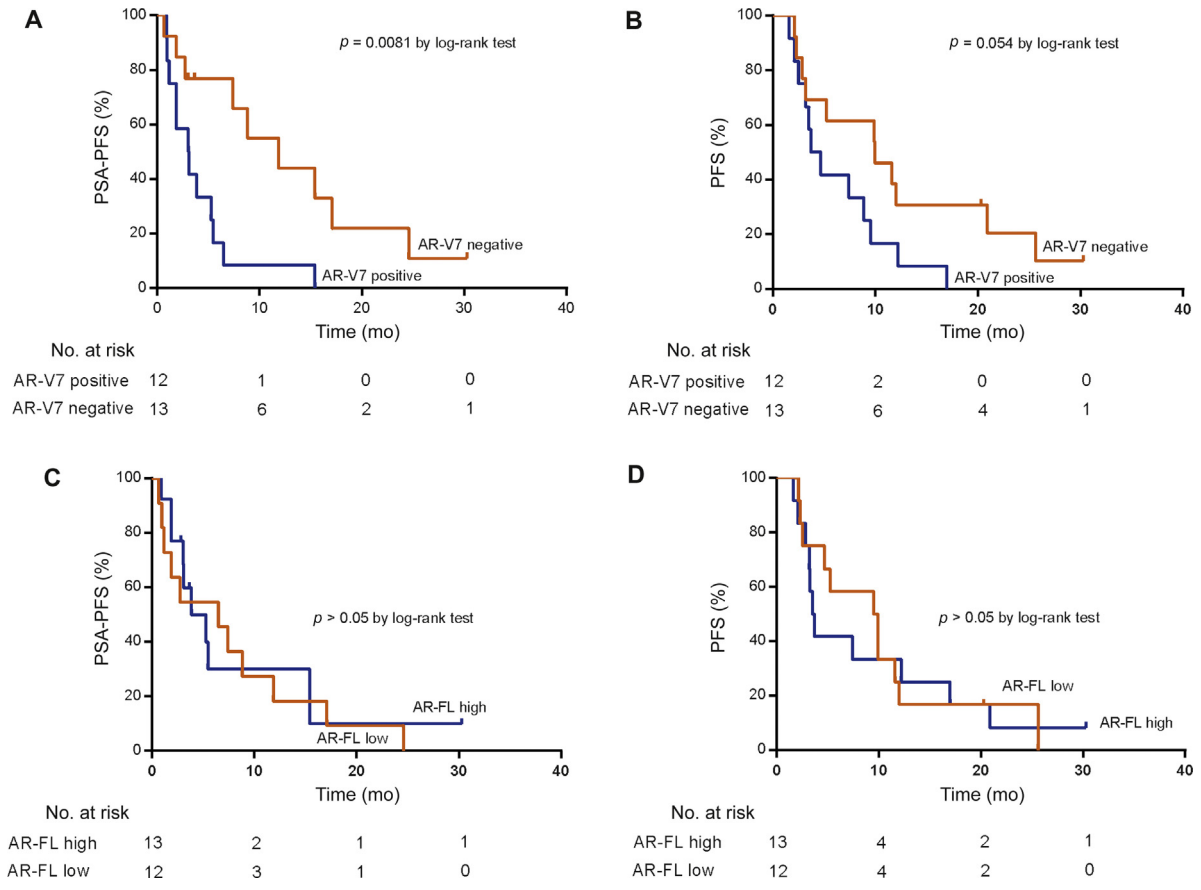


Fig. 4 – Exploratory clinical outcome analysis of AR-V7 and AR-FL status determined by BaseScope assay in patients treated with abiraterone or enzalutamide ($n = 25$). (A) Kaplan-Meier analysis of prostate-specific antigen progression-free survival (PSA-PFS) by AR-V7 status. The median PSA-PFS was 3.1 mo in AR-V7–positive patients and 11.8 mo in AR-V7–negative patients (AR-V7 positivity hazard ratio [HR] for PSA-PFS 2.789, 95% confidence interval [CI] 1.12–6.95; $p = 0.0081$ by log-rank test). (B) Kaplan-Meier analysis of clinical or radiographic progression-free survival (PFS) by AR-V7 status. The median clinical or radiographic PFS was 4.2 mo in AR-V7–positive patients and 9.9 mo in AR-V7–negative patients (AR-V7 positivity HR for PFS 2.118, 95% CI 0.89–5.02; $p = 0.054$ by log-rank test). (C) Kaplan-Meier analysis of PSA-PFS by AR-FL status. The median PSA-PFS was 3.9 mo in AR-FL high patients and 6.5 mo in AR-FL low patients (AR-FL high status HR for PSA-PFS 1.167, 95% CI 0.4957–2.745; $p = 0.7145$ by log-rank test). (D) Kaplan-Meier analysis of clinical or radiographic PFS by AR-FL status. The median clinical or radiographic PFS was 3.6 mo in AR-FL high patients and 9.8 mo in AR-FL low patients (AR-FL high status HR for PFS 1.073, 95% CI 0.464–2.475; $p = 0.8675$ by log-rank test).

remains important [26]. It may be possible to develop treatment or patient selection markers on the basis of a biopsy, as indicated in a recent article suggesting the feasibility of obtaining molecular information representative of the patient by sampling a single metastasis [27]. Therefore, the newly developed capability for detection and quantification of a critical AR aberration in biopsy specimens, upon further work, may address a significant hurdle in measurement science for treatment and patient selection.

In situ detection of AR-V7 can also be achieved by IHC. Two recent studies demonstrated the prognostic value of AR-V7 detection by IHC in tissue specimens or CTCs immobilized on glass slides [8,24]. However, nonspecific signals from this antibody were acknowledged [8]. While antibody-based tests have a number of advantages, development of an optimized antibody is technically challenging and time-consuming. In our comparison of RISH and IHC (Fig. 3), we used a new AR-V7 antibody that was determined to be more specific than those evaluated in previous studies [8,24]. Although the measurements were generally concordant (Fig. 3), discrepancies were found (Supplementary Table 3), potentially reflecting measurement variations that may be related to nonspecific detection by IHC or different regulation of translation from mRNA to protein, as well as protein degradation among cases. Nevertheless, there is merit in further developing IHC-based detection methods for AR-V7, particularly since AR splice variant protein may have a longer half-life than its parent mRNA transcript [28]. Importantly, however, the RISH method described here can also be adapted for application in the CTC platforms described earlier [24] to allow further comparison of RISH and IHC.

Owing to the small sample size limited by difficulty in obtaining an adequate number of pretreatment biopsies, our clinical correlative analysis is exploratory and we did not conduct multivariable analysis adjusting for other prognostic factors. The small sample size also limited our ability to further optimize and validate the cutoff used to define AR-V7 status. As a result of these limitations, the potential clinical utility of the tissue-based RISH test (eg, in CTC-negative patients) remains to be determined. The main goal of the present study was to develop and

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validate a novel in situ AR-V7 test for detection of clinically significant levels of AR-V7, using a novel prototype method that had recently undergone substantial improvement with respect to detection sensitivity (personal communication between J.L. and X.M.). The present study achieved this goal with the clinical resources currently available to the study investigators. Full clinical validation may be conducted in tissue or immobilized CTC specimens collected from ongoing clinical trials, and prospective studies can be designed to evaluate the potential utility of this novel test in drug development and patient management.

5. Conclusions

We demonstrated for the first time a highly specific and quantifiable AR-V7 RISH test for detection of clinically significant levels of AR-V7 mRNA in prostate tissue specimens. Our data lend further credence to the clinical importance of AR splice variants and describe a novel assay that merits further clinical qualification in both tissue and CTCs in future clinical trials.

Author contributions: Jun Luo had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study concept and design: Zhu, Sharp, Anderson, Lu, Ma, Y. Luo, Plymate, Nelson, de Bono, J. Luo.
Acquisition of data: Zhu, Sharp, Anderson, Silberstein, Taylor, Lu, Zhao, Demarzo, Antonarakis, Wang, Wu, Su, Nava Rodrigues, Figueiredo, Coleman, Morrissey.
Analysis and interpretation of data: Zhu, Sharp, Anderson, Lu, Demarzo, Antonarakis, Figueiredo, Welti, Park, Ma, Coleman, Morrissey, de Bono, J. Luo.
Drafting of the manuscript: Zhu, Sharp, de Bono, J. Luo.
Critical revision of the manuscript for important intellectual content: Anderson, Ma, Coleman, Morrissey, Plymate, Nelson.
Statistical analysis: Zhu, Sharp, Nava Rodrigues, Figueiredo, Welti, Coleman, Morrissey, de Bono, J. Luo.
Obtaining funding: J. Luo, de Bono, Ma, Y. Luo, Plymate, Nelson.
Administrative, technical, or material support: Anderson, Silberstein, Taylor, Demarzo, Antonarakis, Wang, Wu, Y. Luo, Su, Nava Rodrigues, Figueiredo, Park, Ma, Coleman, Morrissey, Plymate, Nelson.
Supervision: J. Luo, de Bono, Plymate, Nelson.
Other: None.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.eururo.2017.08.009.

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abrogate AR-V7 signalling and induce robust responses in patients with CRPC. Only then will the biological and clinical significance of AR-V7 be truly confirmed; this remains a priority for the field and an unmet urgent clinical need.

Overcoming treatment resistance

Targeting the core spliceosome complex. Several bacterial fermentation products with potent anticancer activity, owing to an ability to modulate the core spliceosome complex, have been identified using large-scale drug screens. The molecules can be broadly categorized into three classes, namely, pladienolides, herboxidienes, and spliceostatins (TABLE 1). These compounds are structurally distinct, although they also share a common mechanism of action whereby they bind with and inhibit SF3B1 (REF.[110]). Under nonmalignant conditions, SF3B1 interacts with U2AF65 to recruit the snRNP U2 to the 3′ splice site of the intron. However, by binding to SF3B1, these compounds interfere with the early stages of spliceosome assembly and therefore destabilize the interactions between U2 and its pre-mRNA target, thus modifying splice site selection[111]. This perturbation of U2 also causes an accumulation on unspliced pre-mRNA in the nucleus, of which a small proportion has been shown to 'leak out' into the cytoplasm and undergo translation, generating aberrant protein products, which themselves can be cytotoxic[112,113]. In addition, several of these compounds have also been shown to decrease the expression of VEGF, thus inhibiting angiogenesis in chick chorioallantoic membrane assays[114].

The potential clinical utility of bacterial fermentation products has been adequately demonstrated in preclinical studies, as observed, for example, in the dose-dependent inhibition of growth seen in experiments involving prostate cancer xenografts following treatment with pladienolide B[115]. However, the findings of early phase clinical trials have been more mixed. In two phase I, open-label, single-arm, dose-escalation studies, investigators assessed the safety and efficacy of pladienolide E7107 in patients with locally advanced or metastatic solid tumours. Data from both trials showed that E7107 was generally well tolerated and produced both dose-dependent and reversible inhibition of pre-mRNA processing in target genes in vivo[116], although both trials

Table 1 | **Small molecules reported to target the process of splicing**

Agents	Stage of development	Effects and/or mechanism of action	Refs
Targeting the core spliceosome complex			
Pladienolides A–G	Preclinical	Binds with and inhibits SF3B1, thus destabilizing the recruitment of snRNP U2; decreases levels of circulating VEGF and thus inhibits tumour angiogenesis; cell cycle arrest at G1 and G2–M; disrupts spliceosome assembly; generates a truncated form of the cell cycle inhibitor cyclin-dependent kinase inhibitor p27 that remains functional; and reduces the number of nuclear speckles	131,132
E7107	Phase I		114–116
Herboxidiene (GEX1A)	Preclinical		133
FR901463, FR901464, and FR901465	Preclinical		134
Meayamycin B	Preclinical		135
Spliceostatin A	Preclinical		112
H3B-8800	Phase I (NCT02841540)	Small-molecule modulator of SF3B1 that displays preferential lethality towards spliceosome-mutant cancer cells owing to retention of short, GC-rich introns	118
Targeting spliceosomal regulatory proteins			
TG003	Preclinical	Competitive inhibitor of CLK1, CLK2, and CLK4 binding with ATP that inhibits CLK enzymatic phosphorylation, activates splicing factors such as SR proteins and leads to dissociation of nuclear speckles	120
SRPIN340	Preclinical	Competitive antagonist of SRPK1 and SRPK2 binding of ATP and a nicotinamide inhibitor, which inhibits SRPK phosphorylation and activation of splicing factors such as SR proteins and modulates splicing of VEGF	136
Cpd-1, Cpd-2, and Cpd-3	Preclinical	Inhibits both CLKs (CLK1 and CLK2) and SRPKs (SPRK1 and SPRK2), which are components of the splicing machinery that are crucial for exon selection, reduces phosphorylation of SR proteins, causes enlargement of nuclear speckles, and causes widespread splicing alterations	137
GSK525762	Phase I (NCT03150056)	Inhibits the BET family proteins BRD2, BRD3, BRD4, and BRDT, downregulates expression of splicing factors, and decreases alternative splicing events in preclinical models	138
ZEN003694	Phase I/II (NCT02711956)		139
OTX105/MK-8628	Phase I (NCT02259114)		122
Other small-molecule inhibitors			
Isoginkgetin	Preclinical	Biflavonoid natural plant product that interferes with the recruitment of the snRNPs U4, U5, and U6 and prevents transition from spliceosomal complex A to complex B	125
NB-506	Preclinical	Inhibits SRFS1 phosphorylation by topoisomerase I, disrupts early spliceosome assembly in vitro, and produces a cytotoxic effect	124

BET, bromodomain and extraterminal domain; BRD, bromodomain-containing protein; BRDT, bromodomain testis-specific protein; CLK, dual specificity protein kinase CLK; SF3B1, splicing factor 3B subunit 1; snRNP, small nuclear ribonucleoprotein; SR, serine-rich and/or arginine-rich; SRPK, serine and arginine protein kinase; SRPIN340, *N*-(2-(piperidin-1-yl)-5-(trifluoromethyl)phenyl); SRSF1, SR splicing factor 1.

were suspended owing to unexpected incidences of bilateral optic neuritis[116,117].

H3B-8800, a small-molecule modulator of SF3B1 (REF.[118]), has also entered a phase I clinical trial (NCT02841540). This trial aims to determine the safety and recommended phase II dose in patients with myelodysplastic syndromes, acute myeloid leukaemia, or chronic myelomonocytic leukaemia, in which recurrent heterozygous mutations of SF3B1 are thought to have a pathological role. If found to be efficacious in subsequent phase II and phase III trials, H3B-8800 could provide proof of principle that targeting the spliceosome is a valid treatment strategy, and this could also open a variety of new therapeutic avenues. However, the toxicity and tolerability of these agents will equally prove to be important factors that will dictate whether or not these agents ever enter routine clinical use.

Targeting spliceosomal regulatory proteins. Rather than targeting the core spliceosome complex, an alternative approach is to modulate splicing by targeting one or more of the regulatory proteins. For example, a variety of compounds have been identified that can inhibit SR protein phosphorylation, and these have been shown in preclinical models to inhibit splicing[119]. TG-003, a benzothiazole, is one such agent and functions as an inhibitor of CLK1, CLK2, and CLK4, all of which are members of the CDC2-like (or LAMMER) family of dual-specificity protein kinases. These kinases are typically involved in the phosphorylation of SR proteins in the nucleus[120], the inhibition of which results in inhibition of splicing and dissociation of spliceosomal nuclear speckles[120].

In the past 5 years, bromodomain and extraterminal domain (BET) inhibition, a promising therapeutic approach that is currently undergoing clinical evaluation in patients with CRPC (NCT03150056 and NCT02711956), has also been shown to affect alternative splicing by modulating spliceosomal regulators[121]. In a study by Asangani et al.[122], the BET inhibitor JQ1 was found to decrease the expression of AR-V7 in preclinical models of CRPC by downregulating the activity of the splicing factors SRSF1 and U2AF65, and in doing so, re-sensitized enzalutamide-resistant prostate cancer cells to AR-targeted therapy. However, as with therapeutic agents targeting the core spliceosomal complex, the long-term success of BET inhibition as a clinically useful therapeutic modality will hinge on the toxicity profile of BET inhibitors, as determined in the ongoing clinical trials.

Other small-molecule inhibitors of the spliceosome. Several other small molecules have also been identified as being capable of modulating the spliceosome, some of which have been reported to have efficacy in preclinical cancer models. However, these studies have generally been limited by their use of cell-free and non-mammalian models[123], and, as such, the therapeutic application of many of these agents is currently considered limited. Despite this lack of clinical implementation thus far, some interesting results have been seen with a number of these agents. For example, NB-506, a glycosylated indolocarbazole derivative that inhibits the capacity of topoisomerase I to phosphorylate SRFS1, has been shown to disrupt early spliceosome assembly and produces a cytotoxic effect in murine P388 leukaemia cells[124]. In addition, preclinical antitumour activity of the biflavonoid natural plant product isoginkgetin has also been demonstrated, which occurs, at least in part, through the ability of this agent to interfere with the recruitment of the snRNPs U4, U5, and U6 and to inhibit splicing by precluding the transition from spliceosomal complex A to complex B[125].

Targeting the spliceosome in oncogene-driven cancers. As described previously, *MYC* overexpression places considerable oncogenic stress on the spliceosome, resulting in cells becoming equally dependent on the spliceosome for survival as they are on MYC. This observation has led to the hypothesis that, in these tumours, inhibition of the spliceosome might have an anticancer effect. In support of this view, spliceosome dysregulation through inhibition of SF3B1 using sudemycin D has been reported to increase survival and limit the formation of metastases in xenograft models of MYC-dependent breast cancer[69]. Ultimately, although intriguing, whether this principle will be applicable to other similarly important genomic aberrations or whether the clinical utility of this approach will be limited to a subset of MYC-dependent cancers remains to be seen.

Targeting alternatively spliced variants. When devising therapeutic strategies to target pathological alternatively spliced variants, in addition to considering those generated through the action of the spliceosome, taking into account protein variants generated through alternative alterations such as genomic fusions or rearrangements, including proteins that are altered in many cancers such as programmed cell death 1 ligand 1 (PD-L1), is equally important[90]. As such, while targeting the spliceosome remains a key consideration in this process, given the multiple routes through which alternatively spliced variants can arise, the concept of directly targeting these protein variants, rather than their mechanism of origin, seems logical.

Efforts to target alternatively spliced proteins remain attractive, but doing so directly with small-molecule inhibitors has to date proved challenging, often owing to the inherent nature of these alternatively spliced variants. For example, because truncated alternatively spliced AR-Vs generally lack an LBD, alternative target sites are required to facilitate their inhibition. However, the disordered nature of the AR NTD renders a consistent target site difficult to ascertain and has to date hindered drug discovery efforts, thus necessitating the development of novel therapeutic strategies. One such proposed approach involves the use of monoclonal antibodies such as GP369, which specifically blocks the IIIb splice variant of FGFR2 (REF.[126]). GP369 showed efficacy as an inhibitor of tumour growth in preclinical studies involving human cancer cell lines and tumour xenografts driven by activated FGFR2 signalling[127]. A phase I trial involving patients with advanced-stage

solid tumours known to express FGFR2 was opened (NCT02368951) on this basis, although the trial was terminated early owing to safety concerns regarding the development of nephrotic syndrome in two participants during dose-escalation, preventing the attainment of a therapeutic dose. Despite this setback, the ability to target alternatively spliced protein isoforms using monoclonal antibodies could yet help to circumvent the difficulties associated with directly inhibiting splice variants, which have hampered drug discovery efforts in this area to date.

Oligonucleotide therapy. Oligonucleotide-based therapies involve the use of engineered oligonucleotides designed to hybridize with RNA sequences that are known to be responsible for specific splicing events in order to prevent their alternative splicing and the production of erroneous protein products with pathological consequences. The potential of these therapeutic agents has so far been best realized in patients with neurodegenerative conditions, including those with Duchenne muscular dystrophy[128] or spinal muscular atrophy[129], in which late-stage clinical trials are underway. However, an important question remains as to whether oligonucleotide therapy is a viable treatment approach in cancer and particularly in cancers with more diverse splicing events. Evidence supporting the use of oligonucleotide therapy in patients with cancer stems from work by Smith et al.[130], who developed a novel RNA splice-switching oligonucleotide designed to induce skipping of exon 11 in *BRCA1*, which is crucial to the DNA damage repair functions of the protein. In doing so the authors successfully rendered wild-type *BRCA1*-expressing cell lines more susceptible to PARP inhibition[130]. This approach provides a fascinating potential therapeutic strategy for targeting cancers with wild-type *BRCA1*, although the challenge in this setting is to maintain BRCA1 function in nonmalignant cells and thus minimize the potentially widespread risks of toxicity.

Conclusions

Splicing events are a plausible mechanism of treatment resistance and disease progression in patients with CRPC and have been proposed as a potential therapeutic target. Drug discovery efforts to date have, however, been challenging; thus, modulation of the spliceosome as a therapeutic tool represents an attractive alternative option, although, as yet, spliceosome inhibitors have not entered clinical practice in patients with prostate cancer, largely owing to the complexity of the spliceosome and a lack of understanding of its biology. Further research is required in order to identify the exact mechanisms underpinning the splicing abnormalities that are thought to contribute to the progression of CRPC, as well as the consequences of inhibiting these factors, before the true utility of these therapies can be realized.

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Competing interests
A.P., A.S., J.C.W., A.N., and J.S.d.B. are employees of The Institute of Cancer Research, a not-for-profit research organization and independent college of The University of London, which has a commercial interest in abiraterone. A.P., A.S., J.C.W., A.N., and J.S.d.-B. have no personal financial interests in abiraterone. G.V.R. has acted as a consultant of Astellas, Bayer, Pfizer, and Sanofi; receives research funding from Bayer; and has ownership interest in C-diagnostics EtiraRx and GaudiumRx. J.L. has served as a paid consultant and/or adviser of Janssen, Sanofi, and Sun Pharma; has received research funding from Astellas, Constellation, Gilead, Orion, and Sanofi; and is the lead inventor of a technology that has been licensed to A&G, Qiagen, and Tokai. J.S.d.B. has served as an advisory board member for Astellas, AstraZeneca, Bayer, Genentech, Genmab, GlaxoSmithKline, Janssen, Medivation, Merck MSD, Menarini, Orion Pharma, Pfizer, Sanofi–Aventis, and Serono. S.R.P. declares no competing interests.

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Androgen receptor splice variant-7 expression emerges with castration resistance in prostate cancer

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BACKGROUND. Liquid biopsies have demonstrated that the constitutively active androgen receptor splice variant-7 (AR-V7) associates with reduced response and overall survival (OS) from endocrine therapies in castration resistant prostate cancer (CRPC). However, these studies provide little information pertaining to AR-V7 expression in prostate cancer (PC) tissue.

METHODS. Following generation and validation of a novel AR-V7 antibody for immunohistochemistry, AR-V7 protein expression was determined for 358 primary prostate samples and 293 metastatic biopsies. Associations with disease progression, full length AR (AR-FL) expression, response to therapy, and gene expression was determined.

RESULTS. We demonstrated that AR-V7 protein is rarely expressed (<1%) in primary PC but is frequently detected (75% of cases) following androgen deprivation therapy, with further significant ($P = 0.020$) increase in expression following abiraterone acetate or enzalutamide therapy. In CRPC, AR-V7 expression is predominantly (94% of cases) nuclear and correlates with AR-FL expression ($P \leq 0.001$) and AR copy number ($P = 0.026$). However, dissociation of expression was observed suggesting mRNA splicing remains crucial for AR-V7 generation. AR-V7 expression was heterogeneous between different metastases from a patient although AR-V7 expression was similar within a metastasis. Moreover, AR-V7 expression correlated with […]

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Androgen receptor splice variant-7 expression emerges with castration resistance in prostate cancer

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Abstract

Background: Liquid biopsies have demonstrated that the constitutively active androgen receptor splice variant-7 (AR-V7) associates with reduced response and overall survival (OS) from endocrine therapies in castration resistant prostate cancer (CRPC). However, these studies provide little information pertaining to AR-V7 expression in prostate cancer (PC) tissue.

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Conclusion: This study provides impetus to develop therapies that abrogate AR-V7 signaling to improve our understanding of AR-V7 biology, and to confirm its clinical significance.

1. Introduction

Prostate cancer (PC) is the most commonly diagnosed non-cutaneous cancer and second leading cause of male cancer-related death in the Western world [1]. Androgen receptor (AR) signaling is critical for PC development and progression [2-5]. PC patients with advanced disease after primary therapy respond robustly to androgen deprivation therapy (ADT) but nearly all will progress to fatal castration resistant PC (CRPC). There is now mounting evidence that progression to CRPC remains dependent on persistent AR signaling driven by increased androgen synthesis, overexpression of AR co-activators, AR amplification and AR activating point mutations [3, 5-9]. These molecular findings have driven the development of new anti-androgen therapies, such as abiraterone acetate (AA), enzalutamide (E) and apalutamide (AP), that target the AR axis in patients with castration sensitive PC (CSPC) and CRPC. These therapies have led to improved patient outcome and health related quality of life [10-18].

Despite this significant progress, resistance to AA and E is common and on average occurs within a year of starting therapy; this is due, at least in part, to the emergence of constitutively active AR splice variants, of which AR variant-7 (AR-V7) is regarded as the most significant and most extensively characterized [19-32]. AR-V7 is thought to arise from aberrant mRNA splicing of AR exons 1, 2, 3, loss of exons 4-8, and inclusion of the cryptic exon 3 (CE3) into the transcribed AR gene [22, 32]. The resultant protein product is constitutively active in the absence of androgens and drives growth of PC cell lines and patient derived xenografts in the presence of AR directed therapies such as AA or E [23, 24, 32, 33]. AR-V7 forms homodimers with itself and heterodimers with full length AR (AR-FL) and in the absence of androgens binds to AR response elements, facilitating the generation of a pro-tumorigenic transcriptome [34]. In addition, transgenic mice with forced expression of AR-V7 display a pro-tumorigenic phenotype [35]. These pre-clinical studies confirm that AR-V7 may facilitate ligand independent AR signaling to drive resistance to established endocrine therapies.

Insufficient data have been available on AR-V7 mRNA and protein expression in primary PC, although some studies suggest expression [36-39]. AR-V7 protein expression increases as patients develop CRPC and resistance to AA or E [30-32, 40]. A plethora of clinical studies have confirmed AR-V7 expression to be correlated with resistance to AA and E therapy in CRPC; the majority of these measuring AR-V7 mRNA or protein from liquid biopsies (i.e. circulating tumor cells, exosomes, or whole blood) as opposed to utilizing metastatic tumor biopsies [19, 20, 26-31, 41-43]. All clinically licensed therapies modulate AR activity through its ligand-binding domain and therefore conceptually have no activity against AR-V7 mediated oncogenic signaling. Pharmacological inhibitors of bromodomain and extra-terminal proteins and HSP90 suppress AR-V7 generation through inhibition of mRNA splicing and inhibit the growth of CRPC models [33, 44, 45]. However, these therapies target multiple cellular pathways and therefore concerns with regard to clinical utility and safety remain. The development of novel therapies that overcome AR-V7 signaling in CRPC remains an area of urgent unmet clinical need.

In this work, we have performed an extensive cross-institutional study to determine nuclear AR-V7 protein expression in tissue biopsies and autopsies from primary and metastatic PC tumors using a novel AR-V7 antibody. We establish that expression of AR-V7 protein is rare in primary PC. In addition, nuclear AR-V7 expression emerges in response to primary ADT alone in most patients, and further increases in response to AA or E therapy, with nuclear AR-V7 being an important marker of response to these endocrine therapies in CRPC. Furthermore, AR-V7 expression associates with AR-FL expression and AR copy number in CRPC, although many cases with high AR-FL protein expression have undetectable/low AR-V7 protein expression. Moreover, nuclear AR-V7 expression is heterogeneous in different CRPC metastases in the same patient. Finally, nuclear AR-V7 expression is associated with a unique gene signature in CRPC patients. These data support a critical role for AR-V7 in CRPC biology and resistance to established endocrine therapies, providing further impetus for the development of therapeutic strategies that overcome AR-V7 mediated signaling to improve the outcome for patients with this lethal disease.

2. Results

2.1 Validation and optimization of a novel AR-V7 antibody (Clone RM7) for immunohistochemistry.

A novel recombinant rabbit monoclonal antibody (Clone RM7) was developed, in collaboration with RevMAb Biosciences, to CE3 of AR-V7. Antibody validation was performed at The Institute of Cancer Research/Royal Marsden (ICR/RMH) and University of Washington (UW), western blot analysis of AR-V7 positive cell lines (LNCaP95, 22Rv1 and VCaP) demonstrated a strong AR-V7 band at 80KDa (**Figure 1A and Supplementary Figure S1A**). In contrast, no band was seen in AR-V7 negative cell lines (LNCaP, PC3 and DU145) at 80KDa (**Figure 1A and Supplementary Figure S1A**). We next compared RM7 to EPR15656, an AR-V7 antibody that has been studied in PC tissue and circulating tumor cells (CTCs), and utilized for biomarker studies of treatment stratification in CRPC [27, 28, 30]. EPR15656 primarily recognizes AR-V7 but may also bind other proteins demonstrating staining in PC3 cells, colorectal (liver) metastasis, normal lung epithelium and cytoplasmic compartment [27, 28, 30]. EPR15656 demonstrated a strong AR-V7 band at 80KDa in LNCaP95, 22Rv1 and VCaP (**Figure 1A and Supplementary Figure S1A**). However, consistent with reports of positive staining in PC3 cells, EPR15656 demonstrated a strong band at 70KDa in PC3 [30]. Following initial validation, specificity and increased affinity (compared to EPR15656) of RM7 for AR-V7 was confirmed by immunoprecipitation using M12-cumate inducible AR-V7 cells demonstrating a single band at 80KDa (**Figure 1B**). RM7 detected additional bands at approximately 150KDa and 32KDa. However, both the strong 80KDa AR-V7 band and faint 32KDa band disappeared upon shRNA induction by doxycycline suggesting that the 32KDa band is a degradation product of AR-V7 (**Figure 1C**). In addition, the 150KDa band was not seen by Western blot analysis using an alternative extraction method at UW (**Figure 1C**). Furthermore, when RM7 was optimized for immunohistochemistry (IHC); AR-V7 expressing cell lines (22Rv1, LNCaP95 and VCaP) were positive for AR-V7 by IHC and AR-V7 negative cell lines (LNCaP, DU145 and PC3) were negative for AR-V7; confirming that the 150KDa band (present in LNCaP) was not recognized by IHC and RM7 does not stain PC3 cells (**Figure 1D**). In addition, RM7 stained neither a colorectal cancer (liver) metastasis nor normal lung epithelium, which stained positive with EPR15656 previously (**Supplementary Figure S1B**) [30]. Having confirmed that RM7 recognizes AR-V7, we performed IHC on formalin-fixed, paraffin-embedded PC patient tissue biopsies within our study cohorts demonstrating strong, almost exclusively, nuclear staining (**Figure 2 and Figure 3A**). In contrast, EPR15656 demonstrated cytoplasmic staining in PC tissue and CTCs [27, 28, 30]. Taken together, these data demonstrate that RM7 specifically recognizes AR-V7 protein in tissue biopsies from PC patients, with reduced off target liabilities compared to EPR15656.

2.2 Primary prostate cancers rarely express AR-V7 protein.

Previous studies have demonstrated AR-V7 mRNA and protein expression in primary PC [36-39]. Having validated and optimized RM7 for IHC on PC patient samples we

investigated nuclear AR-V7 protein expression in early PC specimens. We utilized the ICR/RMH and UW CSPC cohorts (**Figure 2**). A single biopsy (1.6%) of 63 CSPC biopsies (ICR/RMH CSPC cohort) expressed nuclear AR-V7 (**Figure 3B and Supplementary Table 1**). Similarly, in 295 primary PC specimens (UW CSPC cohort) from men who were treated with a radical prostatectomy, and had not received AR directed therapy, there were no (0%) nuclear AR-V7 positive cases (**Figure 3C**). Clinical data (for PSA progression free survival) was available on 128 patients from the UW CSPC cohort (**Supplementary Table 2**). Of the 128 patients with 5-year follow-up 64 had biochemical recurrence; none of these 64 patients had detectable AR-V7 protein in their initial prostatectomy tissue. These data confirm that AR-V7 is rarely (0.3%; 1 of 358) expressed and therefore nuclear AR-V7 protein expression cannot predict disease recurrence in radically treated primary PC.

2.3 AR-V7 protein emerges as prostate cancer patients and mouse xenografts progress to castration resistant disease and develop resistance to abiraterone acetate or enzalutamide therapy.

Having demonstrated that nuclear AR-V7 protein is infrequently expressed in CSPC we next explored nuclear AR-V7 expression in same patient, matched biopsies, as 63 patients progressed from CSPC to CRPC (**Figure 2**). Nuclear AR-V7 protein significantly ($p<0.001$) increased from CSPC (median H-score, IQR; 0, 0-0) to CRPC (70, 5-130) (**Figure 3B**). Next, we expanded this cohort to 160 CRPC biopsies (**Figure 2**). Median nuclear AR-V7 expression was 75 (5-130) (**Figure 3D and Supplementary Table 3**); 3 (1.9%) mCRPC biopsies of 160 cases had neuroendocrine-like features and were negative for both nuclear AR-FL and nuclear AR-V7 expression. We next determined whether nuclear AR-V7 expression altered as patients progressed through standard of care AR targeting therapies for CRPC (**Figure 3D**). Interestingly, 15 (75%) of 20 biopsies taken after progression on primary ADT (with or without bicalutamide) prior to starting standard systemic therapy for CRPC had detectable nuclear AR-V7 protein expression (40, 1.25-92.5) (**Figure 3D**). Furthermore, nuclear AR-V7 expression was significantly lower ($p=0.020$) in 40 biopsies prior to AA or E therapy (40, 1-107.5) than in 120 biopsies post AA or E therapy (90, 20-150) (**Figure 3D**). There was no clear association between nuclear AR-V7 expression and time of biopsy after starting AA or E therapy ($r=-0.11$ [-0.31-0.09]; $p=0.27$) (**Supplementary Figure S2**). Next we determined whether nuclear AR-V7 expression differed between site of CRPC biopsy. Nuclear AR-V7 expression was higher ($p=0.013$) in lymph node (120, 60-170) metastases compared to bone (50, 1-110), liver (70, 3.75-132.5), prostate (50, 0-70) and other (90, 0-150) sites of metastases (**Figure 3E**). Finally, we investigated whether the same pattern of nuclear AR-V7 expression was observed as VCaP (androgen dependent) mouse xenograft models developed therapeutic resistance. Consistent with our tissue studies, nuclear AR-V7 expression increased in VCaP mouse xenografts as they progressed from the castration sensitive (0, 0-0) to the castration resistance state (155, 102.5-175) to AA/E resistance (180, 160-190) (**Supplementary Figure S3 and S4**). Although nuclear AR-V7 protein is rarely expressed in primary PC, AR-V7 protein expression emerges as patients and mouse xenografts progress to castration resistant disease, and levels increase further as resistance to AA or E therapy develops.

2.4 AR-V7 protein expression associates with response to abiraterone acetate and enzalutamide, but not docetaxel, in castration resistant prostate cancer.

Studies have shown AR-V7 protein and mRNA to be a marker of next generation AR targeted therapy (AA and E) resistance [19, 20, 26-31]. To investigate this further, we determined the response of the ICR/RMH CRPC cohort to AR targeted therapies pre- and post-chemotherapy in AR-V7 negative (nuclear H-score ≤ 10) and AR-V7 positive (nuclear H-score >10) patients. Thirty-six patients received AA or E for CRPC prior to chemotherapy and had fully evaluable response data (**Figure 2**). Patients negative for AR-V7 expression were younger (p=0.04) at the time of starting AR targeting therapy pre-chemotherapy, but no other differences in baseline characteristics were observed (**Table 1**). Patients negative for AR-V7 (n=8) had a greater prostate specific antigen (PSA) 50% nadir (100 vs 68%, p=0.16) and PSA 50% response rate (100 vs 54%, p=0.03) than AR-V7 positive patients (n=28) (**Figure 4A-B**). Patients achieving a 50% PSA fall had significantly lower (p=0.012) nuclear AR-V7 expression (40, 0-100) than those that did not (120, 55-180) (**Supplementary Figure S5**). Furthermore; AR-V7 negative patients had a longer time to PSA progression (11.5 vs 4.8 months (mo), hazard ratio (HR) 0.33 [0.14-0.81], p=0.02), longer time to clinical/radiological progression (13.9 vs 7.2 mo, HR 0.47 [0.20-1.10], p=0.08) and improved overall survival (74.3 vs 25.2mo, HR 0.23 [0.07-0.79], p=0.02) (**Figure 4C-E**). Fifty-four patients received AA or E for CRPC after chemotherapy and had fully evaluable response data (**Figure 2**). There were no differences in the baseline characteristics by AR-V7 status at the time of starting AR targeting therapy post-chemotherapy (**Supplementary Table 4**). Patients negative for AR-V7 (n=17) had a significantly greater PSA 50% nadir (71 vs 24%; p=0.002) and PSA 50% response rate (59 vs 22%; p=0.012) than those positive for AR-V7 (n=37) (**Supplementary Figure S6A-B**). Patients achieving a 50% PSA fall had significantly lower (p=0.011) nuclear AR-V7 expression (3, 0-80) that those that did not (95, 25-125) (**Supplementary Figure S6C**). Interestingly, despite these significant differences in response rates, there was no significant difference in time to PSA progression (2.8 vs 2.3 mo, HR 0.96 [0.54-1.73], p=0.90), time to clinical/radiological progression (4.9 vs 5.1 mo, HR 0.92 [0.51-1.66], p=0.77) or overall survival (14.0 vs 15.7 mo, HR 1.01 [0.56-1.82], p=0.98) (**Supplementary Figure S6D-F**). Having explored response to AR targeted therapy, we next investigated response to docetaxel chemotherapy. Fifty-five patients were treated with docetaxel chemotherapy for CRPC and had fully evaluable response data (**Figure 2**). There was no evidence of a difference in baseline characteristics at the time of starting docetaxel chemotherapy (**Supplementary Table 5**). In contrast to AR targeting therapies, there was no difference in PSA 50% nadir (56 vs 46%, p=0.57) and PSA 50% response rate (39 vs 27%, p=0.54) between AR-V7 negative (n=18) and positive patients (n=37) (**Supplementary Figure S7A-B**). Nuclear AR-V7 expression was not significantly (p=0.14) different in patients achieving a 50% PSA fall with docetaxel (20, 0-85) compared to those that did not (70, 5-132.5) (**Supplementary Figure S7C**). Consistent with this, there was no significant difference in time to PSA progression (4.8 vs 4.7 mo, HR 1.04 [0.57-1.92], p=0.90) and time to clinical/radiological progression (6.9 vs 7.5 mo, HR 1.31 [0.73-2.34], p=0.36) (**Supplementary Figure S7D-E**). However, AR-V7

negative patients had improved overall survival (26.3 vs 18.5 mo, HR 0.50 [0.27-0.95], p=0.03) compared to AR-V7 positive patients (**Supplementary Figure S7F**). Taken together; these data confirm that AR-V7 is a robust prognostic biomarker and an important indicator of sensitivity to AR targeted therapies but not docetaxel treatment in CRPC.

2.5 AR-FL and AR-V7 mRNA and protein expression associate in a high proportion of, but not all, castration resistant prostate cancers.

We and others have shown that AR-FL and AR-V7 mRNA and protein are induced upon castration, and that therapies suppressing mRNA splicing prevent AR-V7 mRNA and protein generation in CRPC [33, 44-47]. Therefore, we next investigated the association between AR-FL and AR-V7 mRNA and protein expression (**Figure 2**). Analysis of RNA-sequencing (RNA-seq) data obtained from 122 CRPC biopsies demonstrated that AR-FL mRNA expression significantly correlated with AR-V7 mRNA expression (r=0.69 [0.58-0.77]; p=<0.001) (**Figure 5A**). In light of RNA quantification not discriminating against cellular localization, we next quantified total (nuclear and cytoplasmic) AR-FL and AR-V7 protein expression in 144 CRPC biopsies (ICR/RMH CRPC cohort) (**Figure 5B and Supplementary Figure S8**). Unlike AR-FL, of which 124 of 144 (86%) biopsies had both cytoplasmic and nuclear AR-FL protein expression, AR-V7 protein was almost exclusively (136/144; 94% of biopsies) nuclear in localization (**Figure 5B)**. There was a significant correlation between total AR-FL and AR-V7 protein expression in 144 CRPC biopsies from the ICR/RMH CRPC cohort (r=0.28 [0.11-0.42], p=<0.001) (**Figure 5C**). However, it is important to recognize that a substantial number of patients with high AR-FL mRNA and protein expression had low or undetectable levels of AR-V7 mRNA and protein. Furthermore, both total AR-FL (r=0.46 [0.28-0.61], p=<0.001) and total AR-V7 (r=0.23 (0.02 to 0.42); p=0.026) protein significantly correlated with AR copy number in 95 CRPC biopsies from the ICR/RMH CRPC cohort (**Figure 5D-E**). Finally, consistent with the demonstration that AR-FL and AR-V7 are differentially localized, there was no significant correlation between nuclear AR-FL and nuclear AR-V7 protein expression in 144 CRPC biopsies from the ICR/RMH CRPC cohort (r=0.11 [-0.06-0.27], p=0.20) (**Figure 5F**). These data demonstrate that taken together, total AR-FL and AR-V7 mRNA and protein expression correlate in CRPC biopsies, although importantly many patients have tumors expressing high levels of AR-FL mRNA and protein but that have low or undetectable AR-V7 mRNA and protein expression. This suggests that the presence of AR-V7 mRNA and protein is not simply a consequence of higher AR-FL levels in all cases.

2.6 AR-V7 protein expression is largely homogenous within metastasis but heterogeneous between metastases from patients with castration resistant prostate cancer.

We have previously shown that in a patient with a genomic rearrangement resulting in the constitutively active AR^{v567es} variant that each of 5 metastases expressed the variant AR in a homogenous fashion [48]. Since AR-V7 is usually not generated from a structural rearrangement of the AR gene but rather from aberrant mRNA splicing, we

next quantitated expression in 133 metastases from 34 CRPC patients that were collected as part of the University of Washington Medical Center Prostate Cancer Donor Rapid Autopsy Program (UW CRPC cohort) (**Figure 2, Figure 6A and Supplementary Table 6**). Automated digital scoring reported as optical density (OD) correlated significantly (r=0.86 [0.83-0.89]; p=<0.001) with manual scoring and was used to determine nuclear AR-V7 expression in the UW CRPC cohort (**Supplementary Figure S9A-C**). Three tissue microarray spots were stained from each metastasis and AR-V7 levels quantified. We found that expression of AR-V7 was largely consistent within each metastasis from a patient and that the variance was not statistically significant (Fligner-Killeen p=0.9999; Levene's p=0.9972) (**Figure 6B and C)**. However, expression of AR-V7 in different metastases in an individual patient differed widely and the variance was statistically significant (Fligner-Killeen p=3.73 x 10^{-06}; Levene's p=3.25 X 10^{-07}) (**Figure 6B and C**). These data suggest that within an individual patient the degree to which AR-V7 may be driving different metastases varies and may result in mixed response to endocrine therapies.

2.7 AR-V7 expression is associated with a unique gene signature in castration resistant prostate cancer.

Having demonstrated inter-patient and intra-patient heterogeneity in nuclear AR-V7 expression we next investigated whether nuclear AR-V7 expression was associated with a specific gene signature in CRPC patients. Forty-one metastatic biopsies from 24 men within the UW CRPC cohort had mRNA expression (RNA-seq) and AR-V7 protein expression (IHC) available [31, 54]. The correlation between AR-V7 protein expression (optical density) and gene mRNA expression (log_2 counts per million) was determined and corrected for multiple testing. We identified 487 genes that correlated (q<0.05) with AR-V7 protein expression; of these 407 positively correlated and 80 negatively correlated (**Figure 7A**). Pathway analysis of the 407 genes that positively correlated with AR-V7 protein expression identified an enrichment for pathways involved in transcription and the androgen response (**Supplementary Figure S10 and Supplementary Table 7**) [49, 50]. We confirmed that AR-V7 mRNA expression and AR-V7 protein expression correlated significantly in 41 UW (p<0.001) and 21 ICR/RMH (p=0.004) CRPC biopsies (data not shown). Next we independently tested the positively correlated 407-gene signature in 21 CRPC metastasis from an ICR/RMH CRPC cohort and 122 CRPC tumor transcriptomes (SU2C/PCF cohort) (**Supplementary Table 8**) [51]. Of the genes identified, 59 were found to be significantly correlated with nuclear AR-V7 protein expression in the ICR/RMH cohort or with AR-V7 mRNA expression in the SU2C/PCF cohort (**Figure 7B-C and Supplementary Table 9**). Following this, pathway analysis of the 59 independently validated genes confirmed a role in transcriptional activity (**Figure 7D and Supplementary Table 10**). Consistent with this finding, 33% were zinc finger containing (ZNF) genes correlated with chromatin binding including HOXB13, ELL2, STEAP2 and BAZ2A. Furthermore, a large number of the genes identified have been associated with PC progression (**Supplementary Table 9**) [52-64]. In addition, genome wide analyses demonstrated that AR-V7 protein expression associates with AR gene expression (**Figure 7C**). Although an association between AR-FL mRNA and AR-V7 mRNA using junction specific reads was confirmed,

there was further confirmation that a substantial number of cases that express high AR-FL mRNA levels have low or undetectable AR-V7 mRNA (**Supplementary Figure S11**). These data suggest that AR-V7 expression is associated with a specific gene signature in a large patient population that may play a key role in transcriptional activity and prostate cancer progression in patients with CRPC.

3. Discussion

Since the pioneering studies of Huggins and Hodges in 1941, the androgen receptor has remained the focus of therapeutic targeting in CRPC. Inhibition of the AR axis with AA and E has improved both overall survival and quality of life for patients with CRPC [10-17, 65]. Although these modalities are initially effective, resistance develops with ongoing AR activity and tumor progression. This is, at least in part, due to the expression of constitutively active AR splice variants of which AR-V7 appears to be most common [19-32]. AR-V7 mRNA and protein expression has been detected at low levels in primary, treatment-naïve PC, and studies have reported a potential association with worse outcome [36-39]. Surprisingly, in two separate patient cohorts we found that nuclear AR-V7 protein was expressed in <1% of PC tumors at diagnosis and therefore it cannot be predictive of outcome after primary therapy. The difference in prevalence of AR-V7 protein expression reported in different studies is likely attributable to the different AR-V7 antibodies used, and in particular, the observation that one antibody used has off-target liabilities as we have previously reported [30]. Consistent with this, we demonstrate AR-V7 protein expression to be almost exclusively nuclear, whereas previous studies have demonstrated cytoplasmic positivity [27, 28, 30]. These data suggest that AR-V7 testing is unlikely to be of use for treatment stratification at time of diagnosis and maybe better utilized beyond first-line treatment [11, 18].

In contrast to primary PC, 75% of CRPC patients who had progressed on primary ADT alone (with or without bicalutamide) expressed nuclear AR-V7 prior to receiving AA or E. Despite AR-V7 expression after primary ADT, subsequent AA or E treatment has significant antitumor activity with response rates ranging from 57-78% [12, 14, 66]. These data indicate that AR-V7 protein expression in biopsies cannot indicate absolute refractoriness to treatment. Although, consistent with previous reports, nuclear AR-V7 levels increased further in response to AA or E; these data suggest that AR-V7 expression is a factor at the initial phase of castration resistance following primary ADT in advanced PC [30, 40]. These data were further confirmed in VCaP mouse xenograft models as they developed resistance to castration and AA/E therapy. Recent studies have shown AA therapy at diagnosis to improve overall survival in PC patients with *de novo* metastatic disease [11, 18]. The demonstration that nuclear AR-V7 expression is rare in primary PC, but emerges with primary therapy, may provide insight into the greater efficacy of AA in CSPC. Importantly, these data show that increased AR-V7 expression is an early event in resistance and if targeted agents to AR splice variants become clinically available, therapy may need to be combined at time of initial ADT.

Critically, we found that AR-V7 protein expression is more prevalent in CRPC biopsies than previously reported from AR-V7 mRNA and protein expression studies in liquid biopsies (11-46%) [19, 20, 27-29]. This important observation is likely due to the differences in sensitivities of the assays used. In addition, CRPC biopsies demonstrate lymph node metastases to express higher levels of AR-V7 than other sites of disease, which may (depending on the source of CTCs) account for the lower incidence of AR-V7 detected in liquid biopsies. Furthermore, we demonstrate intra-patient heterogeneity of nuclear AR-V7 expression between multiple metastases indicating that this is a

further potential source of variation in CTC based AR-V7 assessment. Finally, depending on the biomarkers used to select CTCs, assessment of AR-V7 may be underestimated if not all CTCs are identified. These findings suggest that the detection of AR-V7 in CTCs may not be representative of all metastases, and that while sites of disease expressing AR-V7 may be resistant to current endocrine therapies, those expressing low/no AR-V7 may still respond, within the same subject. Additional studies focusing on the prognostic value of tissue-based AR-V7 detection in CTC-negative patients may be warranted.

The majority of clinical studies have demonstrated that AR-V7 positivity confers resistance and poorer outcome to AR targeting therapies in patients with CRPC [19, 20, 26-31]. We confirmed that AR-V7 positive patients treated with AA or E had a worse PSA response rate in the pre- and post-chemotherapy setting; and those patients who responded had lower levels of AR-V7 expression. Interestingly, despite poorer response rates, only AR-V7 positive patients treated with AA or E prior to chemotherapy had shorter progression free and overall survival. This observation could be multifactorial. Firstly, not all patients had tissue biopsies prior to starting treatment and therefore AR-V7 status may have changed prior to therapy. Secondly, patients without CTCs and therefore no AR-V7 result may be underrepresented in previous studies. In contrast to endocrine therapies, AR-V7 status did not associate with PSA response or progression free survival in patients treated with docetaxel, as previously described [27, 28, 67]. Interestingly, AR-V7 positive patients had shorter overall survival suggesting that AR-V7 positivity may be associated with more aggressive disease, or this group of patients may have derived less benefit from treatment with further novel endocrine therapies. Taken together, these studies will be important to understand, as the landscape of CRPC changes as patients with *de novo* metastatic CSPC receive AA, and as we explore the potential use of AR-V7 to stratify patients to further AR targeted therapies as they progress to CRPC.

The mechanisms by which AR splice variants are generated include genomic rearrangements and/or aberrant alternative mRNA splicing [22, 24, 32, 48, 68-71]. AR-V7 generation has generally been attributed to aberrant splicing of AR pre-mRNA [72]. This does not negate the fact that AR-FL increases under conditions such as castration, and that this leads to the generation of AR-V7 [33, 46, 47]. However, these data indicate that the mechanisms driving increased AR-FL expression and AR signaling in CRPC differ from those required for AR-V7 generation [72-76]. Consistent with this, we demonstrate that although AR-FL and AR-V7 expression associate in CRPC, a substantial number of patients with high levels of AR-FL demonstrate undetectable or low levels of AR-V7 expression. In keeping with this also is evidence emerging that therapies that suppress mRNA splicing decrease AR-V7 generation in CRPC models [33, 45]. JMJD1A has recently been reported to be critical for mRNA splicing and AR-V7 generation but does not impact on AR-FL levels [77]. These data suggest that mRNA splicing is important for AR-V7 generation and is not simply a consequence of increased AR-FL expression. Further understanding of the mechanisms underpinning AR-V7 generation are now required to support the development of therapeutic strategies to suppress splice variant generation in CRPC.

Previous studies examining AR-V7 cistromes and transcriptomes demonstrated proliferative cistomes/transcriptomes which are likely not specific to AR-V7 but a marker of rapidly progressive disease [23, 78-80]. Although a recent study suggested that CRPC transcriptomes are diverse, we identified 59-genes using three independent patient cohorts that associate with AR-V7 expression [79]. One important consideration is that AR-V7 protein expression associates with AR gene expression in genome-wide analysis. However, as both AR-V7 and AR-FL mRNA would be represented in such studies, further junction-specific quantification was performed. Despite this approach confirming a correlation between AR-FL and AR-V7 mRNA, there was further evidence of dissociation in many cases. In addition, a gene signature was derived from AR-V7 protein expression; unlike AR-FL protein, AR-V7 is almost exclusively nuclear and therefore, unlike mRNA analysis, this takes into consideration its likely functional importance. Interestingly, BAZ2A, SRC, STEAP1, STEAP2, DCAF6, TMBIM6, HOXB13, GALANT7, WWC1, SPATS2 and GSTP1 expression associated with AR-V7 expression and all of these have been previously linked to PC progression [52-64]. In addition, HOXB13 has recently been shown to be critical for AR-V7 chromatin binding [79]. Furthermore, we found a number of ZNF contigs to be associated with AR-V7 expression, providing evidence of increased transcriptional activity. These data suggest that AR-V7 protein expression is associated with a unique gene signature important for prostate cancer progression and transcriptional activity. It is important to stress that the 59-gene set derived does not represent the AR-V7 cistrome but is a set of genes associated with AR-V7 expression between cohorts and may identify common characteristics of AR-V7 associated disease. Components of this gene signature, including HOXB13 that has recently been shown to be critical for AR-V7 function, may provide insight into therapeutic targets for novel treatment strategies in patients with high levels of AR-V7 expression [79].

In conclusion, our results show that AR-V7 protein expression, using a validated, highly specific antibody, is not seen in primary CSPC and does not appear until initial resistance to standard ADT occurs, and increases further with AA and E therapy. In addition, AR-V7 protein expression associates with resistance to AR targeted therapies but not taxane treatment in patients with CRPC. Furthermore, although AR-V7 and AR-FL expression levels associate in CRPC, there are many cases in which expression levels are uncoupled suggesting that AR-V7 protein expression is not simply a function of AR-FL protein expression. Moreover, AR-V7 protein is heterogeneously expressed, especially between metastases from the same patient, indicating multiple resistance mechanisms in the same subject. These data suggest that multiple therapeutic modalities may be needed simultaneously to adequately reverse endocrine resistance in AR-V7 positive PC. Finally, AR-V7 protein expression associates with a unique gene signature that may drive transcriptional activity and PC progression. These results further confirm the importance of AR-V7 in CRPC biology and provide impetus for the development of novel therapeutic strategies that abrogate AR-V7 expression at the time of initial ADT in CSPC in order to prevent or delay development of CRPC and improve the outcome for patients with lethal PC.

4. Methods

Cell lines

LNCaP95 cells were kindly provided by Drs. Alan K Meeker and Jun Luo (Johns Hopkins University). 22Rv1 (CRL-2505), VCaP (CRL-2876), DU145 (HTB-81), M12 (a gift from Joy Ware, Medical College of Virginia) and PC3 (CRL-1345) cells were obtained from American Type Culture Collection. Doxycycline inducible cell lines were created using lentiviral vectors in pLKO-Tet-On backbones targeting either GFP (shGFP; 5'-GCAAGCTGACCCTGAAGTTCA-3'), AR-FL exon 8 (shAR-FL; 5'-CCTGCTAATCAAGTCACACAT-3') or AR-V7 cryptic exon 3 (shAR-V7; 5'-GTAGTTGTGAGTATCATGA-3') and lentiviral particles were produced as previously described [81, 82]. Cells were infected with virus and selected with 1 μg/ml puromycin. shRNA expression was induced by treating cells with 1 μg/ml doxycycline for 72 h. All cell lines were grown in recommended media at 37 °C in 5 % CO_2. Cell lines were tested for mycoplasma using the VenorGem One Step PCR Kit (Cambio) and STR-profiled.

Immunoblotting

ICR/RMH antibody validation: Cells were lysed with RIPA buffer (Pierce) supplemented with protease inhibitor cocktail (Roche). Protein extracts (20 μg) were separated on 7% NuPAGE® Tris-Acetate gel (Invitrogen) by electrophoresis and subsequently transferred onto Immobilon-P™ PVDF membranes of 0.45 μm pore size (Millipore). Primary antibodies used were rabbit monoclonal anti-AR-V7 antibody (1 in 1000; RM7; RevMAb biosciences), rabbit monoclonal anti-AR-V7 antibody (1 in 1000; EPR15656; abcam) and mouse monoclonal anti-vinculin antibody (1 in 200000; V9131; Sigma-Aldrich) with species specific secondary antibodies conjugated to horseradish peroxidase. Chemiluminescence was detected on the Chemidoc Touch imaging system (Bio-Rad).

UW antibody validation: Cells were lysed with M-PER™ Mammalian Protein Extraction Reagent (Thermo Fisher Scientific) supplemented with Halt™ Protease Inhibitor and Halt™ Phosphatase Inhibitor Cocktail. Protein extracts (30 μg) were separated on 4–15% Mini-PROTEAN® TGX™ Precast Protein Gel (Bio rad) by electrophoresis and subsequently transferred to a nitrocellulose membrane with an iBlot system. Primary antibodies used were rabbit monoclonal anti-AR-V7 (1 in 2000; RM7; RevMAb biosciences), mouse monoclonal anti-AR N-terminus antibody (1 in 2000; AR441; Santa Cruz Biotechnology) and rabbit monoclonal anti-GAPDH antibody (1 in 10000; #2118; Cell Signaling Technology). The specific signals were visualized on the Blue Ultra Autorad Film (GeneMate) with species specific secondary antibodies conjugated to horseradish peroxidase by chemiluminescence.

AR-V7 immunoprecipitation

Cellular extracts were prepared from cumate-treated M12 cells expressing cumate-inducible AR-V7 lentivirus were prepared using the SparQcumate switch lentivector

system (Systems Bioscience) as previously described [30]. Precleared cell lysate was incubated with rabbit monoclonal anti-AR-V7 antibodies (EPR15656; abcam or RM7; RevMAb biosciences). Rabbit IgG were used as a negative control. Immune complexes were collected using protein A/G Plus agarose beads and analyzed by immunoblotting as described above.

VCaP mouse xenograft models

All animal studies performed in accordance with Beth Israel Deaconess Medical Center IACUC regulations (protocol #086-2016). VCaP mouse xenograft models have been previously described [83]. Briefly, 5 million VCaP cells in 100% Matrigel were injected subcutaneously into 6-week old ICR scid mice (Taconic Biosciences). Xenografts were grown until 1000mm^3, then mice were castrated. For AA and E resistant xenograft model, when castrate tumors exceeded 150% nadir volume they were treated with AA (30mg/kg) and E (50mg/kg). Tumors were biopsied prior to castration resistance, at castration resistance and when resistant to AA and E therapy.

Institute of Cancer Research and Royal Marsden Hospital (ICR/RMH) and University of Washington (UW) tissue samples

The ICR/RMH CSPC and CRPC cohort was identified from men with CRPC treated at the Royal Marsden NHS Foundation Trust. The ICR/RMH CSPC cohort contained 63 patients with sufficient formalin-fixed, paraffin embedded (FFPE) diagnostic (archival) CSPC biopsies; all biopsies demonstrated adenocarcinoma and were from either prostate needle biopsies (47), transurethral resection of the prostate (TURP; 5), transurethral resection of the bladder (TURBT; 1), prostatectomy (8), bone (1) and rectal (1). The ICR/RMH CRPC cohort contained 160 patients (which included all 63 patients in the CSPC cohort) with sufficient FFPE CRPC biopsies from metastatic biopsies of bone (81), lymph node (51), soft tissue (8), liver (10) and TURP (7), TURBT (1) or prostate (2). All tissue blocks were freshly sectioned and only considered for IHC analyses if adequate material was present (author DNR.). Demographic and clinical data for each patient were retrospectively collected from the hospital electronic patient record system.

The UW CSPC cohort was identified from men who received radical prostatectomy without neoadjuvant therapy. Tissue microarrays (TMAs) of FFPE tissue from primary prostate acinar adenocarcinomas was generated. The tissue came from the radical prostatectomy samples of 295 patients, none of whom had received neoadjuvant therapy. The TMAs consisted of single cores of 12 carcinomas, duplicate cores of 167 carcinomas, triplicate cores of 44 carcinomas and quadruplicate cores of 72 carcinomas. The UW CRPC cohort was identified from men who died from their prostate cancer and were part of the University of Washington GU Cancer Rapid autopsy program [5]. The cohort consisted of TMA 83 generated from biopsies of 133 metastases from 34 patients. Triplicate cores of the 133 metastases were placed on the TMA.

Immunohistochemistry (IHC)

ICR/RMH CSPC and CRPC cohort: AR-V7 and AR-FL IHC was performed as previously described [30, 31]. Briefly, AR-V7 IHC was performed using recombinant rabbit monoclonal anti-AR-V7 antibody (Clone RM7; RevMAb biosciences). Biopsies were first deparaffinized prior to antigen retrieval by microwaving (in Tris/EDTA buffer, pH 8.1) for 18 minutes at 800W and anti-AR-V7 antibody (1:500 dilution in Dako REAL diluent, Agilent Technologies) was incubated with tissue for 1 hour at room temperature. After washes, bound antibody was visualized using Dako EnVision Detection System (Agilent Technologies). Sections were counterstained with hematoxylin. Cell pellets from 22Rv1 (positive) and PC3 (negative) were used as controls. Rabbit IgGs were used as a further negative control. AR-FL IHC was performed using mouse monoclonal anti-AR antibody (AR441, Agilent Technologies). Biopsies were first deparaffinized prior to antigen retrieval using pH 8.1 Tris/EDTA solution heated in a water bath and anti-AR antibody (1:1000 dilution in Dako REAL diluent, Agilent Technologies) was incubated with tissue for 1 hour at room temperature. After washes, bound antibody was visualized using Dako EnVision Detection System (Agilent Technologies). Sections were counterstained with hematoxylin. Cell pellets from VCaP (positive) and PC3 (negative) were used as controls. Mouse IgGs were used as a further negative control.

UW CSPC and CRPC cohort: Briefly, AR-V7 IHC was performed using recombinant rabbit monoclonal anti-AR-V7 antibody (Clone RM7; RevMAb biosciences). Deparaffinization, antigen retrieval (Cell conditioner 1; Ventana Medical Systems) and immunostaining were performed on the Ventana Benchmark automated stainer (Ventana Medical Systems). Sections were incubated for 2 hours at 37^{o}C with anti-AR-V7 antibody (1:50 in antibody diluent, Ventana Medical Systems). After washes, bound antibody was visualized using Ventana Optiview DAB detection kit (Ventana Medical Systems). Sections were counterstained with hematoxylin. Controls were sections of a TMA made of cell lines known to express AR-FL and/or AR-V7 (LNCaP, 22Rv1, VCaP), to not express AR-FL or AR-V7 (DU145, PC3, M12) and cells engineered to stably express both AR-FL (M12 AR-FL) and AR-V7 (M12 AR-V7) by transfection.

IHC quantification

ICR/RMH CSPC and CRPC cohort: AR-V7 and AR-FL protein expression was determined for each case by a pathologist (author DNR) blinded to clinical data using the modified H score (HS) method; a semi-quantitative assessment of staining intensity that reflects antigen concentration. HS was determined according to the formula: [(% of weak staining) × 1] + [(% of moderate staining) × 2] + [(% of strong staining) × 3], yielding a range from 0 to 300 [84].

UW CSPC and CRPC cohort: AR-V7 protein expression in the UW CSPC cohort was determined for each case by a pathologist (author LDT) as described above. AR-V7 protein expression in the UW CRPC cohort was determined using automated digital scoring as follows: TMA slides were scanned with an Aperio ScanScope (Leica Biosystems) at 40x (0.25 microns/pixel). Using Aperio ImageScope software, the AR-V7

stained TMA slides were annotated to create regions of interest (ROI) for analysis. Quantitative image analysis of the annotated ROIs was performed using Aperio Brightfield Image Analysis Toolbox software (Leica Biosystems). The analysis data for each TMA spot was extracted into Microsoft Excel for further analysis. The quantitative analysis data for each TMA spot included total numbers and percentages of nuclei (positive and negative), average positive intensity, average positive optical density, and area of analysis. The intensity is a measurement of the light transmission, or brightness, of the positive staining in the nuclei and is logarithmically related to the optical density. The optical density is a measurement of absorbance and is linearly related to the amount of staining present. Automated scores for AR-V7 protein expression were reviewed and confirmed by a pathologist (author LDT); and have been shown to correlate highly with manual scoring (**Supplementary Figure S9**) [84-86].

RNA-seq analysis

UW CRPC cohort: A set of 41 metastatic tumors from 24 men with CRPC were obtained through the University of Washington Prostate Cancer Donor Autopsy Program and used for transcript profiling by RNA-seq, as described using frozen tissues [31, 87]. RNA sequencing data are deposited in the Gene Expression Omnibus database under the accession number GSE118435. These tissues were from metastases where we had tissue available from the same block that had been used to spot the tissue microarray. Tissue microarray AR-V7 IHC scores were matched to mRNA samples by block ID. We then computed the Pearson correlation between AR-V7 expression (automated digital scoring) and gene mRNA expression (log_2 counts per million), controlling for multiple testing using the cor.test and qvalue functions in R. There were 487 genes with q-value <0.05, 407 of which correlated with higher expression in AR-V7 expressing tumors. This 407-gene signature was independently tested in a set of 21 CRPC biopsies (RMH/ICR cohort; see below) and 122 CRPC transcriptomes (SU2C/PCF cohort; see below). AR-FL and AR-V7 mRNA expression in spliced reads per million mapped reads (SRPM) for 41 CRPC transcriptomes from the UW cohort were calculated as previously described [88]. Junction reads spanning the AR exon 3 to exon 4 junction were used to estimate AR-FL specific expression, while reads spanning the AR exon 3 to cryptic exon 3 junction were used to estimate AR-V7 specific reads, normalized by total spliced reads (genome-wide) to correct for sequencing depth.

ICR/RMH cohort: Twenty-one patients with AR-V7 protein expression by IHC and RNA-seq analysis (from the SU2C/PCF consortium) from same biopsy were used. Data from 21 transcriptomes generated by the International Stand Up To Cancer/Prostate Cancer Foundation (SU2C/PCF) Prostate Cancer Dream Team were downloaded and re-analyzed [3]. Paired-end transcriptome sequencing reads were aligned to the human reference genome (GRCh37/hg19) using Tophat2 (v2.0.7). Gene expression, Fragments Per Kilobase of transcript per Million mapped reads (FPKM), was calculated using Cufflinks [89]. For those genes identified in the UW CRPC cohort; association between nuclear AR-V7 protein expression (IHC) and each gene mRNA expression (RNA-seq) from the same biopsy was determined using Pearson correlation coefficient.

SU2C/PCF cohort: Data from 122 CRPC transcriptomes generated by the International Stand Up To Cancer/Prostate Cancer Foundation (SU2C/PCF) Prostate Cancer Dream Team were downloaded and re-analyzed as described above. For those genes identified in the UW CRPC cohort; association between AR-V7 mRNA expression and each gene mRNA expression was determined using Pearson correlation coefficient.

Pathway enrichment analysis

Out of the 407 genes positively associated with higher AR-V7 levels with a q-value <0.05 in the UW CRPC cohort (described above), 59 were found to be positively associated and significant with p-value <0.05 in either the ICR/RMH cohort or the SU2C/PCF cohort. The UpSetR R package was used to plot overlap between cohorts. Pathway over-representation analysis of the 407 and 59 gene sets were conducted using the compute overlaps tool with MSigDBv6.2 (H – Hallmark, CP – Canonical Pathways, C4 – Computational Gene Sets, C5 – GO and C6 – Oncogenic Pathways) [49, 50, 90].

Statistical analysis

All statistical analyses were conducted using Stata v13.1 or GraphPad Prism v6 and are indicated within all figures and tables. H-scores (HS) were reported as median values with interquartile range (IQR). For ICR/RMH CSPC and CRPC cohort, Mann-Whitney test was used to compare differences in nuclear AR-V7 protein expression levels. Spearman's rank correlation coefficient was used to determine the association between nuclear AR-V7 protein expression and timing of CRPC biopsy after starting AA or E therapy. Nonparametric equality-of-medians test was used to determine the difference in nuclear AR-V7 protein expression between metastatic sites. Wilcoxon matched-pairs signed rank test was used to determine the difference between nuclear AR-V7 protein expression as VCaP mouse xenografts progressed from castration sensitive through castration resistant to AA/E resistance. Spearman's rank correlation coefficient was used to determine associations between AR-FL and AR-V7 mRNA expression, total AR-V7 and total AR-FL protein expression, total AR-V7 protein expression and AR copy number, total AR-FL protein expression and AR copy number, nuclear AR-V7 and nuclear AR-FL protein expression, and optical density and HS quantification for nuclear AR-V7 expression. Fligner-Killeen and Levene's tests for homogeneity of variances between-tumors and within-tumors were performed in R using the fligner.test and leveneTest functions. Patients response to AR targeted therapy (AA or E) pre- and post-chemotherapy, and docetaxel were determined. For each therapy, PSA nadir was calculated as the lowest PSA level on therapy and 12-week PSA response was calculated as the % change in PSA between the start of therapy (baseline) and 12-weeks treatment (or closest available PSA reading). Time to PSA progression was defined as time from start of therapy to first PSA increase that is ≥ 25% and ≥ 2µg/L above the PSA nadir. Time to clinical/radiological progression was defined as time from start of therapy to documented radiological progression or clinical progression (including change of therapy, addition of investigational medicinal product or stopping treatment). Overall survival was defined as time from start of therapy to date of death or last follow

up/contact. Patients baseline characteristics and clinical outcomes were compared by positive (nuclear AR-V7 HS > 10) or negative (nuclear AR-V7 HS ≤ 10) AR-V7 status. Patient's baseline characteristics were compared using Fisher's exact test, Student's t-tests (2 tailed) and Wilcoxon rank-sum test as indicated. 50% PSA nadir and 12 week 50% PSA response rates were compared using Fisher's exact test. The difference between nuclear AR-V7 expression by 50% PSA response rate was compared using Mann-Whitney test. Median time to PSA progression, time to clinical/radiological progression and overall survival were estimated using the Kaplan-Meier method. Association with AR-V7 status (positive vs negative) was tested using univariable Cox proportional hazards models. For all statistical analysis, a p-value of less than 0.05 was considered to be statistically significant.

Study approvals

All animal studies were performed in accordance with Beth Israel Deaconess Medical Center IACUC regulations (protocol #086-2016). All patients treated at the Royal Marsden NHS Foundation Trust had provided written informed consent and were enrolled in institutional protocols approved by the Royal Marsden NHS Foundation Trust Hospital (London, UK) ethics review committee (reference no. 04/Q0801/60). All procedures involving human subjects at the University of Washington (Seattle, Washington) and Fred Hutchinson Cancer Research Center (Seattle, Washington) were approved by the Institutional Review Board at those institutions.

5. Author contributions

AS, IC, WY, CS, DNR, JWR, IF, CB, DD, GS, RR, TU, AN, JW, CM, SC, PSN, SPB, LDT, JSdB and SRP designed the research studies. AS, IL, WY, CS, DNR, JWR, IF, CB, RR, TU, AN, JW, SC and LDT conducted experiments and acquired data. AS, IC, WY, CS, DD, DNR, JWR, IF, CB, GS, TU, AN, JW, SC, PSN, SPB, LDT, JSdB and SRP analyzed the data. AS, IC, WY, CS, DD, DNR, JWR, IF, CB, GS, RR, TU, AN, JW, CM, SC, JL, PSN, SPB, LDT, JSdB and SRP wrote and critically reviewed the manuscript.

6. Competing interests

AS, WY, DD, DNR, IF, CB, GS, RR, AN, JW, SC and JSdB are employees of The Institute of Cancer Research, which has a commercial interest in abiraterone. AS has served as an advisory member for Sanofi. JL is an inventor of a relevant technology that has been licensed to A&G, Tokai, and Qiagen. PSN has served as an advisory member for Janssen, Astellas and Roche. SPB has served as an advisory member for Janssen, Sanofi and Astellas. JSdB has served as an advisory member for Astellas, Janssen, Taiho, Vertex, AstraZeneca, Bayer, Genmab, Genentech, GlaxoSmithKline, Merck, Pfizer, Roche and Sanofi-Aventis. IC, CS, JWR, TU, CM, LDT and SRP have no competing interests.

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9. Figures and figure legends

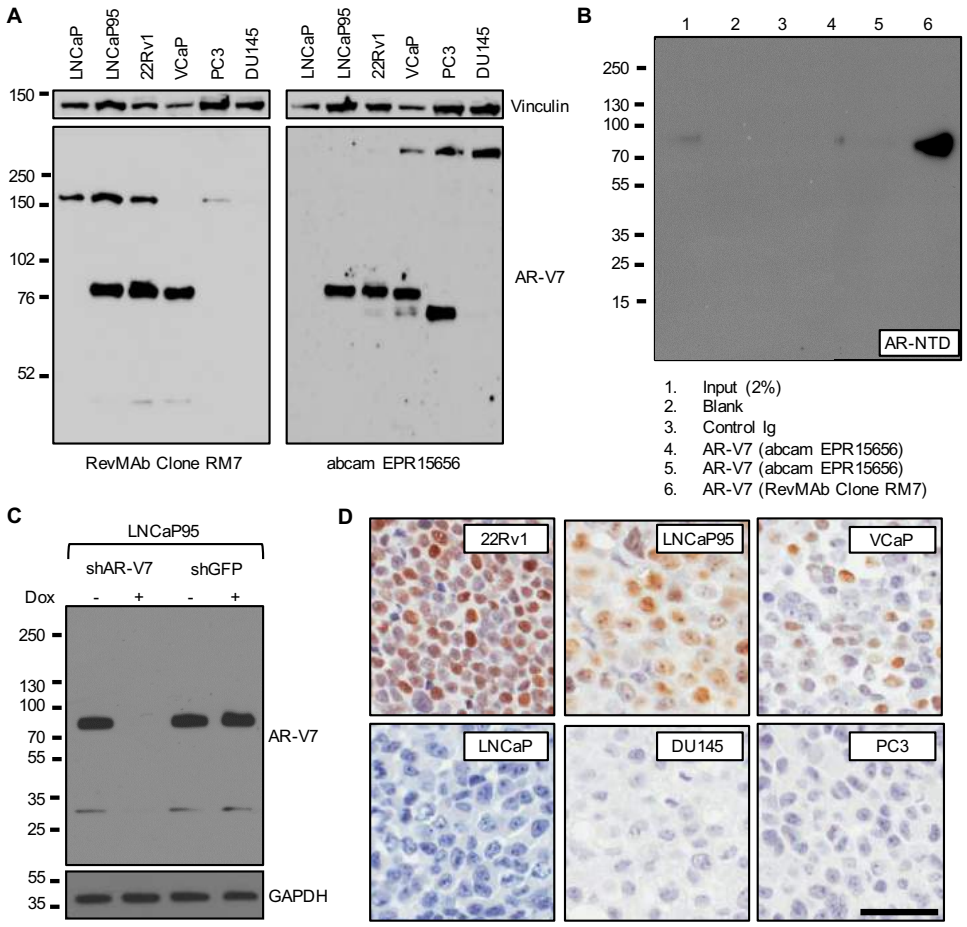


Figure 1: Validation and optimization of a novel AR-V7 antibody (Clone RM7) for immunohistochemistry.

(A) Western blot (long exposure) of AR-V7 positive (LNCaP95, 22Rv1 and VCaP) and negative (LNCaP, PC3 and DU145) PC cell lines using a novel recombinant rabbit monoclonal anti-AR-V7 antibody (Clone RM7) and a previously reported anti-AR-V7 antibody (EPR15656). All cell lines except for LNCaP95 (10% charcoal stripped serum) were grown in 10% fetal bovine serum. **(B)** Immunoprecipitation of AR-V7 from M12-cumate inducible AR-V7 cells using same concentration of AR-V7 antibodies and western blot performed with AR N-terminal domain (AR-NTD) antibody. **(C)** LNCaP95 cells with (doxycycline) inducible shRNA to AR-V7 were treated with (or without) doxycycline and western blot performed with AR-V7 antibody (RM7). **(D)** Micrographs of AR-V7 detection by IHC using AR-V7 antibody (RM7) in cell line pellets positive (22Rv1, LNCaP95 and VCaP) and negative (LNCaP, DU145 and PC3) for AR-V7 (magnification 200x; scale bar 50 μm).

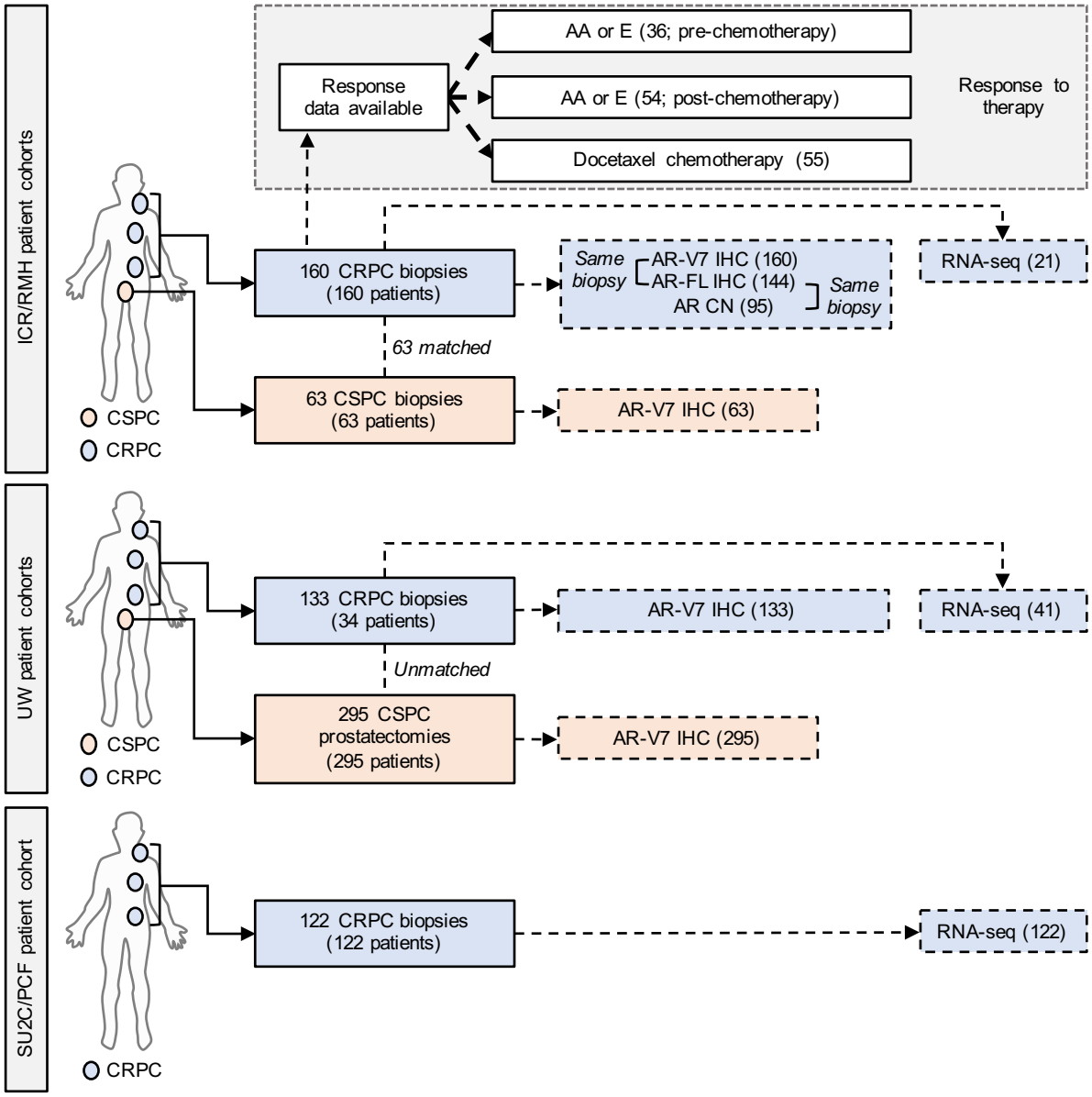


Figure 2: Summary of clinical samples analyzed.

Overview of the ICR/RMH, UW and SU2C/PCF patient cohorts utilized for this study. ICR/RMH patient cohort included 63 CSPC biopsies and 160 CRPC biopsies stained for nuclear AR-V7 expression. Of the 160 biopsies with AR-V7 expression; AR-FL expression (144 biopsies), AR copy number (95 biopsies) and RNA-seq (21 biopsies) was available. Response data was available for abiraterone acetate (AA) or enzalutamide (E) pre-chemotherapy (36 patients), AA or E post-chemotherapy (54 patients) and docetaxel chemotherapy (55 patients). UW patient cohort included 295 CSPC tissues (from 295 patients) who had radical prostatectomy as primary therapy and 133 CRPC biopsies of metastases (from 34 patients). Of 133 CRPC biopsies from 34 patients with AR-V7 expression; RNA-seq (41 biopsies) was available. SU2C/PCF patient cohort included 122 CRPC biopsies with RNA-seq analysis.

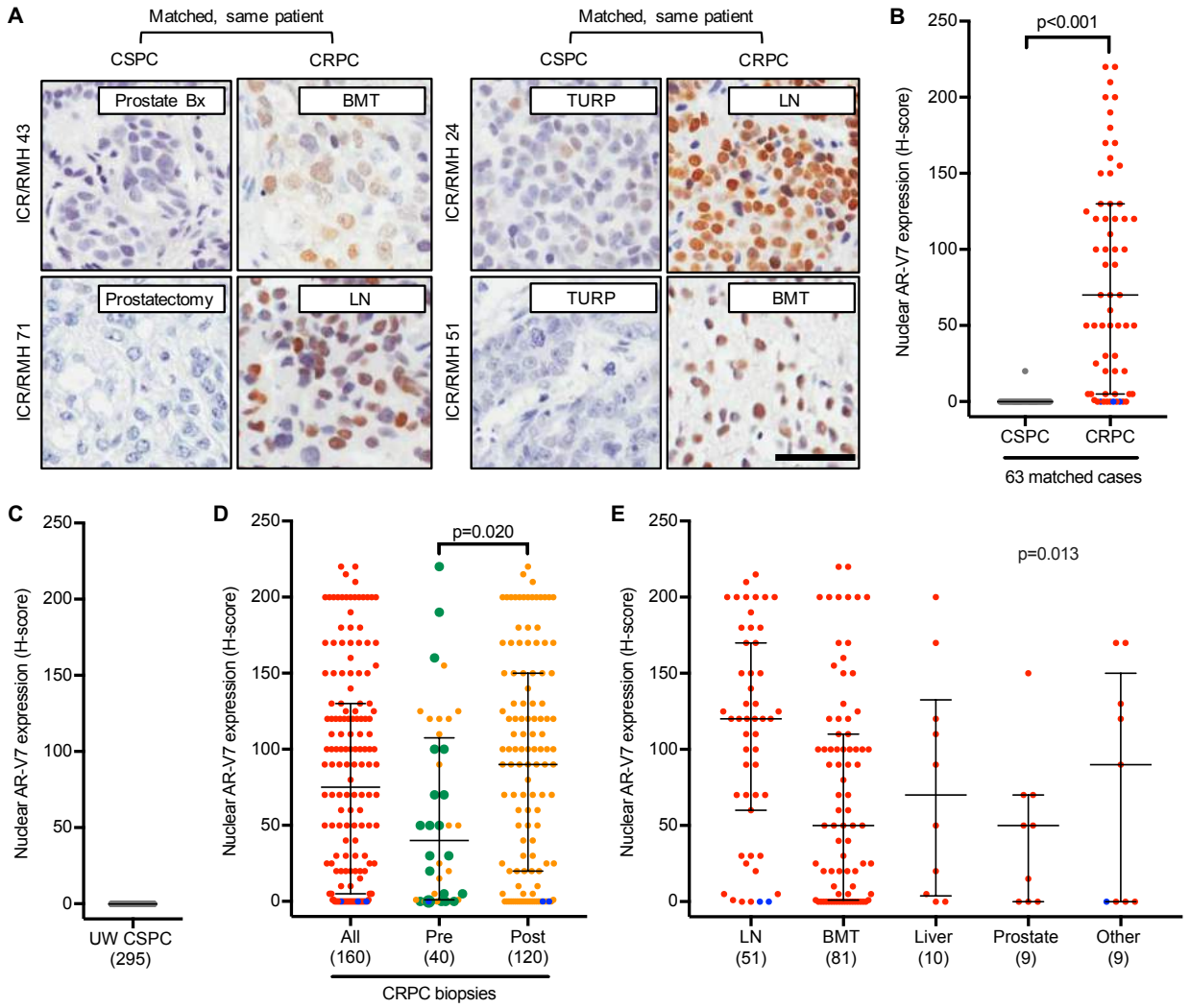


Figure 3: AR-V7 protein expression in prostate cancer.

(A) Representative micrographs of AR-V7 detection by immunohistochemistry (IHC) in four ICR/RMH patients with matched castration sensitive prostate cancer (CSPC) and castration resistant prostate cancer (CRPC) biopsies (magnification 200x; scale bar 50 µm). Prostate (Prostate Bx), prostatectomy, transurethral resection of the prostate (TURP), lymph node (LN) and bone marrow trephine (BMT) biopsies are shown. **(B)** Expression (H-score; HS) of nuclear AR-V7 expression in 63 same patient matched CSPC (grey) and CRPC (red) biopsies from ICR/RMH cohort. Three AR null CRPC cases with neuroendocrine features are shown (blue). Median HS and interquartile range is shown. p-value was calculated using Wilcoxon signed-rank test. **(C)** Expression (HS) of nuclear AR-V7 expression in 295 prostatectomy samples prior to any AR targeted therapy. Median HS and interquartile range is shown. **(D)** Expression (HS) of nuclear AR-V7 expression in 160 CRPC biopsies (red) and dichotomized (orange) by pre (40 biopsies) and post (120 biopsies) abiraterone acetate (AA) or enzalutamide (E) treatment. Three AR null CRPC cases with neuroendocrine features are shown (blue). Twenty biopsies taken after progression on primary ADT (with or without bicalutamide)

and prior to standard therapy for CRPC are shown (green). Median HS and interquartile range is shown. p-value was calculated using Mann-Whitney test. **(E)** Expression (HS) of nuclear AR-V7 expression in 160 CRPC biopsies (red) from lymph node (LN), bone (BMT), liver, prostate and other sites of metastases. Three AR null CRPC cases with neuroendocrine features are shown (blue). Median HS and interquartile range is shown. p-value was calculated using Nonparametric equality-of-medians test.

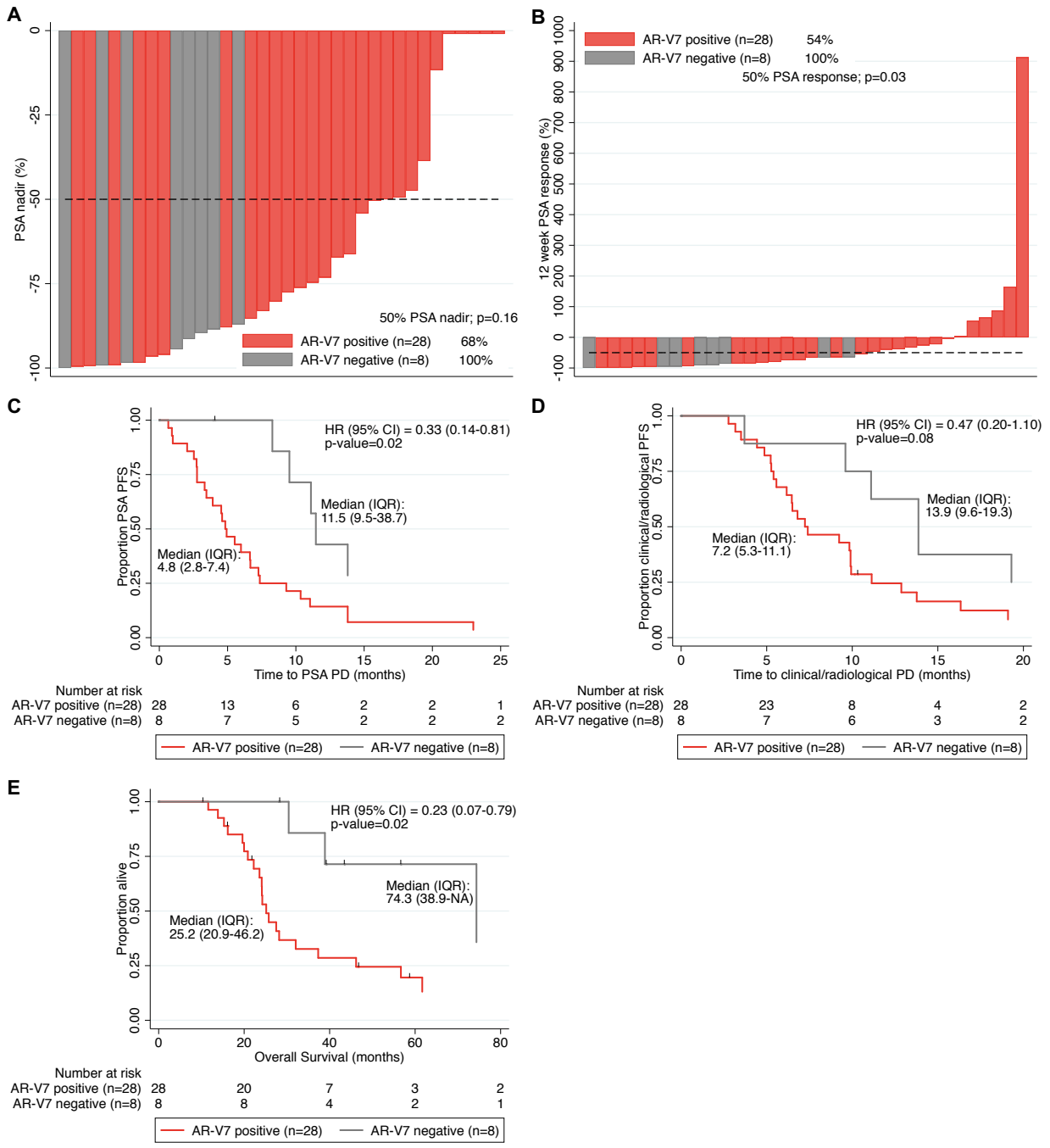


Figure 4: AR-V7 status and response to AR targeting therapies (abiraterone acetate or enzalutamide) prior to chemotherapy in castration resistant prostate cancer.

Thirty-six patients received AR targeting therapies (abiraterone acetate or enzalutamide) prior to chemotherapy for CRPC. **(A)** Percentage PSA nadir on AR targeting therapies for AR-V7 negative (H-score ≤ 10; grey) and AR-V7 positive (H-score > 10; red) CRPC patients is shown. 50% PSA nadir rate is shown. p-value was

calculated using Fisher's exact test. **(B)** Percentage 12-week 50% PSA response rate on AR targeting therapies for AR-V7 negative (grey) and AR-V7 positive (red) CRPC patients is shown. Twelve-week 50% PSA response rate is shown. p-value was calculated using Fisher's exact test. **(C-E)** Kaplan-Meier curves show time to PSA progression (C), time to clinical/radiological progression (D) and overall survival (E) from starting AR targeting therapy. Hazard ratios (HR) with 95% confidence intervals (CI) are shown. p-value was calculated using univariate Cox proportional hazards model.

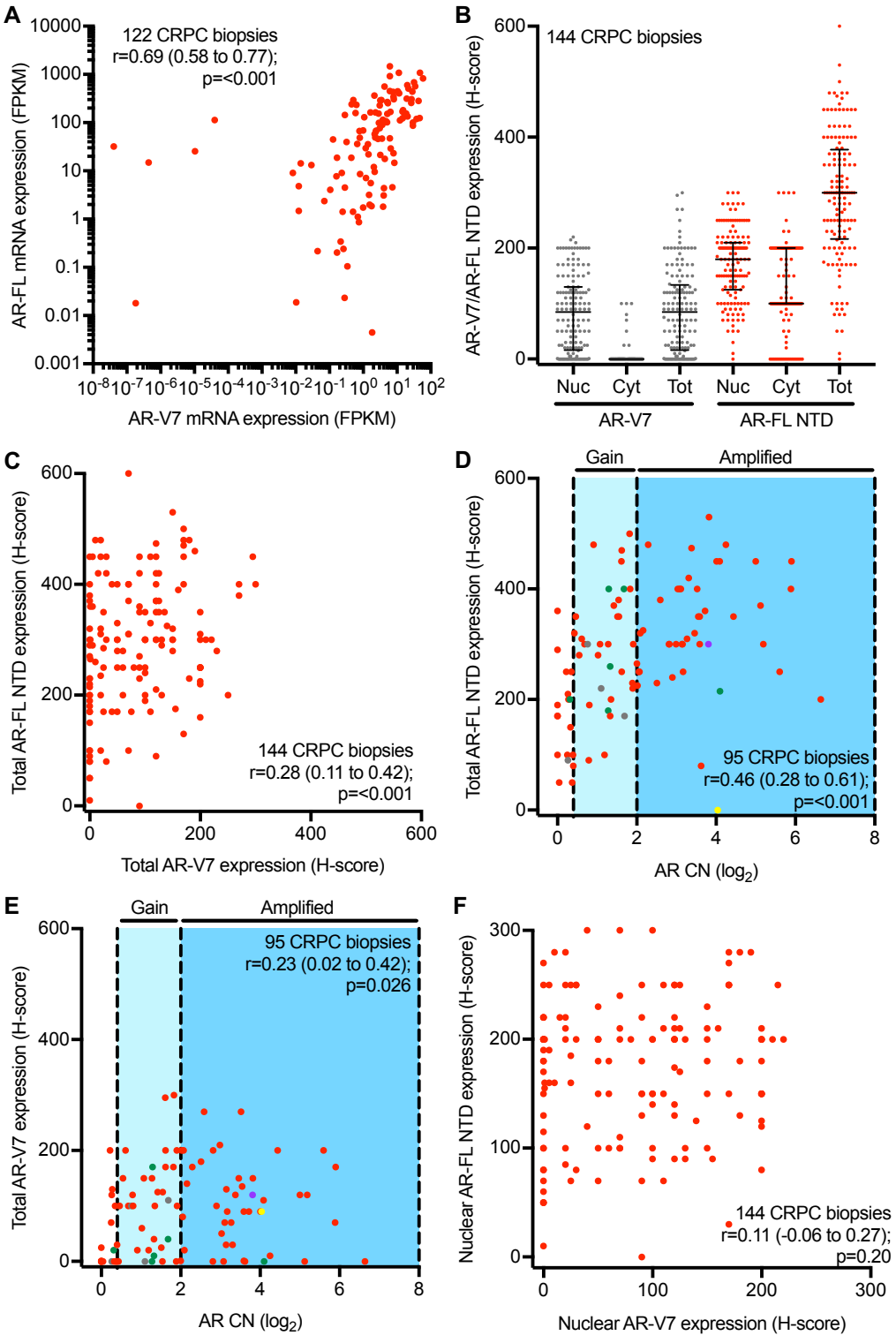


Figure 5: AR-FL and AR-V7 mRNA and protein quantification with AR copy number analysis in castration resistant prostate cancer.

(A) AR-FL and AR-V7 mRNA expression in fragments per kilobase of transcript per million mapped reads (FPKM) for 122 CRPC transcriptomes from the PCF/SU2C cohort is shown. Spearman's rank correlation is shown. **(B)** Expression (H-Score; HS) for nuclear, cytoplasmic and total (nuclear + cytoplasmic) AR-V7 (grey) and AR-FL N-terminal domain (NTD; red) is shown. Median HS and interquartile range is shown. **(C)** Expression (HS) of total AR-FL NTD protein and total AR-V7 protein in 144 CRPC biopsies from the ICR/RMH CRPC cohort is shown. Spearman's rank correlation is shown. **(D)** Expression (HS) of total AR-FL NTD protein and AR copy number (log_2) in 95 CRPC biopsies from the ICR/RMH CRPC cohort is shown. Cases with AR mutations are shown (L702H grey, T878A green, H875Y purple, K313E yellow). Spearman's rank correlation is shown. **(E)** Expression (HS) of total AR-V7 protein and AR copy number (log_2) in 95 CRPC biopsies from the ICR/RMH CRPC cohort is shown. Cases with AR mutations are shown. Spearman's rank correlation is shown. **(F)** Expression (HS) of nuclear AR-FL NTD protein and nuclear AR-V7 protein in 144 CRPC biopsies from the ICR/RMH CRPC cohort is shown. Spearman's rank correlation is shown.

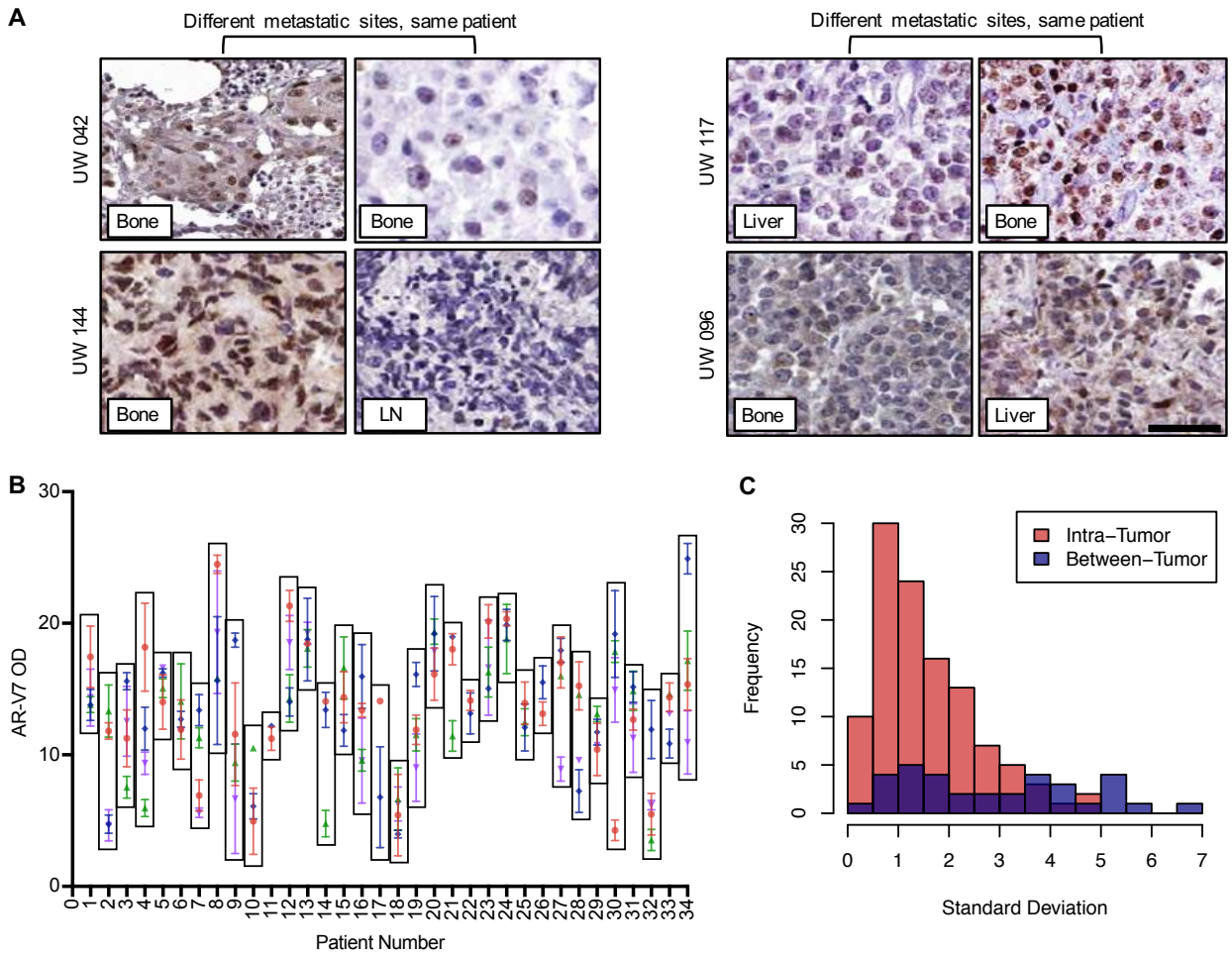


Figure 6: AR-V7 protein expression variability within metastasis and between metastases from individual patients with castration resistant prostate cancer.

(A) Representative micrographs of AR-V7 detection by immunohistochemistry in four UW patients with multiple castration resistant prostate cancer (CRPC) biopsies (magnification 200x; scale bar 50 μm). **(B)** Expression (optical density; OD) of nuclear AR-V7 expression in 133 metastases from 34 CRPC patients from UW CRPC cohort. Mean OD and standard deviation (SD) for three measurements from each metastasis is shown. Each box encloses all metastases from a patient. Different colors for each patient represent an individual metastasis. **(C)** Frequency distribution of SD within a metastasis (Intra-Tumor; comparison of triplicates in a metastasis; red) and between metastases (Between-Tumor; comparison of multiple metastasis within a patient; blue) is shown. Median SD for Intra-Tumor measurements is 1.2 and Between-Tumor measurements 2.9.

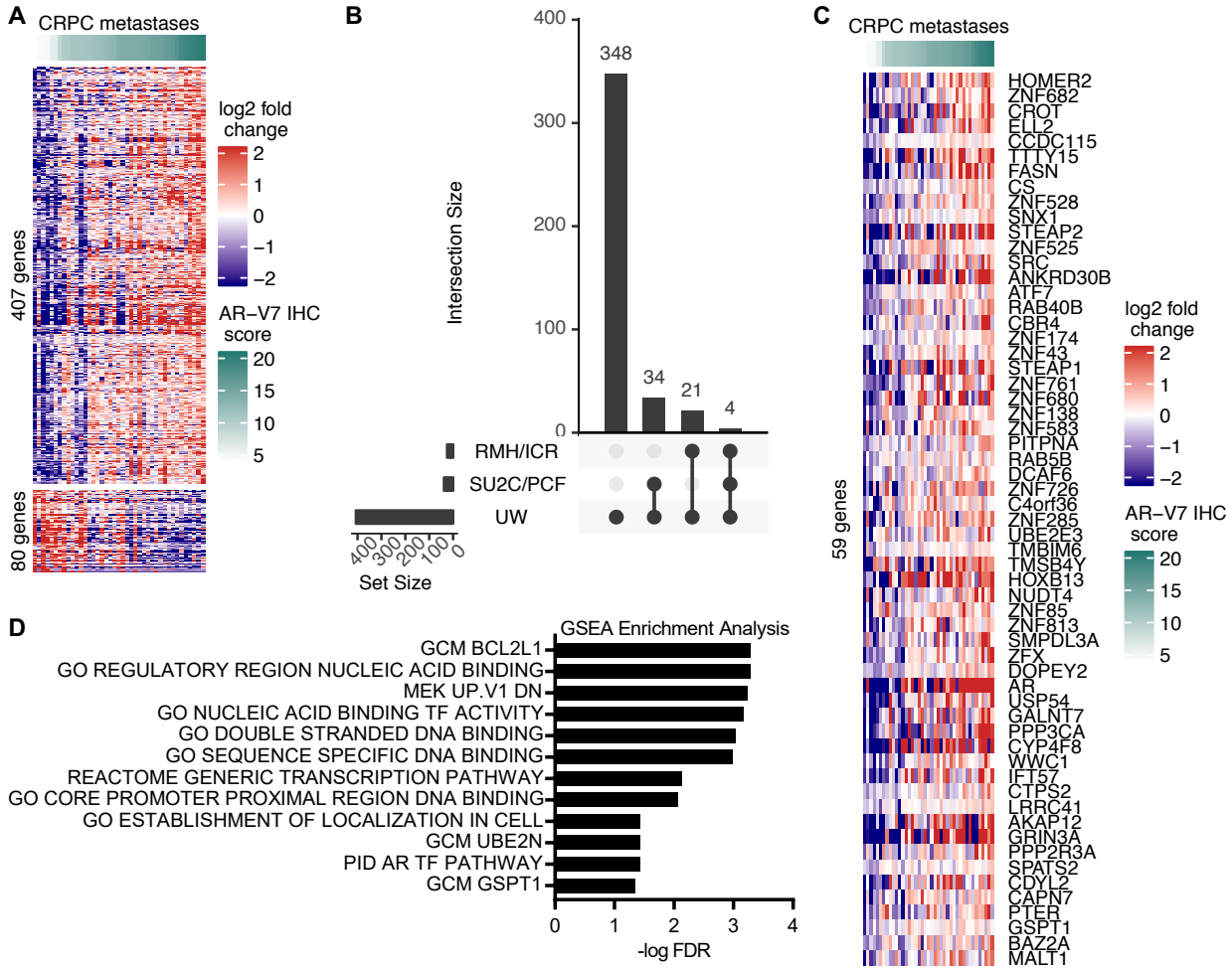


Figure 7: AR-V7 protein expression is associated with a unique gene signature in metastatic castration resistant prostate cancer.

(A) Expression (optical density; OD) of nuclear AR-V7 protein correlated (q<0.05) with gene mRNA expression of 487 (407 upregulated and 80 downregulated) genes in 41 metastases from 24 patients from UW CRPC cohort. Heatmap shows metastasis ranked in order of nuclear AR-V7 expression (OD) and mean-centered $\log_2$ fold change in gene mRNA expression. **(B)** Fifty-nine of the 407 upregulated genes were validated in either 21 CRPC metastasis from ICR/RMH CRPC cohort or 122 CRPC transcriptomes from SU2C/PCF cohort. Figure shows overlap of significantly correlated genes between the three cohorts. **(C)** Heatmap shows metastasis ranked in order of nuclear AR-V7 expression (OD) and mean-centered $\log_2$ fold change in gene mRNA expression of the 59-gene signature in the UW CRPC cohort (n = 41). **(D)** Pathway over-representation analysis using MSigDBv6.2 (H – Hallmark, CP – Canonical Pathways, C4 – Computational Gene Sets, C5 – GO and C6 – Oncogenic Pathway) in the 59-gene set. Pathways with FDR <0.05 are shown.

10. Table

	AR-V7 negative (H-score ≤ 10) N=8	AR-V7 positive (H-score > 10) N=28	p value
Age, years			
Mean (SD)	63.0 (4.8)	69.0 (7.5)	0.04[1]
Performance status, N (%)			
0	4 (50)	14 (50)	
1	4 (50)	14 (50)	1.00[2]
2	0 (0)	0 (0)	
>2	0 (0)	0 (0)	
Bloods			
Hb (g/L), Mean (SD)	133.0 (8.7)	127.6 (12.2)	0.25[1]
ALT (U/L), Mean (SD)	21.4 (6.1)	22.0 (8.5)	0.84[1]
ALP (U/L), Median (IQR)	90.0 (80.3-170.5)	90.0 (63.5-176.8)	0.83[3]
Albumin (g/L), Mean (SD)	36.5 (2.9)	38.4 (3.3)	0.16[1]
LDH (U/L), Median (IQR)	163.0 (144.0-169.0)^	168.0 (156.0-184.0)^^	0.29[3]
PSA (ng/mL), Median (IQR)	154.0 (8.9-238.3)	87.5 (35.5-272.5)	0.62[3]
Metastatic, N (%)			
Node only	3 (38)	4 (14)	
Visceral (with/without bone)	1 (13)	1(4)	0.12[2]
Bone	4 (50)	23 (82)	
AR therapy N (%)			
Abiraterone	5 (63)	19 (68)	1.00[2]
Enzalutamide	3 (38)	9 (32)	
Prior CRPC treatments, N (%)			
Abiraterone	0 (0)	0 (0)	
Enzalutamide	0 (0)	0 (0)	NA
Docetaxel	0 (0)	0 (0)	
Cabazitaxel	0 (0)	0 (0)	

Table 1: Baseline characteristics at time of staring AR targeting therapy (pre-chemotherapy).

N – number, SD – standard deviation, IQR – interquartile range, Hb – hemoglobin, ALT – alanine aminotransferase, ALP – alkaline phosphatase, LDH – lactate dehydrogenase, PSA – prostate specific antigen, NA – not applicable

^1 patient missing LDH value
^^1 patient missing LDH value

1 t-test
2 Fisher's exact test
3 Rank-sum test

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Research Paper

Ipilimumab plus nivolumab and DNA-repair defects in AR-V7-expressing metastatic prostate cancer

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ABSTRACT

AR-V7-expressing metastatic prostate cancer is an aggressive phenotype with poor progression-free survival (PFS) and overall survival (OS). Preliminary evidence suggests that AR-V7-positive tumors may be enriched for DNA-repair defects, perhaps rendering them more sensitive to immune-checkpoint blockade. We enrolled 15 metastatic prostate cancer patients with AR-V7-expressing circulating tumor cells into a prospective phase-2 trial. Patients received nivolumab 3 mg/kg plus ipilimumab 1 mg/kg every 3 weeks for four doses, then maintenance nivolumab 3 mg/kg every 2 weeks. Targeted next-generation sequencing was performed to determine DNA-repair deficiency (DRD) status. Outcomes included PSA response rates, objective response rates (ORR), PSA progression-free survival (PSA-PFS), clinical/radiographic PFS and OS. Median age of participants was 65, median PSA was 115 ng/mL, 67% had visceral metastases, and 60% had ≥4 prior systemic therapies. Six of 15 men (40%) had DRD mutations (three in *BRCA2*, two in *ATM*, one in *ERCC4*; none had microsatellite instability). Overall, the PSA response rate was 2/15 (13%), ORR was 2/8 (25%) in those with measurable disease, median PSA-PFS was 3.0 (95%CI 2.1–NR) months, PFS was 3.7 (95%CI 2.8–7.5) months, and OS was 8.2 (95%CI 5.5–10.4) months. Outcomes appeared generally better in DRD+ vs. DRD− tumors with respect to PSA responses (33% vs. 0%; *P*=0.14, nonsignificant), ORR (40% vs. 0%; *P*=0.46, nonsignificant), PSA-PFS (HR 0.19; *P*<0.01, significant), PFS (HR 0.31; *P*=0.01, significant), and OS (HR 0.41; *P*=0.11, nonsignificant). There were no new safety concerns. Ipilimumab plus nivolumab demonstrated encouraging efficacy in AR-V7-positive prostate cancers with DRD mutations, but not in the overall study population.

INTRODUCTION

Androgen-receptor splice variant 7 (AR-V7) is a constitutively-active isoform of the androgen receptor that is associated with a particularly aggressive form of advanced prostate cancer [1]. Because AR-V7 lacks the androgen-receptor ligand-binding domain, AR-V7-positive prostate cancers are generally resistant to novel hormonal therapies including abiraterone and enzalutamide [2, 3]. In addition, prostate cancers expressing AR-V7 often show poor responses to taxane chemotherapies including docetaxel and cabazitaxel [4, 5]. To this end, patients with AR-V7-positive prostate cancer generally have a median progression-free survival (PFS) of only 3-4 months and a median overall survival (OS) of 7-9 months. Therefore, developing effective therapies for AR-V7-expressing advanced prostate cancer represents an urgent unmet need.

Immune-checkpoint blockade may be one potential strategy to treat such patients. In many cancer types, inhibition of cytotoxic T-lymphocyte–associated antigen 4 (CTLA-4) and/or the programmed death 1 (PD-1) receptor has resulted in meaningful antitumor responses [6]. In some settings, combined blockade of both PD-1 (mediating T-cell exhaustion in peripheral tissues) and CTLA-4 (involved in earlier phases of T-cell activation) has proven more efficacious than inhibition of either pathway alone [7, 8]. Furthermore, tumors harboring DNA mismatch-repair defects or those with hypermutation may be particularly sensitive to immune-checkpoint inhibition [9, 10]. While prostate cancer is generally regarded as a low–mutation-burden tumor [11] and immune-checkpoint blockade has resulted in only modest benefits as a monotherapy [12, 13], recent data have suggested that AR-V7-expressing prostate cancers may be associated with a greater number of DNA-repair gene mutations and a higher mutation load [14].

We hypothesized that metastatic castration-resistant prostate cancer patients with AR-V7-positive circulating tumor cells (CTCs) would be susceptible to treatment with combined immune-checkpoint blockade, and that this approach would be safe and tolerable. We also sought to determine (in an exploratory fashion) whether treatment efficacy was associated with presence of DNA-repair gene mutations. To test these hypotheses, we conducted a phase-2 clinical trial testing ipilimumab plus nivolumab in patients with AR-V7-positive advanced prostate cancer.

RESULTS

Patient characteristics

From March 2016 through December 2016, a total of 36 patients underwent clinical-grade AR-V7 testing for eligibility purposes, 26 (72%) had detectable CTCs, and 16 men (44%) were AR-V7-positive. One patient failed screening, leaving 15 patients that comprised our study cohort. Supplementary Table 1 summarizes the baseline characteristics of the study participants. Median age was 65 years, 47% had ECOG performance-status of 1, median PSA was 115 ng/mL, 67% had visceral (liver or lung) metastases, and 60% had received ≥4 prior regimens for metastatic castration-resistant prostate cancer (mCRPC). All patients received at least one dose of the study drugs. At the time of data cutoff (October 2017), median follow-up was 8.6 (range, 1.9–17.9) months, and two patients remained alive.

Overall clinical outcomes

All patients were evaluable for efficacy (summarized in Table 1, Supplementary Figure 1). Overall, 2 of 15 men (13.3%, 95%CI 3.7–37.9%) achieved a PSA response. Among the 8 patients with measurable soft-tissue disease, the objective response rate (ORR) was 25.0% (95%CI 7.2–59.1%). Median PSA-PFS was 3.0 (95%CI 2.1–NR) months, and median PFS was 3.7 (95%CI 2.8–7.5) months. Three of 15 patients (20.0%, 95%CI 7.1–45.2%) achieved a "durable PFS". Median OS was 8.2 (95%CI 5.5–10.4) months.

DNA-repair defects and outcomes

Six of 15 patients (40%) harbored potentially deleterious somatic and/or germline mutations in a least one DNA-repair gene (Table 2, Supplementary Table 7C), and were considered DNA-repair deficient (DRD+). Patient 3 had a germline *BRCA2* mutation, patient 4 had somatic mutations in both *BRCA2* and *MSH6*, patient 6 had a somatic *ATM* mutation, patient 8 had a germline *BRCA2* and a somatic *FANCM* mutation, patient 9 had a somatic *ATM* mutation, and patient 14 had a somatic *ERCC4* mutation. Baseline characteristics and clinical outcomes of the DRD+ and DRD– patients are summarized in Supplementary Tables 1 and 2. Two patients (3 and 8) had germline mutations in *BRCA2*, and two patients (4 and 8) had biallelic *BRCA2* alterations resulting from LOH of the wild-type allele. No patient demonstrated microsatellite instability. Mean tumor mutational load was estimated at 3.2 (range, 0.8–7.8) mutations/Mb in DRD+ patients and 1.6 (range, 0.8–3.1) mutations/Mb in DRD– patients.

To further examine whether DNA-repair defects are enriched in AR-V7-positive patients, we interrogated the StandUp2Cancer (SU2C) database comprising whole-exome and transcriptome sequencing from 150 mCRPC biopsies [11], of which 143 had adequate RNA yields. Of these, 17.5% of cases (25/143) had AR-V7/AR-FL ratios on RNA sequencing of >10%, and were designated as AR-V7-high; while the remaining 82.5% (118/143) were designated as AR-V7-low. This threshold was set so that the prevalence of an AR-V7-positive tissue-based test would be broadly similar to that of a positive CTC-based AR-V7 test. To this end,

Table 1: Overall outcomes for all patients, and according to DNA-repair deficiency (DRD) status

	Overall (N=15)	DRD Negative (N=9)	DRD Positive (N=6)	HR (95%CI)	*P* value
PSA_{50}, N (%) (95% CI)	2/15 (13.3%) (3.7–37.9)	0/9 (0%) (0–29.9)	2/6 (33.3%) (9.7–70.0)	–	0.14
ORR, N (%) (95% CI)	2/8 (25.0%) (7.2–59.1)	0/3 (0%) (0–56.2)	2/5 (40.0%) (11.8–76.9)	–	0.46
Durable PFS (95% CI)	3/15 (20.0%) (7.1–45.2)	0/9 (0%) (0–29.9)	3/6 (50.0%) (18.8–81.2)	–	0.044
PSA-PFS (mo), (95% CI)	2.96 (2.07–NR)	2.07 (1.74–NR)	5.82 (4.24–NR)	0.19 (0.06–0.62)	0.0003
PFS (mo), (95% CI)	3.68 (2.76–7.52)	2.83(1.87–NR)	6.51 (3.88–NR)	0.31 (0.10–0.92)	0.014
OS (mo), (95% CI)	8.18 (5.52–10.41)	7.23 (3.45–NR)	9.04 (8.18–NR)	0.41 (0.14–1.21)	0.11

NR: upper 95% confidence limit of survival probability not reached.

pathogenic DRD mutations were found in 36.0% (9/25) of AR-V7-high cases but only in 18.6% (22/118) of AR-V7-low cases (*P*=0.056), suggesting a possible (but non-significant) association between AR-V7 and DNA-repair defects. In the AR-V7-high SU2C cohort, the altered DNA-repair genes were *BRCA2* (x4), *ATM* (x2), *CDK12* (x2) and *MSH2* (x1).

We then compared clinical outcomes in DRD+ and DRD– patients from our trial (Table 1). Response measures appeared generally better in DRD+ versus DRD– cases (Figure 1) with respect to PSA responses (33% vs. 0%; *P*=0.14, nonsignificant), ORR (40% vs. 0%; *P*=0.46, nonsignificant) and "durable PFS" (50% vs. 0%; *P*=0.04, significant). Interestingly, both patients who achieved PSA responses (4 and 8) had biallelic *BRCA2* alterations. Similarly, time-to-event outcomes also appeared better in DRD+ versus DRD– patients (Figure 2) with respect to PSA-PFS (HR 0.19, 95%CI 0.06–0.62; *P*<0.001, significant), PFS (HR 0.31, 95%CI 0.10–0.92; *P*=0.01, significant), and OS (HR 0.41, 95%CI 0.14–1.21; *P*=0.11, nonsignificant).

Other biomarkers and outcomes

To examine the prognostic impact of CTC phenotypic heterogeneity, we compared outcomes in patients with a high (≥1.5) versus low (<1.5) Shannon index (Supplementary Table 3). Five (33%) and 10 men (67%) were classified as Shannon-high and Shannon-low, respectively. There were numerically more Shannon-high cases among DRD+ compared to DRD– patients (50% [3/6] vs. 22% [2/9] respectively, *P*=0.26, nonsignificant). Outcomes appeared generally better in Shannon-high vs. Shannon-low patients with respect to PSA responses (20% vs. 10%; *P*=1.0, nonsignificant), ORR (100% vs. 0%; *P*=0.04, significant), "durable PFS" (40% vs. 10%; *P*=0.24, nonsignificant), PSA-PFS (HR 0.67, 95%CI 0.23–1.99; *P*=0.44, nonsignificant), PFS (HR 0.43, 95%CI 0.15–1.22; *P*=0.11, nonsignificant), and OS (HR 0.34, 95%CI 0.11–0.99; *P*=0.07, nonsignificant) (Figure 3). Interestingly, both men with RECIST-defined objective responses (6 and 14) had high Shannon indices. CTC pleomorphism (high vs. low) was also assessed in relation to clinical outcomes. No statistical trends were observed (Supplementary Table 4, Supplementary Figure 2), although both patients with PSA responses (4 and 8) were classified as pleomorphism-high.

Eight patients underwent new metastatic biopsies and were evaluable for PD-L1 status. Five (62%) and 3 men (38%) were PD-L1–positive and -negative, respectively. Representative immunostains are shown in Supplementary Figure 3. There were numerically more PD-L1–positive cases among DRD+ compared to DRD– tumors (80% [4/5] vs. 33% [1/3] respectively, *P*=0.19). No statistical trends between PD-L1 status and clinical outcomes were observed (Supplementary Table 5, Supplementary Figure 4), although both patients with objective responses (6 and 14) had PD-L1–expressing tumors.

Safety and adverse events

The most common toxicities that developed during or after treatment were fatigue, AST elevation, diarrhea and anorexia (Supplementary Table 6). Seventeen grade 3-4 adverse events occurred in 7 of 15 patients (46%). There were two cases of grade 3-4 fatigue, two cases of grade 3-4 diarrhea/colitis, and two cases of grade 3-4 elevated lipase. Immune-related adverse events were of

Table 2: Summary of DNA-repair deficiency (DRD) status among the 15 patients treated with ipilimumab plus nivolumab

Patient no.	DRD status	DNA-repair gene	Pathogenic DNA-repair mutations	Germline vs. somatic	Loss of heterozygosity (LOH)	MSI markers shifted	Mutational load (muts/Mb)	Source of tumor DNA
1	–	-	-	-	-	N/A	1.1	Plasma
2	–	-	-	-	-	N/A	2.4	Prostate
3	+	*BRCA2*	E1646Qfs*23	Germline	No	0/5	1.6	Liver mass
4	+	*BRCA2* *MSH6*	P3189H E192X	Somatic Somatic	Yes No	0/5	7.8	Lymph node
5	–	-	-	-	-	N/A	3.1	Plasma
6	+	*ATM*	D2708N	Somatic	No	0/5	1.6	Lymph node
7	–	-	-	-	-	0/5	1.4	Epidural mass
8	+	*BRCA2* *FANCM*	D3095E R579H	Germline Somatic	Yes No	0/5	0.8	Prostate
9	+	*ATM*	E2039X	Somatic	No	0/5	1.1	Plasma
10	–	-	-	-	-	N/A	1.1	Plasma
11	–	-	-	-	-	0/5	1.3	Prostate
12	–	-	-	-	-	0/5	0.8	Prostate
13	–	-	-	-	-	0/5	1.3	Lymph node
14	+	*ERCC4*	D762V	Somatic	No	0/5	5.6	Lymph node
15	–	-	-	-	-	0/5	1.8	Liver mass

particular interest. There were five events (affecting 33% of patients) that were possibly or probably related to autoimmune phenomena and that required treatment with corticosteroids: two episodes of colitis, two episodes of pneumonitis, and one episode of hepatitis; hypophysitis was not observed. There were no treatment-related deaths.

DISCUSSION

Prostate cancer expressing AR-V7 represents a lethal phenotype with inadequate treatment options. Here, we report data from the first trial specifically targeting AR-V7-positive disease and the first trial of ipilimumab plus nivolumab in prostate cancer. Although sufficient clinical activity was not observed in the overall study population (and the primary endpoint was not met), encouraging clinical activity using combined immune-checkpoint blockade was seen in the subset of patients harboring germline and/or somatic mutations in DNA-repair genes (and not restricted to mismatch-repair genes). Moreover, there appeared to be a positive correlation between AR-V7 detection and the presence of sequence alterations in DNA-repair genes, further supporting an immunotherapy approach in these patients.

It is now appreciated that approximately 20-25% of metastatic prostate cancers harbor somatic mutations involving DNA-repair genes, primarily homologous-recombination repair genes (e.g. *BRCA2*, *ATM*) and, to a lesser extent, mismatch-repair genes (e.g. *MSH2*, *MSH6*) [11, 21]. The current study, coupled with our secondary analysis of the StandUp2Cancer dataset, suggests that DNA-repair defects (DRD) may be further enriched in AR-V7-positive prostate cancers with a prevalence approaching 40%. These DRD+ patients may benefit from alternative treatment strategies including poly–ADP-ribose polymerase (PARP) inhibitors [22] or other genetically-targeted approaches [23, 24]. The potential association between AR-V7 detection and DRD mutations has also been suggested by a previous study,[14] but still requires further confirmation.

The correlation between DNA mismatch-repair deficiency (resulting in microsatellite instability) and

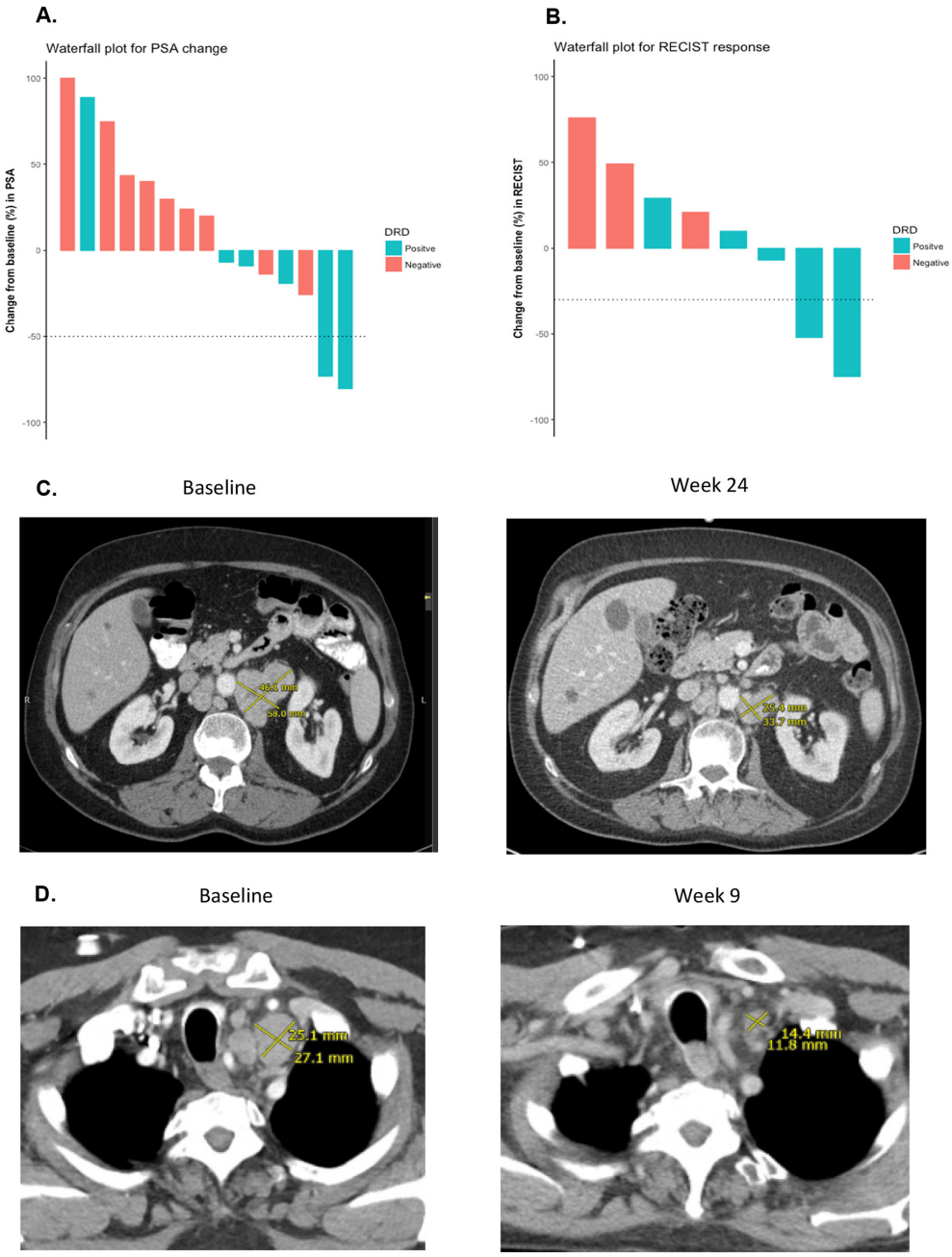


Figure 1: PSA responses and radiographic responses according to DRD status. (A) Waterfall plot showing PSA responses according to DRD status. The two patients with PSA_{50} responses (#4 and #8) both had biallelic *BRCA2* gene mutations. Patient #4 had a mixed soft-tissue response (some measurable lesions decreased while others increased) and achieved a durable PFS. Patient #8 did not have any measurable disease, but also achieved a durable PFS, and experienced complete resolution of malignant bone pain (pain score 7/10 decreased to 0/10 after 12 weeks of therapy); he is still alive after 17.5+ months of follow-up. **(B)** Waterfall plot showing objective RECIST responses according to DRD status. The two patients with soft-tissue responses (#6 and #14) had mutations in *ATM* and *ERCC4*, respectively. Patient #6 achieved a durable PFS, and is still alive after 17.9+ months of follow-up. **(C)** CT scan of radiographic response for patient #6 (with somatic *ATM* mutation) at baseline and after 24 weeks of treatment. The sum diameter of his target lesions decreased by 52% at the time of his best response. **(D)**. CT scan of radiographic response for patient #14 (with somatic *ERCC4* mutation) at baseline and after 9 weeks of treatment. The sum diameter of his target lesions decreased by 75% at the time of his best response.

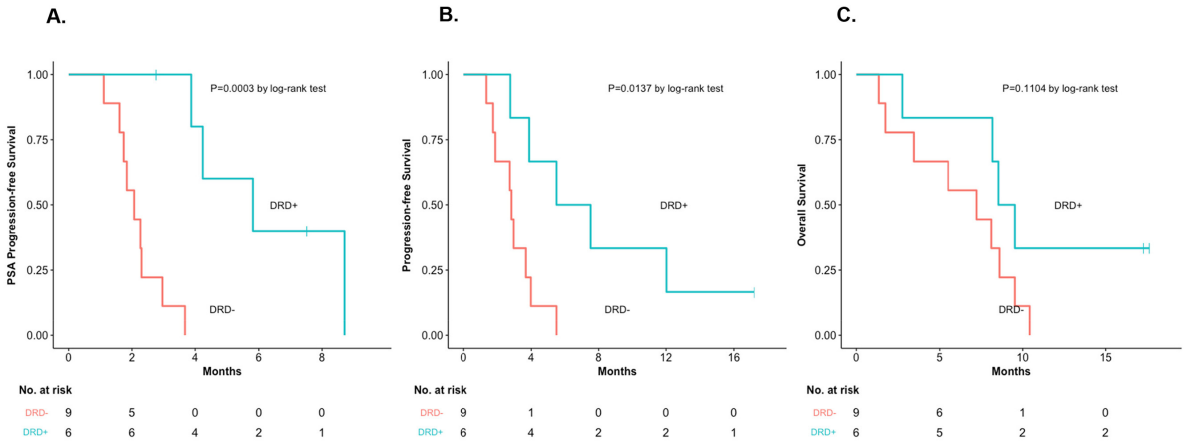


Figure 2: Time-to-event outcomes, according to DRD status. **(A)** PSA-PFS, according to DRD status [HR 0.19, 95%CI 0.06–0.62, *P*=0.0003]. **(B)** PFS, according to DRD status [HR 0.31, 95%CI 0.10–0.92, *P*=0.014]. **(C)** OS, according to DRD status [HR 0.41, 95%CI 0.14–1.21, *P*=0.11].

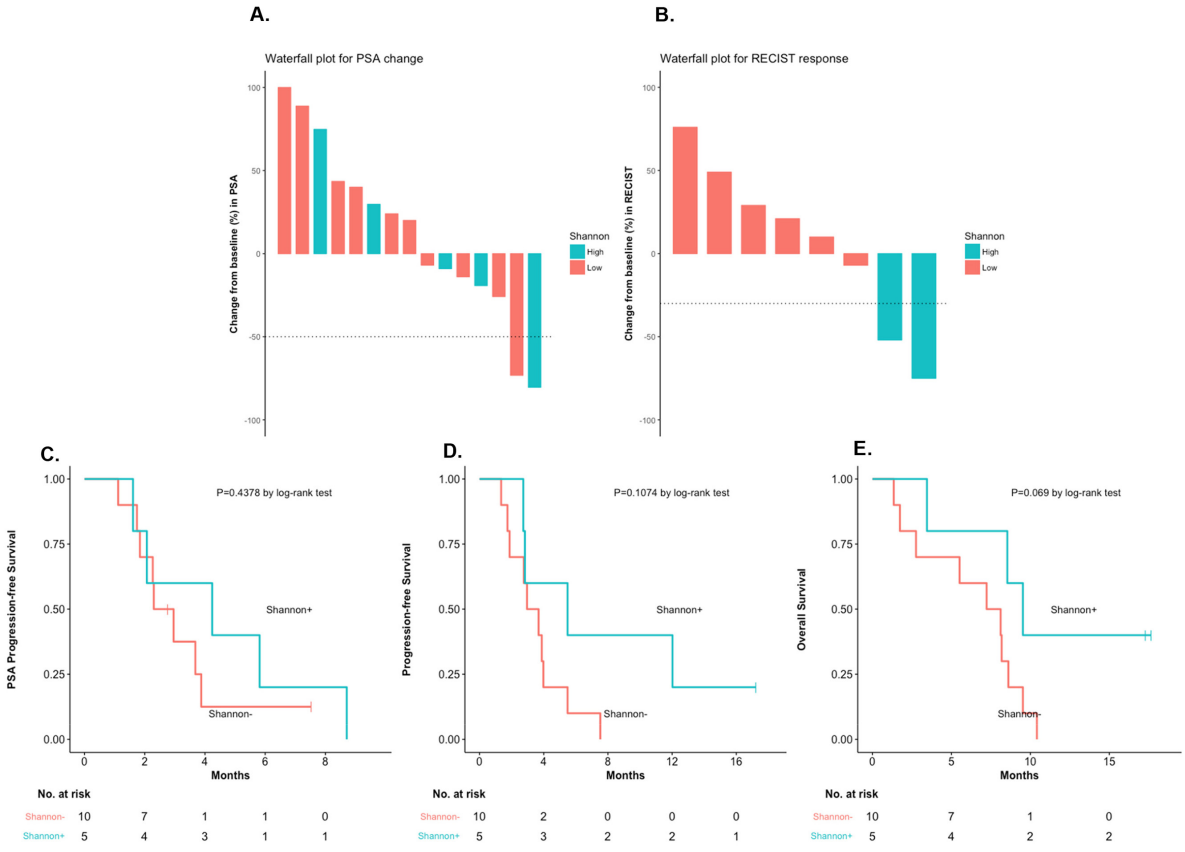


Figure 3: Clinical outcomes, according to Shannon index (low vs. high). **(A)** PSA responses, according to Shannon Index. **(B)** RECIST responses, according to Shannon index. **(C)** PSA-PFS, according to Shannon index [HR 0.67, 95%CI 0.23–1.99, *P*=0.44]. **(D)** PFS, according to Shannon index [HR 0.43, 95%CI 0.15–1.22, *P*=0.11]. **(E)** OS, according to Shannon index [HR 0.34, 95%CI 0.11–0.99, *P*=0.07].

responsiveness to PD-1 inhibitor therapy is now well established, although MMR mutations are only observed in 2-3% of advanced prostate cancers [9]. Our data suggest that sensitivity to immune-checkpoint inhibitors may perhaps be expanded to other types of DNA-repair alterations, particularly homologous-recombination deficiency (HRD) mutations. Among the six DRD+ patients in this study, five had HRD lesions (three in *BRCA2*, two in *ATM*) and one had a nucleotide-excision repair (*ERCC4*) lesion. Interestingly, mean tumor mutational burden was approximately 2-fold higher in DRD+ versus DRD– cancers, although none of these patients demonstrated microsatellite instability. These findings are consistent with two prior studies (including one in prostate cancer) that reported a modestly higher mutational load in *BRCA2*-mutant vs. wild-type tumors [25, 26]. Two other studies in *BRCA1*-deficient breast cancers and *BRCA1/2*-deficient ovarian cancers, respectively, demonstrated that these tumors may have higher predicted neoantigen loads, more tumor-infiltrating lymphocytes and increased expression of PD-1 and CTLA-4 as compared to their homologous-repair–proficient counterparts [27, 28]. Furthermore, a recent study combining durvalumab (a PD-L1 inhibitor) with olaparib (a PARP inhibitor) in mCRPC patients reported high response rates in men with HRD mutations [29]. Finally, a recent clinical study in advanced urothelial carcinoma suggested that outcomes to PD-1 or PD-L1 inhibitors were superior in patients with vs. without HRD mutations [30]. Taken together, these data imply that HRD alterations, not just MMR alterations, may sensitize patients to immune-checkpoint blockade. In addition, the current study is the first to suggest that defects in nucleotide-excision repair (e.g. *ERCC4*) may also be associated with immunotherapy sensitivity.

We also observed a trend between high phenotypic CTC heterogeneity (Shannon index) and favorable responses to combination immunotherapy. In addition, DRD+ patients demonstrated a trend towards higher CTC heterogeneity compared to DRD– patients. Previous studies showed that mCRPC patients with Shannon-high CTCs respond poorly to novel hormonal therapies and better to taxane chemotherapies [20]. Interestingly, the two patients with the highest Shannon indices (6 and 14) both had objective tumor responses, both harbored DRD alterations, and both expressed PD-L1. This suggests a theoretical model whereby DRD mutations result in greater genomic heterogeneity, manifesting as greater phenotypic CTC heterogeneity, and increasing the likelihood of a favorable response to immune-checkpoint inhibition. This hypothesis remains to be proven.

In conclusion, our data suggest that the combination of nivolumab plus ipilimumab demonstrates acceptable safety and encouraging efficacy in men with AR-V7-expressing advanced prostate cancer who also harbor DNA-repair alterations, but not in the overall study population. Moreover, the prevalence of these DNA-repair defects appears to be higher in AR-V7-positive patients. Both of these findings require large-scale prospective validation.

MATERIALS AND METHODS

Patient eligibility

Eligible patients had histologically confirmed, progressive, metastatic castration-resistant prostate cancer (mCRPC) with detectable AR-V7 transcripts using the Johns Hopkins CTC-based clinical-grade AR-V7 assay (see below) [15, 16]. Additional eligibility criteria included an ECOG performance-status of 0-1, at least 18 years of age, serum testosterone <50 ng/dL with ongoing androgen-deprivation therapy, adequate organ (liver, kidney, bone marrow) function, and availability of new or archival tumor tissue for biomarker analysis. Key exclusion criteria included a second active malignancy within 5 years, prior immune-checkpoint inhibitor therapy, active brain or meningeal metastases, history of autoimmune disease, or requirement for systemic corticosteroids. Complete eligibility criteria are available in the Supplementary Materials.

Study design

This was a single-institution one-arm open-label phase 2 study conducted at Johns Hopkins. Patients received treatment by intravenous infusion consisting of 3 mg per kilogram of nivolumab plus 1 mg per kilogram of ipilimumab every 3 weeks for 4 doses, followed by a maintenance regimen of 3 mg per kilogram of nivolumab every 2 weeks thereafter. Treatment continued until radiographic progression, unequivocal clinical progression, development of unacceptable toxicity, or withdrawal of consent. Suspected immune-related toxicities were managed using available guidelines. Patients were not permitted to receive nivolumab maintenance therapy unless they tolerated all four doses of combination immunotherapy.

The primary endpoint was the PSA response rate, defined as a ≥50% decline in PSA from baseline maintained for ≥4 weeks. Secondary endpoints included freedom-from-PSA-progression (PSA-progression-free-survival; PSA-PFS), freedom-from-clinical/radiographic-progression (progression-free-survival; PFS), objective response rate (ORR) according to RECIST1.1 criteria [17] in patients with measurable disease, PFS lasting >24 weeks (termed "durable PFS"), and overall survival (OS). PSA-progression was defined as a ≥25% increase in PSA from baseline or nadir, requiring confirmation ≥4 weeks later (PCWG2 criteria [18]). Clinical/radiologic-progression was defined as unequivocal symptomatic progression (worsening disease-related symptoms or new

cancer-related complications), or radiographic progression (CT scan showing ≥20% enlargement in sum diameter of soft-tissue target lesions [RECIST1.1]; bone scan showing ≥2 new osseous lesions not related to bone flare) or death, whichever occurred first. Safety and adverse effects were also assessed.

Study assessments were prospectively defined. PSA measurements were obtained at baseline and every 4 weeks on study. Radiographic evaluations (CT of chest/abdomen/pelvis and technetium-99 bone scans) were performed at baseline and every 12 weeks. Physical examination, toxicity assessments, and laboratory studies (complete blood count, comprehensive metabolic panel, thyroid function) were performed every 4 weeks. Safety was assessed by collecting and grading adverse events according to CTCAE v4.0 criteria.

This was an investigator-initiated trial (NCT02601014) designed by the principal investigators (E.S.A. and C.G.D.) and funded by Bristol Myers-Squibb who also provided both study drugs free of cost. The study was approved by the Johns Hopkins University IRB, and was overseen by an independent scientific review committee and an independent data and safety monitoring committee. All patients provided written informed consent before participation.

DNA sequencing

All 15 patients underwent prospective tumor DNA sequencing. Details of targeted next-generation sequencing methods performed on pre-treatment tumor, matched normal and circulating-tumor (ct)DNA samples, and bioinformatic analyses, are provided in the Supplementary Materials. We performed targeted sequencing on 8 matched tumor-normal and 3 tumor-only cases (Supplementary Table 7A). In 4 patients, where tumor tissue was not available, we performed next-generation sequencing of cell-free ctDNA (Supplementary Table 7B). In addition to examining sequence alterations and microsatellite instability, we generated estimates of mutation burden for each tumor. We subsequently focused on sequence alterations in DNA-repair genes, identified somatic and germline variants and assessed allele-specific copy-number and loss-of-heterozygosity events for these loci. Putative pathogenic variants were determined by an ensemble of bioinformatic platforms, as described in the Supplementary Materials. To correlate genomic findings with clinical outcomes, patients were classified as "positive" or "negative" for potentially pathogenic mutations in DNA-repair genes. Patients were considered to be DNA repair-deficient (DRD-positive [DRD+]) if they had at least one pathogenic mutation in a gene involved in DNA-damage repair [22]; otherwise they were classified as DRD-negative (DRD–).

AR-V7 and CTC analyses

A modified AdnaTest assay (Qiagen, Hannover, Germany) conducted in our CLIA-certified laboratory was used to interrogate CTCs for AR-V7 mRNA detection [15], and a positive test was required for eligibility. Briefly, this employs EpCAM-based CTC capture followed by multiplexed reverse-transcription polymerase-chain-reaction (qRT-PCR) using custom primers to detect full-length androgen receptor (AR-FL) mRNA and AR-V7 mRNA, as previously described [2, 15]. In addition, all patients underwent collection of CTCs at baseline using the Epic Sciences platform (San Diego, CA) [19], and these cells were analyzed for phenotypic heterogeneity (Shannon index)[20] and degree of pleomorphism, as described in the Supplementary Materials. Clinical outcomes were compared among patients with high versus low CTC heterogeneity and high versus low pleomorphism.

PD-L1 analysis

In patients undergoing a new metastatic tumor biopsy, expression of PD-L1 protein was assessed using immunohistochemistry (rabbit monoclonal antibody, Ventana, Tucson, AZ), as described in the Supplementary Materials. A positive test was defined as any percentage of PD-L1 staining on tumor cells.

Statistical analyses

The primary endpoint was PSA response, and a response rate above 5% was considered clinically meaningful in this AR-V7-positive population. Accordingly, a sample size of 15 patients with ≥3 PSA responses would produce a 90% confidence interval of 6–44%, which would be above the 5% threshold. A positive study would therefore be defined as ≥3 of 15 patients achieving a PSA response.

Analyses of response endpoints (*e.g.* PSA response, ORR) were expressed as proportions with 2-sided Wilson binomial 95% confidence intervals. Time-to-event endpoints (*e.g.* PFS, OS) were analyzed using the Kaplan-Meier method and 95% confidence intervals were generated using the generalized Brookmeyer-Crowley method after log-transformation. Clinical outcomes were compared among patients who were DRD+ and DRD– (primary biomarker analysis), as well as according to other biomarker categories (CTC heterogeneity, CTC pleomorphism, tumor PD-L1 expression). To examine associations between clinical outcomes and biomarker status, response endpoints were compared using Fisher's exact test, and time-to-event endpoints were compared using the log-rank test with Cox proportional-hazards models to derive hazard

ratios. All tests were two-sided, and P values ≤0.05 were considered significant; we did not correct for multiple hypotheses. Statistical analyses were performed using *R* (version 3.4.3).

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CONFLICTS OF INTEREST

E.S.A. is a paid consultant/advisor to Janssen, Astellas, Sanofi, Dendreon, Medivation, ESSA, AstraZeneca, Clovis and Merck; has received research funding to his institution from Janssen, Johnson & Johnson, Sanofi, Dendreon, Genentech, Novartis, Tokai, Bristol Myers-Squibb, AstraZeneca, Clovis and Merck; and is the co-inventor of a biomarker technology that has been licensed to Qiagen. S.A.T. has served as a consultant and received honoraria from Roche/ Ventana Medical Systems, Almac Diagnostics, Janssen, AbbVie and Astellas/Medivation; he is also a co-founder of, consultant for and Laboratory Director of Strata Oncology. Y.W., A.J., R.P.G. and R.D. are employees of Epic Sciences. V.E.V. is a founder of Personal Genome Diagnostics (PGDx), is a member of its Scientific Advisory Board and Board of Directors, and owns PGDx stock, which is subject to certain restrictions under university policy; he is also on the Scientific Advisory Board for Ignyta; the terms of these arrangements are managed by the Johns Hopkins University in accordance with its conflict of interest policies. The remaining authors disclose no relevant conflicts of interest.

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original report

Clinical Utility of CLIA-Grade AR-V7 Testing in Patients With Metastatic Castration-Resistant Prostate Cancer

abstract

Purpose A splice variant of the androgen receptor, AR-V7, confers resistance to AR-targeted therapies (ATTs) but not taxane chemotherapies in patients with metastatic castration-resistant prostate cancer. Since August 2015, a clinical-grade assay to detect AR-V7 messenger RNA expression in circulating tumors cells (CTCs) has been available to providers through a Clinical Laboratory Improvement Amendments–certified laboratory at Johns Hopkins University.

Methods We contacted ordering providers of the first 150 consecutive tests by using a questionnaire-based survey to determine how the results of AR-V7 testing were used to influence clinical practice.

Results In all, 142 (95%) of 150 questionnaires were completed by 38 providers from 29 sites across the United States and Canada. AR-V7 test results were reported either as CTC– (28%), CTC+/AR-V7– (30%), or CTC+/AR-V7+ (42%). Prevalence of AR-V7 detection increased with prior exposure to ATTs (abiraterone and enzalutamide naïve, 22%; after abiraterone or enzalutamide, 35%; after abiraterone and enzalutamide, 43%). Overall, management was affected by AR-V7 testing in 53% of the patients and even more often with CTC+/AR-V7+ results. AR-V7+ patients were commonly switched from ATT to taxane chemotherapy (43%) or were offered a clinical trial (43%); management remained unchanged in only 14% of these patients. Overall, patients who had a change in management on the basis of AR-V7 testing were significantly more likely to achieve a physician-reported 50% decline in prostate-specific antigen response on next-line therapy than those who did not change treatment (54% *v* 31%; $P = .015$).

Conclusion Providers used AR-V7 testing to influence clinical decision making more often than not. Physicians reported that men with AR-V7+ results had the most treatment changes, and such men were preferentially managed with taxane therapy or offered a clinical trial, which may have improved outcomes.

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INTRODUCTION

Metastatic castration-resistant prostate cancer (mCRPC) remains dependent on androgen receptor (AR) signaling for survival in the presence of low testosterone levels.[1] Two novel AR-targeted therapies (ATTs), abiraterone and enzalutamide, induced high objective response rates and improved overall survival in patients with mCRPC.[2,3] Treatment with abiraterone or enzalutamide in the prechemotherapy setting led to a 50% decline in prostate-specific antigen (PSA_{50}) response in 62% and 78% of these patients, respectively.[2,3] However, sequential use of abiraterone and enzalutamide has resulted in much lower PSA response rates with the use of the second ATT agent (ie, abiraterone after enzalutamide, 10% to 15%; enzalutamide after abiraterone, 20% to 30%).[4-6] These data underscore the importance of identifying a predictive biomarker of resistance to ATT to prevent the use of subsequent futile therapy.

Potential mechanisms of resistance to ATT include AR amplification and mutation, as well as the expression of AR splice variants.[7] A well-characterized

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splice variant, AR-V7, is a truncated form of full-length AR (AR-FL) that lacks the ligand-binding domain but retains both the transactivation and DNA-binding domains, allowing for constitutive AR signaling in the absence of androgen.[8-10] In patients with mCRPC, detection of AR-V7 in circulating tumors cells (CTCs) was shown to predict resistance to novel ATTs.[11-13] Moreover, AR-V7+ patients had a significantly shorter overall survival, suggesting a prognostic value of AR-V7 in addition to its use as a predictive biomarker.[11,13]

Chemotherapy is an alternative to ATT for AR-V7+ patients with mCRPC. Detection of AR-V7 has been shown not to preclude response to taxane-based chemotherapy.[14,15] Further prospective investigation found a significant survival benefit with the use of taxanes versus ATTs in patients with AR-V7+ disease.[16] Interestingly, the presence of this splice variant is a dynamic feature with possible conversion from AR-V7+ to AR-V7– status after chemotherapy with taxanes.[14,17] These data suggest that serial AR-V7 testing may guide the clinical treatment of patients with mCRPC. Those patients with AR-V7– prostate cancer may continue to benefit from ATT, whereas chemotherapy may be more effective in patients with detectable AR-V7 transcript.[18]

If the clinical utility of AR-V7 testing can be confirmed, it may also have an economic benefit. In a recent study, we modeled the cost of treating all patients with mCRPC with abiraterone or enzalutamide versus using AR-V7 testing to direct treatment.[19] By using a clinical scenario in which AR-V7+ patients were changed from treatment with abiraterone and/or enzalutamide to chemotherapy thus avoiding the cost of futile ATT therapy, AR-V7 testing resulted in a theoretical cost savings to the health care system of $150 million per year. To this end, since August 2015, clinical-grade AR-V7 testing performed in a Clinical Laboratory Improvement Amendments (CLIA)–certified laboratory at Johns Hopkins University has been available to health care providers for clinical use.[20] However, despite the commercial availability of this AR-V7 test, its clinical utility is unknown. Here, we have retrospectively compiled questionnaire-based data on how ordering providers are applying the results of AR-V7 testing in their clinical practice to influence decision making.

METHODS

The analytical validation and test characteristics of our CLIA-grade AR-V7 assay have been described previously.[20] Our molecular pathology database was queried for all AR-V7 tests ordered by internal and external providers for clinical purposes. We identified patients by name, date of birth, date of testing, AR-V7 status, and the ordering provider of each test. Clinical results of this test were reported as CTC–, CTC+/AR-V7–, or CTC+/AR-V7+, because each of these categories is associated with different outcomes.[12] A clinical utility questionnaire (Data Supplement) was generated for each AR-V7 test ordered and was mailed or e-mailed to each ordering provider. We defined a biomarker-based change in treatment as a confirmation of treatment choice or a change from one therapy to another after AR-V7 testing. The institutional review board at Johns Hopkins University approved this study and granted a waiver of consent to contact the provider of each AR-V7 test ordered because that was considered a clinical audit. The ordering provider was then contacted for participation and asked to complete a questionnaire pertaining to treatment decisions that were made on the basis of results of that specific AR-V7 test. Participation in this study was voluntary. If a provider did not wish to participate or the questionnaire was not returned after two attempts to contact the provider, data for that patient were not included in the analysis.

One hundred fifty consecutive AR-V7 clinical test results were obtained between August 31, 2015, and August 31, 2016, representing the first 150 tests ordered. From these, 142 questionnaires (95%) were completed and returned by 38 providers across 29 sites (28 in the United States [in 22 states] and one in Canada). Statistical analyses for this project were largely descriptive. In specified cases, a two-tailed Fisher's exact test was used to compare proportions between two or more groups. The significance level was set at $P < .05$, and corrections were not performed for multiple comparisons.

RESULTS

Information from 142 of 150 questionnaires sent (95% participant response rate) were included in this analysis. All 142 AR-V7 tests were ordered for patients with mCRPC. The number of lines of additional systemic therapies for mCRPC among these patients was reported as follows: 24% (n = 33) had received no other lines, 27% (n = 39) had received one line, 27% (n = 39) had received two lines, 13% (n = 18) had received three lines, and 9% (n = 13) had received four or more lines of systemic therapy before testing. Eighteen percent of men (n = 26) had previously received bicalutamide, 46% (n = 66) had received abiraterone, 49% (n = 70) had received enzalutamide, 48% (n = 69) had received docetaxel, and 13% (n = 18) had received cabazitaxel.

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Table 1. Summary of AR-V7 Test Results According to the Number of Novel ATTs Previously Received

	Total No. of Tests		Abiraterone and Enzalutamide		Abiraterone or Enzalutamide		Abiraterone and Enzalutamide Naïve	
Test Result	n/N	%	n/N	%	n/N	%	n/N	%
CTC–	40/142	28.17	8/40	20.0	11/40	27.5	21/40	52.5
CTC+/AR-V7–	42/142	29.58	8/42	19.0	20/42	47.62	14/42	33.33
CTC+/AR-V7+	60/142	42.25	26/60	43.33	21/60	35.0	13/60	21.66

Abbreviations: AR-V7, androgen receptor splice variant 7; ATT, AR-targeted therapy; CTC, circulating tumor cell; n/N, number of patients in that category divided by total number of patients.

Overall, the prevalence of a CTC– result was 28%, the prevalence of a CTC+/AR-V7– result was 30%, and the prevalence of a CTC+/AR-V7+ result was 42%. We then subdivided test results according to physician-reported prior treatment with a novel ATT (Table 1). The majority of patients without detectable CTCs were naïve to both abiraterone and enzalutamide (53%). Patients who were treated with abiraterone and/or enzalutamide resulted in a higher prevalence of AR-V7 detection compared with patients who were not treated with an ATT: 22% of treatment-naïve patients were AR-V7+; after treatment with abiraterone or enzalutamide, 35% of patients were AR-V7+; and after treatment with abiraterone and enzalutamide, 43% of patients were AR-V7+.

To assess the clinical utility of AR-V7 testing, providers were asked whether the AR-V7 status influenced their decision making. The majority of AR-V7– tests (CTC– or CTC+/AR-V7–) did not change the clinical practice of the providers (Table 2). However, almost two thirds (62%) of AR-V7+ tests resulted in a change in management. In patients for whom treatment was changed, providers were then asked to specify the type of therapy selected on the basis of the test result. We also stratified those responses by AR-V7 test result (Table 3). Patients with an AR-V7– result (CTC– or CTC+/AR-V7–) were preferentially treated with an ATT agent (confirmed AR treatment, or changed from taxane to AR therapy). A smaller subset of AR-V7– patients were treated on a clinical trial or changed to chemotherapy. Conversely, after an AR-V7+ result, most patients were changed from an ATT agent to taxane chemotherapy (43%) or were enrolled in a clinical trial (43%). A list of these AR-V7–directed clinical trials is provided in Appendix Table A1. Providers were next asked to self-report whether each patient achieved a PSA_{50} response on next-line systemic therapy (ie, the subsequent therapy selected after the AR-V7 test result (Table 4). The physician-reported PSA_{50} response rate (PSA_{50} RR) was significantly higher among patients in whom management was changed on the basis of AR-V7 testing compared with those in whom treatment was not altered (54% *v* 31%; $P = .015$). PSA_{50} RR data were missing from 16% (n = 12) and 22% (n = 15) of questionnaires in which management was changed or not changed, respectively.

Table 2. Clinical Utility of AR-V7 Testing in Patients With mCRPC

	Did the AR-V7 Assay Result in a Change in Management for This Patient?			
	Yes		No	
Test Result	n/N	%	n/N	%
CTC–	18/40	45.0	22/40	55.0
CTC+/AR-V7–	20/42	47.62	22/42	52.38
CTC+/AR-V7+	37/60	61.67	23/60	38.33

Abbreviations: AR-V7, androgen receptor splice variant 7; CTC, circulating tumor; mCRPC, metastatic castration-resistant prostate cancer; n/N, number of patients in that category divided by total number of patients.

We also investigated which systemic therapy was used in patients with mCRPC after progression on both abiraterone and enzalutamide, according to AR-V7 status (Table 5). For patients with an AR-V7– result, most (44%) were offered standard taxane-based chemotherapy, and 19% enrolled on a clinical trial. By contrast, AR-V7+ patients who had already received abiraterone and enzalutamide were more often treated on a clinical trial (54%) compared with treatment using chemotherapy (19%). For patients who had received abiraterone and enzalutamide, we investigated the prevalence of AR-V7 positivity after physician-reported treatment with docetaxel (Appendix Table A2). No significant difference in prior docetaxel treatment was observed between AR-V7– (56% [nine of 16]) and AR-V7+ (73% [19 of 26]; $P = .32$) patients. In the chemotherapy-naïve group, no numerical difference was noted in the reported clinical trial enrollment based on AR-V7 status. In the patients who were treated with docetaxel, those who were AR-V7– were commonly treated with standard chemotherapy (ie, cabazitaxel; 67% [six of nine]) whereas AR-V7+ patients were more frequently placed on a clinical trial (58% [11 of 19]).

Finally, we examined provider treatment preferences for AR-V7+ patients irrespective of the prior therapies received (Table 6). These patients were most commonly treated on either a clinical trial (35%) or with taxane chemotherapy (32%), similar to those patients who had previously exhausted

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Table 3. Change in Management on the Basis of AR-V7 Testing in Patients with mCRPC

	How Did the AR-V7 Assay Change Your Management?											
	AR → Taxane		Taxane → AR		Confirmed AR		Confirmed Taxane		Clinical Trial		No Response	
Test Result	n/N	%	n/N	%	n/N	%	n/N	%	n/N	%	n/N	%
CTC–	0		7/18	38.89	6/18	33.33	1/18	5.56	4/18	22.22	0	
CTC+/AR-V7–	0		6/20	30.0	10/20	50.0	2/20	10.0	1/20	5.0	1/20	5.0
CTC+/AR-V7+	16/37	43.24	0		1/37	2.70	3/37	8.11	16/37	43.24	1/37	2.70

Abbreviations: AR, androgen receptor; AR-V7, androgen receptor splice variant 7; CTC, circulating tumor; mCRPC, metastatic castration-resistant prostate cancer; n/N, number of patients in that category divided by total number of patients.

all ATT options. A minority of patients (7%) received either enzalutamide or abiraterone despite an AR-V7+ result, whereas all AR-V7+ patients managed with observation (10%) enrolled in hospice shortly thereafter.

To summarize the data compiled from providers' real-world experience with AR-V7 testing, we propose a hypothetical treatment algorithm for making decisions regarding patients with mCRPC using AR-V7 as a potential treatment-selection biomarker (Fig 1). After first-line systemic therapy with abiraterone or enzalutamide, AR-V7+ patients would preferentially cross over to taxane-based therapy, whereas those who are AR-V7– may continue on a second ATT. Because of occasional conversions from AR-V7+ to AR-V7– status, men progressing on taxane treatment can be retested and could potentially consider treatment with an ATT if the AR-V7 status reverts to negative. Finally, even patients progressing after treatment with abiraterone and enzalutamide may be considered for AR-V7 testing if an AR-V7–directed clinical trial is available.

DISCUSSION

The optimal sequencing of therapeutic agents in patients with mCRPC is unknown and remains a major challenge. The recent discovery of CTC-based AR-V7 detection as a potential predictive biomarker of ATT resistance (but not taxane resistance) may aid in such treatment decisions. To this end, the National Comprehensive Cancer Network prostate cancer guidelines now suggest that AR-V7 testing can be considered and may play a role in guiding therapy selection in mCRPC, but at this time, these guidelines have not gone as far as recommending testing to determine treatment choice. In another recent consensus report, the majority of prostate cancer specialists polled (59%) stated that AR-V7 testing would be useful for some (majority or minority of) patients with mCRPC.[21] Although further prospective validation of the predictive ability of AR-V7 is currently ongoing, here we investigated the clinical utility of AR-V7 testing in a real-world setting.

We asked providers whether the result of the AR-V7 test influenced their clinical practice for that specific patient. Overall, more than 50% of providers stated that the AR-V7 test changed their treatment decision. Providers were also asked to self-report whether patients achieved a PSA_{50} response on their next-line therapy. Importantly, we observed a significantly higher PSA_{50} RR in patients whose providers used the AR-V7 result to change their therapy. Although our findings are retrospective, they suggest that AR-V7 testing may possibly lead to improved clinical responses to treatment, at least in terms of PSA_{50} RR. We did not assess for radiographic progression-free or overall survival, which may not have correlated with PSA_{50} RR.

The statistically significant PSA_{50} RR difference between the change and no-change groups is largely driven by the AR-V7+ subgroup (described in Appendix Table A3). AR-V7+ patients for whom management did not change had a PSA_{50} RR of 5% (*v* 39% in AR-V7+ patients for whom treatment was changed). A clear limitation to our study is that we did not explicitly ask providers to list the specific next-line therapy for those patients

Table 4. PSA_{50} Response Rate to Next-Line Therapy Based on Change in Clinical Practice After AR-V7 Testing

	Did the Patient Achieve a PSA_{50} Response on Next-Line Therapy?			
	Yes		No	
Management	n/N	%	n/N	%
Changed	34/63	53.97	29/63	46.03
Did not change	16/52	30.77	36/52	69.23

NOTE. Fisher's exact test $P = .015$.
Abbreviations: AR-V7, androgen receptor splice variant 7; n/N, number of patients in that category divided by total number of patients; PSA_{50}, 50% decline in prostate-specific antigen.

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Table 5. Next-Line Systemic Therapy in Patients After Treatment With Abiraterone and Enzalutamide on the Basis of AR-V7 Status

	Next-Line Therapy After Abiraterone and Enzalutamide							
	Taxane		Clinical Trial		Observation		Unknown	
Status	n/N	%	n/N	%	n/N	%	n/N	%
AR-V7–	7/16	43.75	3/16	18.75	1/16	6.25	5/16	31.25
AR-V7+	5/26	19.23	14/26	53.84	3/26	11.54	4/26	15.38

Abbreviation: AR-V7, androgen receptor splice variant 7; n/N, number of patients in that category divided by total number of patients.

who did not have a treatment change based on the AR-V7 test. Therefore we cannot directly compare between these groups. We hypothesize that AR-V7+ patients in the change group were more commonly treated with chemotherapy compared with the no-change group, resulting in improved outcomes. The no-change group may also have been less clinically fit for chemotherapy or did not meet eligibility criteria for enrolling on a clinical trial. We also acknowledge a high PSA_{50} RR in AR-V7+ patients treated with chemotherapy and AR-V7– patients treated with ATT (both in the change group). Another potential weakness is the subjective definition of changing clinical practice and including patients whose treatment choice was confirmed on the basis of AR-V7 testing. This may have inflated the perceived clinical utility of the biomarker. Nonetheless, these data suggest that further prospective investigation is warranted to study biomarker-driven clinical outcomes.

By using the treatment history captured by our questionnaires, we were able to observe an increasing prevalence of AR-V7 detection with prior exposure to ATTs, as expected. This finding is consistent with previously published data[12,13,16] and suggests that the clinical data obtained in this study are representative. In addition, we observed interesting nonsignificant trends in our physician-reported data regarding prior treatment. For instance, in ATT-naïve patients, prior treatment with docetaxel increased the incidence of AR-V7 to 40% (*v* 21% in the chemotherapy-naïve group). This finding is corroborated by the recent biomarker data from the ARMOR3-SV (A Study of Galeterone Compared with Enzalutamide in Men Expressing Androgen Receptor Splice Variant-7 mRNA [AR-V7] Metastatic CRPC) trial, in which first-line patients with mCRPC who had previously received docetaxel for metastatic hormone-sensitive disease had a higher prevalence of AR-V7 detection compared with chemotherapy-naïve patients.[22] Moreover, 79% of men with newly developed mCRPC had prior exposure to bicalutamide. Interestingly, the incidence of AR-V7 was 27% in patients who had received bicalutamide compared with 0% in patients with no prior bicalutamide treatment. The higher trend of AR-V7+ tests after treatment with docetaxel or bicalutamide, in the absence of a novel ATT, may suggest that total number of therapies (ie, more advanced disease) contributes to AR-V7 expression in addition to the known relationship with prior ATT exposure. Another provocative hypothesis might be that docetaxel works as an AR-modulating therapy in prostate cancer, inhibiting microtubule-dependent nuclear transport of wild-type AR but not AR-V7, thereby selecting for the emergence of AR-V7–expressing clones during or after chemotherapy treatment.

This study had some additional limitations. First, the prevalence of AR-V7 was probably overestimated compared with earlier reports because providers were more likely to order a test if the clinical scenario suggested that the test might be positive. Other significant weaknesses of this analysis were its retrospective nature and reliance on self-reporting by providers. To this end, we did not review the medical records of all patients to confirm their prior treatment history or their PSA_{50} response. An audit of 14 randomly selected questionnaires determined that providers gave accurate responses to the questionnaire in most instances (see Appendix), suggesting that our data represent the true clinical course for each patient. Finally, we concede that many patients will receive all ATTs approved by the US Food and Drug Administration, regardless of AR-V7 status, because clinical responses to ATTs are sometimes observed in AR-V7+ patients.[13] However, the clinical utility of AR-V7 testing may potentially be clearest in AR-V7+ patients who have additional ATTs still available for treatment. By identifying high-risk patients (ie, AR-V7+) who have adequate performance status earlier in their mCRPC treatment, this would allow them to be treated with taxane therapy before the chemotherapy window closes. AR-V7– tests are probably less clinically useful because patients could

Table 6. Next-Line Systemic Therapy Selected by Treating Physicians for Patients With AR-V7+ mCRPC

	Next-Line Therapy for AR-V7+ Patients									
AR Targeted		Taxane		Clinical Trial		Observation		Unknown	
n/N	%	n/N	%	n/N	%	n/N	%	n/N	%
4/60	6.67	19/60	31.67	21/60	35.0	6/60	10.0	10/60	16.67

Abbreviations: AR, androgen receptor; AR-V7, androgen receptor splice variant 7; mCRPC, metastatic castration-resistant prostate cancer; n/N, number of patients in that category divided by total number of patients.

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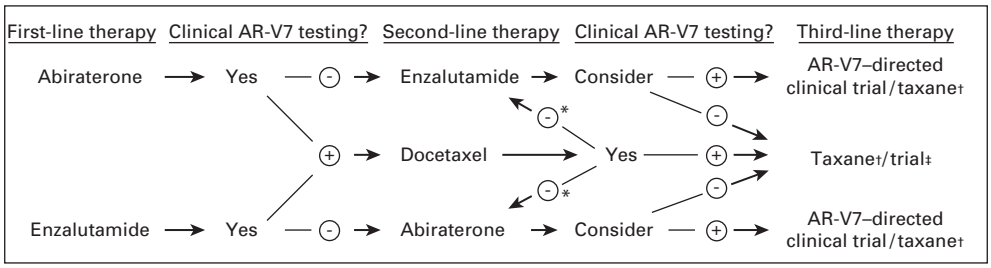


Fig 1. Potential decision algorithm based on serial androgen receptor splice variant 7 (AR-V7) testing across the castration-resistant prostate cancer landscape. After each line of therapy, we propose an algorithm for consideration of AR-V7 testing. Patients with a positive AR-V7 test could be changed from an AR-targeted therapy (ATT) agent to taxane-based chemotherapy. After taxane treatment, repeat AR-V7 testing may be clinically helpful, and patients with a negative AR-V7 test can be considered for additional ATT. After abiraterone and enzalutamide treatment, patients should be subject to AR-V7 testing only if an AR-V7–directed clinical trial is available. (*) Indicates limited clinical data supporting the use of ATTs in AR-V7+ patients that subsequently convert to AR-V7–. (†) Denotes either docetaxel (first-line) or cabazitaxel (second-line), depending on prior treatment. (‡) Denotes a clinical trial that does not require a positive AR-V7 test for AR-V7– patients, whereas AR-V7+ patients should consider an AR-V7–directed trial, if available.

receive either chemotherapy or ATTs at their physician's discretion.

We propose a decision algorithm using serial AR-V7 testing across the mCRPC landscape. This algorithm is based largely on published data suggesting that the presence of AR-V7 confers resistance to ATTs but does not influence response to taxane chemotherapy.[11,13,14,16] We note that at this time, there is no clinical trial evidence to support the use of ATTs in AR-V7+ patients who convert to AR-V7– status after chemotherapy. In the setting of patients who have received abiraterone and enzalutamide, AR-V7 testing may be considered if AR-V7-selected clinical trials are available. In our study, 17 of 22 AR-V7+ patients were enrolled on a clinical trial that mandated AR-V7 detection as an entry criterion, suggesting that many of the tests in this study were ordered for the purpose of screening for a clinical trial. In these instances, the clinical utility of AR-V7 testing is primarily to allow enrollment on an AR-V7–directed trial. Finally, there is ongoing debate in the prostate cancer community about whether AR-V7 detection is merely a proxy for AR amplification or overexpression and not an independent predictor of response to therapy or clinical outcomes.[21] Although several studies have shown that AR-V7 detection is indeed correlated with AR-FL expression, AR-V7 still remains independently prognostic in multivariable analysis after controlling for AR-FL levels.[11,13,23]

In conclusion, to our knowledge, this is the first study to examine the preliminary real-world clinical utility of CLIA-grade AR-V7 testing in patients with mCRPC. We show that AR-V7 testing influenced clinical decision making overall (regardless of test results) but that its utility was greatest in the setting of AR-V7+ results. Additional prospective studies are needed and are ongoing (eg, NCT02269982; Prospective Circulating Prostate Cancer Predictors in Higher Risk mCRPC Study [PROPHECY]).

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APPENDIX

Analysis of PSA_{50} Response Rate Stratified by AR-V7 Status and Change/No Change Designation

We further queried our database to investigate the difference in the 50% decline in prostate-specific antigen (PSA_{50}) response rate (RR) between the change and no-change groups on the basis of androgen receptor splice variant 7 (AR-V7) status. For patients with AR-V7+ disease, the PSA_{50} RR in those who did not change therapy was 5.3% (one of 19) compared with 38.7% (12 of 31) in the change group (Table A3). In the AR-V7− patients, the PSA_{50} RR was 45.45% (15 of 33) in the no-change group versus 68.88% (22 of 32) in the change group. We asked whether there was a difference in the number of lines of therapy (ie, more advanced disease) between the change and no-change groups. Each group was subdivided by AR-V7 status (Table A3). Within both the AR-V7+ and AR-V7− groups, there was no discernible difference in number of lines of therapy between the change and no-change group. We also examined PSA_{50} RR by treatment choice in those patients who had a change in treatment (these data were not solicited for patients who did not have a treatment change in the questionnaire). In AR-V7+ patients, the PSA_{50} RR to chemotherapy (either changed or confirmed) was 73.33% (11 of 15). The PSA_{50} RR in clinical trials for AR-V7+ patients was one (6.7%) of 15 in the change group. In AR-V7− patients who had treatment changed (or confirmed), the PSA_{50} RR was 70.8% (17 of 24) on AR-targeted therapy (chemotherapy, 100% [three of three]; clinical trial, 0% [zero of three]; observation, 100% [one of one]; unknown, 100% [one of one].

Audit of Randomly Selected Questionnaires Completed by Johns Hopkins University Providers

We (M.C.M.) performed a medical record audit of 14 randomly selected questionnaires completed by Johns Hopkins University providers to assess for accuracy. Clinical data from other sites were not accessible for audit. We investigated only objective questionnaire responses: clinical stage, prior therapies, most recent treatment, PSA_{50} response on next-line therapy, and clinical trial eligibility. In the 14 questionnaires audited, we found two minor discrepancies: (1) bicalutamide was listed in error as a prior therapy (one time), and (2) the correct clinical trial was misidentified for a patient (one time). Subjective responses (ie, clinical helpfulness and change in management) were not assessed. Although this audit reflects a small sample size (approximately 10% of the entire population included in this study), it suggests that providers documented accurate responses to the questionnaire in almost all instances.

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Table A1. AR-V7–Directed Clinical Trials for Patients With mCRPC

Identifier	Title	Selection
NCT02438007	A Study of Galeterone Compared with Enzalutamide in Men Expressing Androgen Receptor Splice Variant-7 mRNA (AR-V7) Metastatic CRPC (ARMOR3-SV)	AR-V7 specific
NCT02532114	Niclosamide and Enzalutamide in Treating Patients With Castration-Resistant, Metastatic Prostate Cancer	
NCT02601014	Biomarker-Driven Therapy With Nivolumab and Ipilimumab in Treating Patients With Metastatic Hormone-Resistant Prostate Cancer Expressing AR-V7 (STARVE-PC)	AR-V7 specific
NCT03050866	Cabazitaxel in mCRPC Patients With AR-V7 Positive Circulating Tumor Cells (CTCs) (CABA-V7)	AR-V7 specific

Abbreviations: AR-V7, androgen receptor splice variant 7; mCRPC, metastatic castration-resistant prostate cancer.

Table A2. Next-Line Systemic Therapy in Patients After Abiraterone and Enzalutamide on the Basis of AR-V7 Status and Prior Treatment With Docetaxel

	Next-Line Therapy in Patients After Abiraterone and Enzalutamide							
	Taxane		Clinical Trial		Observation		Unknown	
Docetaxel Status	n/N	%	n/N	%	n/N	%	n/N	%
Docetaxel naïve								
AR-V7–	1/7	14.2	3/7	42.86	0		3/7	42.86
AR-V7+	2/7	28.57	3/7	42.86	2/7	28.57	0	
After docetaxel								
AR-V7–	6/9	66.67	0		1/9	11.11	2/9	22.22
AR-V7+	3/19	15.79	11/19	57.89	1/19	5.26	4/19	21.05

Abbreviation: AR-V7, androgen receptor splice variant 7; n/N, number of patients in that category divided by total number of patients.

Table A3. PSA_{50} RR and Lines of Therapy Subgrouped by AR-V7 Status in Patients With mCRPC Who Had a Change or No Change in Management

			Lines of Therapy									
	PSA_{50} RR		0		1		2		3		4+	
Management	n/N	%	n/N	%	n/N	%	n/N	%	n/N	%	n/N	%
Change												
AR-V7–	22/32	68.75	11/32	34.4	9/32	28.1	8/32	25	2/32	6.25	2/32	6.25
AR-V7+	12/31	38.7	4/31	12.9	8/31	25.8	8/31	25.8	8/31	25.8	3/31	9.7
No change												
AR-V7–	15/33	45.5	10/33	30.3	14/33	42.4	5/33	15.2	2/33	6	2/33	6
AR-V7+	1/19	5.3	2/19	10.5	4/19	21.0	5/19	26.3	5/19	26.3	3/19	15.8

Abbreviations: AR-V7, androgen receptor splice variant 7; mCRPC, metastatic castration-resistant prostate cancer; n/N, number of patients in that category divided by total number of patients; PSA_{50}, 50% decline in prostate-specific antigen; RR, response rate.

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ORIGINAL REPORT

Clinical Significance of Androgen Receptor Splice Variant-7 mRNA Detection in Circulating Tumor Cells of Men With Metastatic Castration-Resistant Prostate Cancer Treated With First- and Second-Line Abiraterone and Enzalutamide

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ABSTRACT

Purpose
We reported previously that the detection of androgen receptor splice variant-7 (AR-V7) mRNA in circulating tumor cells (CTCs) correlated with poor outcomes from the use of abiraterone and enzalutamide in patients with castration-resistant prostate cancer (CRPC). Here, we expanded our cohort size to better characterize the prognostic significance of AR-V7 in this setting.

Methods
We prospectively enrolled 202 patients with CRPC starting abiraterone or enzalutamide and investigated the prognostic value of CTC detection (+ *v* –) and AR-V7 detection (+ *v* –) using a CTC-based AR-V7 mRNA assay. We examined ≥ 50% prostate-specific antigen (PSA) responses, PSA progression-free survival, clinical and radiologic progression-free survival, and overall survival. We constructed multivariable models adjusting for PSA, Gleason sum, number of prior hormone therapies, prior abiraterone or enzalutamide use, prior taxane use, presence of visceral metastases, and Eastern Cooperative Oncology Group score. We also separately examined the first-line and second-line novel hormonal therapy (NHT) settings.

Results
Median follow-up times were 15.0, 21.7, and 14.6 months for CTC–, CTC+/AR-V7– and CTC+/AR-V7+ patients, respectively. CTC+/AR-V7+ patients were more likely to have Gleason scores ≥ 8 ($P = .05$), metastatic disease at diagnosis ($P = .01$), higher PSA ($P < .01$), prior abiraterone or enzalutamide use ($P = .03$), prior taxane use ($P = .02$), and Eastern Cooperative Oncology Group ≥ 1 ($P = .01$). Outcomes for the overall cohort (and separately for the first-line and second-line NHT cohorts) were best for CTC– patients, intermediate for CTC+/AR-V7– patients, and worse for CTC+/AR-V7+ patients. These correlations remained significant in multivariable models.

Conclusion
This expanded analysis further characterizes the importance of CTC-based AR-V7 mRNA detection in predicting outcomes in patients with CRPC receiving first- and second-line NHT and, to the best of our knowledge, is the first to suggest that this assay be interpreted using three separate prognostic categories: CTC–, CTC+/AR-V7–, and CTC+/AR-V7+.

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INTRODUCTION

Treatment-specific biomarkers (eg, markers that help select or exclude a particular therapy) for metastatic castration-resistant prostate cancer (CRPC) are currently lacking. It has emerged recently that detection of androgen receptor splice variant-7 (AR-V7) in circulating tumor cells (CTCs) may represent one such treatment-selection marker in men with metastatic CRPC.[1] AR-V7 is an abnormally spliced mRNA isoform of the androgen receptor, producing a protein product lacking the C-terminal ligand-binding domain but retaining the transcriptionally active *N*-terminal domain. Despite its inability to bind ligand (eg, dihydrotestosterone), AR-V7 remains constitutively active in a ligand-independent manner and is capable of driving CRPC growth.[1]

ASSOCIATED CONTENT

See accompanying Editorial on page 2103

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Accumulating evidence suggests that CTC-based detection of AR-V7 may be associated with a lack of benefit of novel hormonal therapies (NHT) including abiraterone[2] and enzalutamide.[3] An initial pilot study conducted by our group (n = 62) suggested that AR-V7 mRNA detection was associated with resistance to abiraterone and enzalutamide.[4] This was confirmed subsequently by additional studies showing a similar lack of benefit of NHT in patients with detectable AR-V7 mRNA or protein.[5,6] In addition, it has been suggested that CTC-based AR-V7 detection is compatible with sensitivity to taxane chemotherapies such as docetaxel and cabazitaxel.[7,8] Furthermore, the relative benefit of taxane chemotherapy over NHT may be greater in AR-V7+ patients than in AR-V7– patients where chemotherapy and NHT seem to have comparable efficacy.[6,7]

These prior studies suffered from two significant limitations. First, because of small sample sizes, these studies were generally not able to explore the prognostic value of AR-V7 separately in the first-line and second-line NHT settings (and typically included a mix of patients, some of whom had received prior NHT). Indeed, the usefulness of the AR-V7 biomarker could be different in the first-line and second-line settings. Second, none of the above-mentioned studies included data on patients without detectable CTCs. This is problematic because some patients with CRPC may not harbor CTCs and therefore cannot be evaluated for AR-V7. The purpose of our analysis was to expand on our study examining AR-V7 in men receiving abiraterone or enzalutamide (currently, n = 202). By doing so, we aimed to better understand the clinical significance of AR-V7 in both the first-line and second-line NHT settings and to explore the prognostic value of CTC– results compared with CTC+/AR-V7– and CTC+/AR-V7+ results.

METHODS

Patients

We prospectively enrolled men with metastatic CRPC who were beginning treatment with enzalutamide or abiraterone. Patients had to have histologically confirmed prostate adenocarcinoma, progressive disease despite castration levels of serum testosterone (< 50 ng/dL), and radiographic metastases on computed tomography (CT) or technetium-99 bone scans. Patients had to have three or more rising serum prostate-specific antigen (PSA) values taken ≥ 2 weeks apart, consistent with Prostate Cancer Working Group guidelines.[9] Patients were excluded if they planned to receive additional concurrent anticancer therapies. Prior taxane chemotherapy was permitted, as was previous treatment with the alternative NHT (ie, prior abiraterone in enzalutamide-treated patients, and vice versa). This study was approved by the Johns Hopkins University institutional review board, and patients provided written informed consent.

Study Design

This was a prospective study evaluating the ability of baseline CTC status (+ *v* –) and AR-V7 status (+ *v* –) to predict clinical benefit from NHT. Patients were asked to provide peripheral blood CTC samples at baseline (before beginning NHT) and at the time of progression. Enzalutamide was administered at 160 mg once daily, and abiraterone was administered at 1,000 mg once daily (with prednisone 5 mg twice a day). Follow-up was prospectively defined: patients had PSA measurements every 1 to 2 months, as well as CT (chest, abdomen, and pelvis) and technetium-99 bone scans every 2 to 4 months. Therapy with enzalutamide or abiraterone was continued until PSA progression, clinical or radiographic progression, or unmanageable drug-related toxicity.

CTC Assay and AR-V7 Detection

CTC analyses were conducted using a modified AdnaTest platform (QIAGEN, Hannover, Germany), as described previously.[4] Capture of CTCs was performed using the EpCAM-based ProstateCancerSelect kit, and mRNA expression analyses were performed using the ProstateCancerDetect kit with multiplexed reverse-transcription polymerase chain reaction primers to establish the presence or absence of CTCs. Custom primers were designed to detect full-length androgen receptor (AR-FL) mRNA and AR-V7 mRNA, as described previously.[4] The relative abundance of AR-V7 was determined by calculating the ratio of AR-V7 transcript to AR-FL transcript.

In our previous reports,[4,7] we presented data only on CTC+ patients, whereas CTC– patients were excluded from analysis (because their AR-V7 status could not be determined). Here, we aimed to consider our biomarker readout in three separate categories: CTC– (AR-V7 agnostic), CTC+/AR-V7–, and CTC+/AR-V7+. In this way, all enrolled patients, even those without detectable CTCs, would contribute data to our study.

Outcome Measures

The primary end point was clinical and radiographic progression-free survival (PFS); progression was defined as symptomatic progression (worsening disease-related symptoms or new cancer-related complications) or radiologic progression (on CT scan: ≥ 20% enlargement in sum diameter of target lesions [Response Evaluation Criteria in Solid Tumors[9]]; on bone scan: two or more new bone lesions not caused by flare), or death, whichever occurred first.[10] Secondary end points included PSA response rate, PSA progression-free survival (PSA-PFS), and overall survival (OS). PSA response was defined as the proportion of patients with a ≥ 50% PSA decline from baseline at any time point after therapy (and maintained for ≥ 3 weeks); best PSA response (maximal percentage decrease from baseline) was also determined. PSA progression was defined as a ≥ 25% increase in PSA from nadir (and by ≥ 2 ng/mL), requiring confirmation ≥ 3 weeks later (Prostate Cancer Working Group criteria).[10] OS was defined as the interval from enrolment to death from any cause.

Statistical Analyses

Clinical outcomes were analyzed separately in the three biomarker groups: CTC–, CTC+/AR-V7–, and CTC+/AR-V7+. PSA response rates were compared using Fisher's exact test. Time-to-event outcomes (PFS, PSA-PFS, and OS) were evaluated using Kaplan-Meier analysis, and survival-time differences were compared using the log-rank test. Univariable and multivariable logistic regressions (for PSA response) and Cox regressions (for time-to-event end points) were used to assess the independent effect of biomarker status on clinical outcomes. Covariates included in the multivariable models were baseline PSA, number of prior hormonal therapies, presence of visceral metastases, Gleason sum, Eastern Cooperative Oncology Group score, prior taxane chemotherapy, and prior abiraterone or enzalutamide. These variables were strongly associated with clinical outcomes in our prior AR-V7 studies.[4,7] Because of fewer events in the multivariable models for OS, only three covariates were included in these models (baseline PSA, prior chemotherapy, and prior abiraterone or enzalutamide).

Statistical analyses were performed for the cohort as a whole (n = 202; primary analysis) and also separately for the first-line NHT (n = 124) and second-line NHT (n = 78) cohorts. Sample size was determined on the basis of the primary comparison of PFS between CTC+/AR-V7– and CTC+/AR-V7+ patients in the overall cohort. Assuming a 30% prevalence of AR-V7 detection among CTC+ men, 148 CTC+ patients provided 90% power to detect a hazard ratio of 2.0 when median PFS in CTC+/AR-V7– men was 8.0 months, using a two-sided log-rank test at a significance level of .05. This calculation assumed 36 months of accrual time and a 2% dropout rate. Fifty-three of the enrolled patients were CTC– when the

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study reached the required sample size of 148 men with CTC+, and these patients were included in the analysis as a separate group. The first-line NHT cohort included patients beginning abiraterone or enzalutamide who had not previously received the alternative agent. The second-line NHT cohort included men beginning abiraterone or enzalutamide who had previously received the alternative drug. All statistical tests were two sided, and *P* values were not corrected for multiple comparisons. Statistical analyses were performed using *R* (version 2.15.1).

RESULTS

Patients

Between December 2012 and November 2015, 202 men (95 starting abiraterone, 107 starting enzalutamide) were prospectively enrolled. As of February 2016, median follow-up times for the at-risk population, calculated using the reverse Kaplan-Meier method, were 15.0, 21.7, and 14.6 months for CTC–, CTC+/AR-V7–, and CTC+/AR-V7+ patients, respectively. Baseline patient characteristics are summarized in Table 1. Overall, 53 of the 202 men (26.2%) were CTC–, 113 of the 202 men (56.0%) were CTC+/AR-V7–, and 36 of the 202 men (17.8%) were CTC+/AR-V7+. CTC+/AR-V7+ patients were more likely to have Gleason scores ≥ 8 ($P = .05$), metastatic disease at diagnosis ($P = .01$), higher PSA levels ($P < .01$), higher alkaline phosphatase levels ($P < .01$), prior abiraterone or enzalutamide use ($P = .03$), prior taxane use ($P = .02$), presence of pain ($P < .01$), and Eastern Cooperative Oncology Group status ≥ 1 ($P = .01$).

One hundred twenty-four patients had not received abiraterone or enzalutamide previously (first-line NHT cohort). Of these, 36 (29.0%) were CTC–, 73 (58.9%) were CTC+/AR-V7–, and 15 (12.1%) were CTC+/AR-V7+. Seventy-eight men had received abiraterone or enzalutamide previously (second-line NHT cohort). Of these, 17 (21.8%) were CTC–, 40 (51.3%) were CTC+/AR-V7–, and 21 (26.9%) were CTC+/AR-V7+. The prevalence of CTC– patients was lower and the prevalence of CTC+/AR-V7+ patients was higher in the second-line compared with the first-line NHT cohorts.

PSA Responses

Overall (n = 202), PSA response rates to enzalutamide or abiraterone were 75.5% (40 of 53) in CTC– patients, 52.2% (59 of 113) in CTC+/AR-V7– patients, and 13.9% (5 of 36) in CTC+/AR-V7+ patients ($P < .001$; Fig 1A). In multivariable logistic regression analysis (Data Supplement), biomarker status remained an independent predictor of PSA response. To understand the clinical characteristics that may permit PSA response to abiraterone or enzalutamide despite detection of AR-V7, we compared baseline characteristics among all CTC+/AR-V7+ patients stratified by whether they achieved a PSA response (Data Supplement). PSA responders had more favorable clinical characteristics (less prior abiraterone or enzalutamide use, less prior docetaxel use, less visceral metastases, less bone pain, lower PSAs, lower AR-FL levels, and lower AR-V7/AR-FL ratios) than did PSA nonresponders. We also discovered that two of these PSA responders received concurrent palliative radiotherapy to an osseous metastatic site, which may have influenced PSA trends.

Among first-line NHT patients (n = 124), PSA response rates in CTC– patients, CTC+/AR-V7– patients, and CTC+/AR-V7+ patients were 86.1% (31 of 36), 65.8% (48 of 73), and 26.7% (four of 15), respectively ($P < .001$; Fig 2A). In multivariable logistic regression analysis (Data Supplement), biomarker status remained an independent predictor of PSA response. Among second-line NHT patients (n = 78), PSA response rates in CTC– men, CTC+/AR-V7– men, and CTC+/AR-V7+ men were 52.9% (nine of 17), 27.5% (11 of 40), and 4.8% (one of 21), respectively ($P = .003$; Fig 3A). In multivariable logistic regression analysis (Data Supplement), biomarker status generally remained an independent predictor of PSA response.

Table 1. Baseline Characteristics of Patients With CRPC Starting Treatment With Abiraterone or Enzalutamide (N = 202)

Baseline Characteristic	CTC– (n = 53 [26.2%])	CTC+/AR-V7– (n = 113 [56.0%])	CTC+/AR-V7+ (n = 36 [17.8%])	*P**
Median age, years	70	71	70	.888
Nonwhite ethnicity, %	9	14	17	.575
Years since diagnosis, mean	7.9	7.3	5.6	.098
Gleason sum ≥ 8 at diagnosis, %	68	60	83	.052
Type of primary local therapy, %				.011
Surgery only	12	24	25	
Radiation only	31	30	19	
Both	35	20	8	
None	23	26	47	
M1 disease at diagnosis, %	16	22	47	
No. prior hormonal therapies, median	2	2	3	.158
Time to castration resistance, median no. months	23.0	20.5	14.0	.148
Prior use of abiraterone or enzalutamide, %	32	35	58	.025
Prior use of docetaxel, %	19	25	44	.022
Presence of bone metastases, %	76	83	100	.010
Presence of visceral metastases, %	23	27	34	.559
ECOG performance status ≥ 1, %	41	24	53	.006
Presence of pain, %	32	40	72	.002
Baseline PSA, ng/mL, median	13.7	31.4	92.0	< .001
Baseline alkaline phosphatase, U/L, median	80	96	120	< .001

Abbreviations: AR-V7, androgen receptor splice variant-7; CRPC, castration-resistant prostate cancer; CTC, circulating tumor cell; ECOG, Eastern Cooperative Oncology Group; PSA, prostate-specific antigen.
**P* values are based on Fisher's exact test and the Mann-Whitney *U* test for categorical and continuous variables, respectively.

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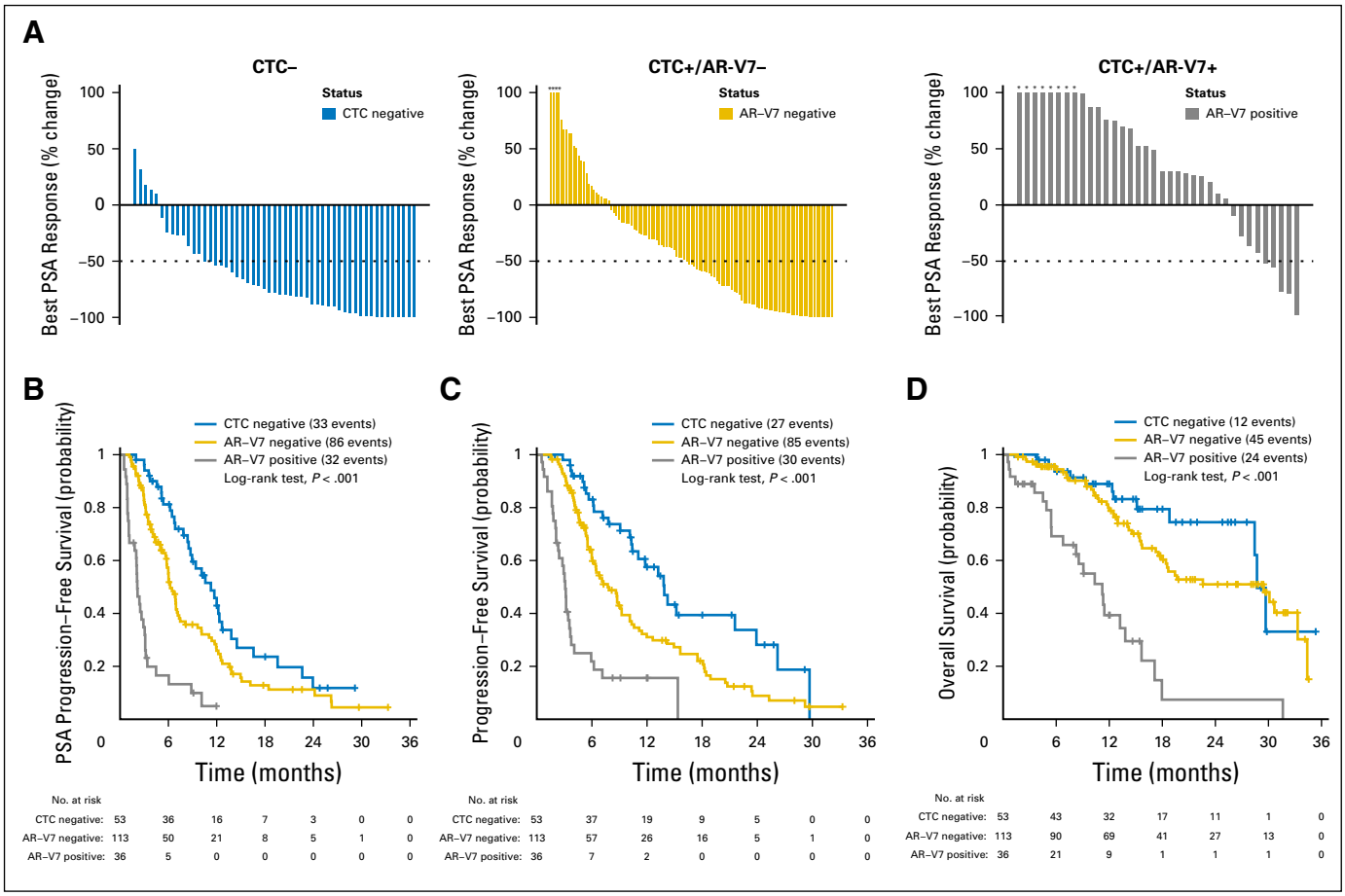


Fig 1. Clinical outcomes in the overall cohort of men starting treatment with abiraterone or enzalutamide (N = 202), according to CTC status and AR-V7 status. (A) Waterfall plots depicting best PSA responses according to CTC status and AR-V7 status, expressed in three categories: CTC–, CTC+/AR-V7–, and CTC+/AR-V7+. The dotted line illustrates the threshold for defining a PSA response (≥ 50% PSA reduction from baseline). PSA response rates in CTC– patients, CTC+/AR-V7– patients, and CTC+/AR-V7+ patients were 75.5% (40 of 53), 52.2% (59 of 113), and 13.9% (5 of 36), respectively ($P < .001$). All three groups were significantly different from each other (CTC– *v* CTC+/AR-V7–, $P = .005$; CTC– *v* CTC+/AR-V7+, $P < .001$; CTC+/AR-V7– *v* CTC+/AR-V7+, $P < .001$). (B) Kaplan-Meier curves indicating PSA progression-free survival according to CTC status and AR-V7 status. Median PSA progression-free survival in CTC– patients, CTC+/AR-V7– patients, and CTC+/AR-V7+ patients was 11.3 months (95% CI, 8.7 to 13.8), 6.2 months (95% CI, 5.8 to 7.3), and 2.1 months (95% CI, 1.9 to 3.1), respectively ($P < .001$). (C) Kaplan-Meier curves indicating clinical and radiographic progression-free survival according to CTC status and AR-V7 status. Median progression-free survival in CTC– patients, CTC+/AR-V7– patients, and CTC+/AR-V7+ patients was 13.9 months (95% CI, 11.0 to not reached), 7.7 months (95% CI, 6.2 to 10.1), and 3.1 months (95% CI, 2.3 to 3.7), respectively ($P < .001$). (D) Kaplan-Meier curves indicating overall survival according to CTC status and AR-V7 status. Median overall survival in CTC– patients, CTC+/AR-V7– patients, and CTC+/AR-V7+ patients was 28.7 months (95% CI, 28.4 to not reached), 29.5 months (95% CI, 18.4 to not reached), and 11.2 months (95% CI, 8.3 to 17.1), respectively ($P < .001$). AR-V7, androgen receptor splice variant-7; CTC, circulating tumor cell; PSA, prostate-specific antigen.

PSA-PFS

In the overall cohort, median PSA-PFS to enzalutamide or abiraterone was 11.3 months (95% CI, 8.7 to 13.8) in CTC– patients, 6.2 months (95% CI, 5.8 to 7.3) in CTC+/AR-V7– patients, and 2.1 months (95% CI, 1.9 to 3.1) in CTC+/AR-V7+ patients ($P < .001$; Fig 1B). In multivariable Cox regression analysis (Data Supplement), biomarker status remained independently prognostic for PSA-PFS.

In the first-line NHT cohort, median PSA-PFS in CTC– patients, CTC+/AR-V7– patients, and CTC+/AR-V7+ patients was 12.7 months (95% CI, 11.7 to 23.9), 7.3 months (95% CI, 6.2 to 12.0), and 2.9 months (95% CI, 2.0 to not reached), respectively ($P < .001$; Fig 2B). In multivariable Cox regression analysis (Data Supplement), biomarker status remained independently prognostic for PSA-PFS. In the second-line NHT cohort, median PSA-PFS in CTC– patients, CTC+/AR-V7– patients, and CTC+/AR-V7+ patients was 6.4 months (95% CI, 5.1 to not reached), 4.4 months (95% CI, 3.2 to 6.0), and 1.1 months (95% CI, 1.0 to 3.1), respectively ($P < .001$; Fig 3B). In multivariable Cox regression analysis (Data Supplement), biomarker status remained independently prognostic for PSA-PFS.

PFS

In the overall cohort, median PFS was 13.9 months (95% CI, 11.0 to not reached) in CTC– patients, 7.7 months (95% CI, 6.2 to 10.1) in CTC+/AR-V7– patients, and 3.1 months (95% CI, 2.3 to 3.7) in CTC+/AR-V7+ patients ($P < .001$; Fig 1C). In multivariable Cox regression analysis (Data Supplement), biomarker status remained independently prognostic for PFS.

In the first-line NHT cohort, median PFS in CTC– patients, CTC+/AR-V7– patients, and CTC+/AR-V7+ patients was 21.6 months (95% CI, 13.9 to not reached), 10.1 months

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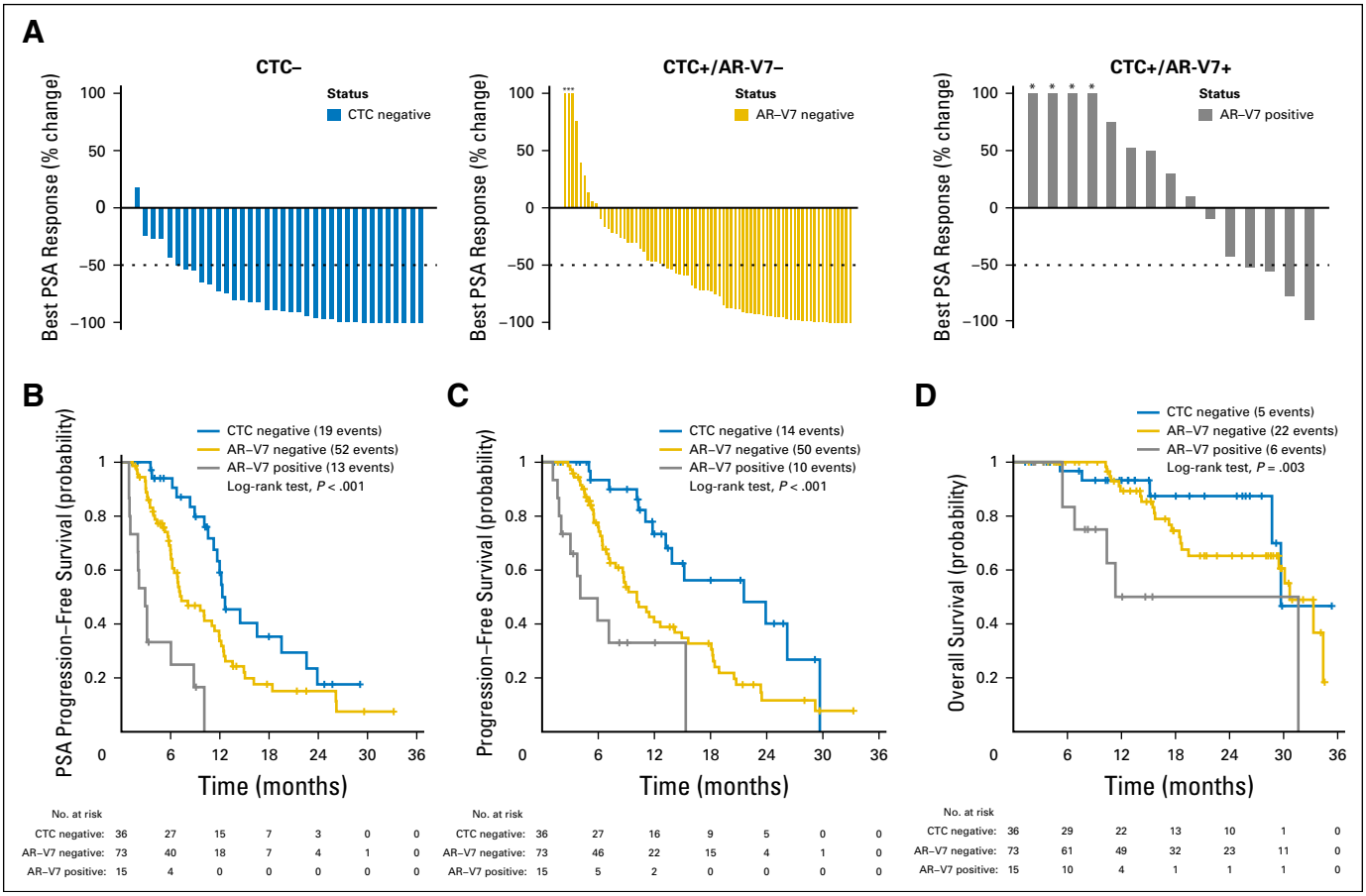


Fig 2. Clinical outcomes for men starting abiraterone or enzalutamide in the first-line novel hormonal therapy setting (N = 124), according to CTC status and AR-V7 status. (A) Waterfall plots depicting best PSA responses according to CTC status and AR-V7 status, expressed in three categories: CTC–, CTC+/AR-V7–, and CTC+/AR-V7+. The dotted line illustrates the threshold for defining a PSA response (≥ 50% PSA reduction from baseline). PSA response rates in CTC– patients, CTC+/AR-V7– patients, and CTC+/AR-V7+ patients were 86.1% (31 of 36), 65.8% (48 of 73), and 26.7% (4 of 15), respectively ($P < .001$). All three groups were significantly different from each other (CTC– *v* CTC+/AR-V7–, $P = .03$; CTC– *v* CTC+/AR-V7+, $P < .001$; CTC+/AR-V7– *v* CTC+/AR-V7+, $P = .009$). (B) Kaplan-Meier curves indicating PSA progression-free survival according to CTC status and AR-V7 status. Median PSA progression-free survival in CTC– patients, CTC+/AR-V7– patients, and CTC+/AR-V7+ patients was 12.7 months (95% CI, 11.7 to 23.9), 7.3 months (95% CI, 6.2 to 12.0), and 2.9 months (95% CI, 2.0 to not reached), respectively ($P < .001$). (C) Kaplan-Meier curves indicating clinical and radiographic progression-free survival according to CTC status and AR-V7 status. Median progression-free survival in CTC– patients, CTC+/AR-V7– patients, and CTC+/AR-V7+ patients was 21.6 months (95% CI, 13.9 to not reached), 10.1 months (95% CI, 7.9 to 14.9), and 4.1 months (95% CI, 3.0 to not reached), respectively ($P < .001$). (D) Kaplan-Meier curves indicating overall survival according to CTC status and AR-V7 status. Median overall survival in CTC– patients, CTC+/AR-V7– patients, and CTC+/AR-V7+ patients was 29.7 months (95% CI, 28.7 to not reached), 30.7 months (95% CI, 29.5 to not reached), and 21.5 months (95% CI, 10.4 to not reached), respectively ($P = .003$). AR-V7, androgen receptor splice variant-7; CTC, circulating tumor cell; PSA, prostate-specific antigen.

(95% CI, 7.9 to 14.9), and 4.1 months (95% CI, 3.0 to not reached), respectively ($P < .001$; Fig 2C). In multivariable Cox regression analysis (Data Supplement), biomarker status generally remained independently prognostic for PFS. In the second-line NHT cohort, median PFS in CTC– patients, CTC+/AR-V7– patients, and CTC+/AR-V7+ patients was 6.2 months (95% CI, 5.4 to not reached), 5.3 months (95% CI, 4.1 to 7.7), and 2.8 months (95% CI, 2.1 to 3.4), respectively ($P < .001$; Fig 3C). In multivariable Cox regression analysis (Data Supplement), biomarker status generally remained independently prognostic for PFS.

OS

In the overall cohort, median OS was 28.7 months (95% CI, 28.4 to not reached) in CTC– patients, 29.5 months (95% CI, 18.4 to not reached) in CTC+/AR-V7– patients, and 11.2 months (95% CI, 8.3 to 17.1) in CTC+/AR-V7+ patients ($P < .001$; Fig 1D). In multivariable Cox regression analysis (Data Supplement), biomarker status generally remained independently prognostic for OS.

In the first-line NHT cohort, median OS in CTC– patients, CTC+/AR-V7– patients, and CTC+/AR-V7+ patients was 29.7 months (95% CI, 28.7 to not reached), 30.7 months (95% CI, 29.5 to not reached), and 21.5 months (95% CI, 10.4 to not reached), respectively ($P = .003$; Fig 2D). In multivariable Cox regression analysis (Data Supplement), biomarker status generally remained independently prognostic for OS. In the second-line NHT cohort, median OS in CTC– patients, CTC+/AR-V7– patients, and CTC+/AR-V7+ patients was 18.8 months (95% CI, 12.5 to not reached), 13.0 months (95% CI, 10.0 to 22.6), and 8.5 months (95% CI, 4.9 to 15.6), respectively ($P < .001$; Fig 3D). In multivariable Cox regression analysis (Data Supplement), biomarker status generally remained independently prognostic for OS.

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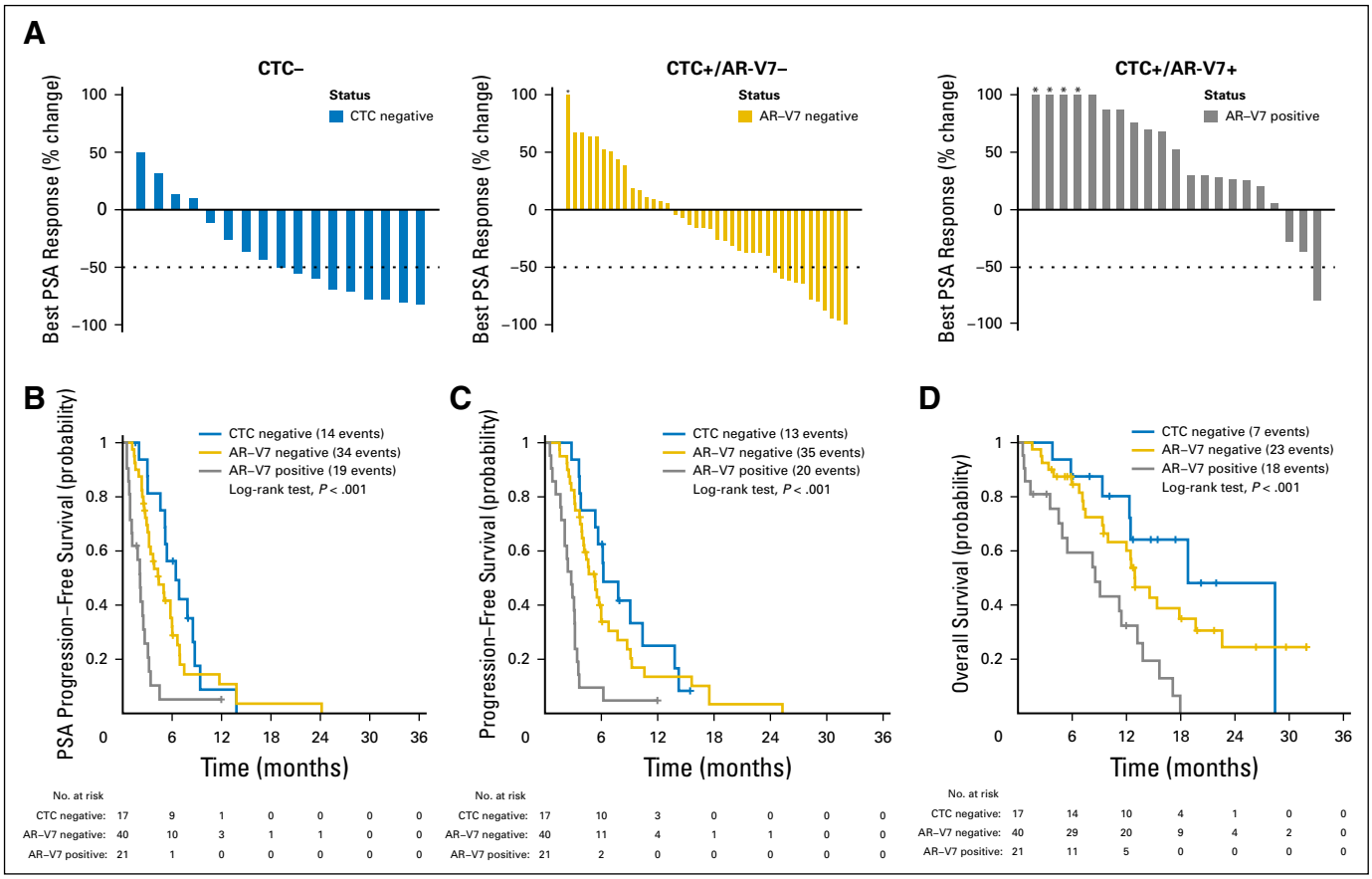


Fig 3. Clinical outcomes for men starting abiraterone or enzalutamide in the second-line novel hormonal therapy setting (N = 78), according to CTC status and AR-V7 status. (A) Waterfall plots depicting best PSA responses according to CTC status and AR-V7 status, expressed in three categories: CTC–, CTC+/AR-V7–, and CTC+/AR-V7+. The dotted line illustrates the threshold for defining a PSA response (≥ 50% PSA reduction from baseline). PSA response rates in CTC– patients, CTC+/AR-V7– patients, and CTC+/AR-V7+ patients were 52.9% (nine of 17), 27.5% (11 of 40), and 4.8% (one of 21), respectively ($P = .003$). All three groups were significantly different from each other (CTC– vs CTC+/AR-V7–, $P = .078$; CTC– vs CTC+/AR-V7+, $P = .002$; CTC+/AR-V7– vs CTC+/AR-V7+, $P = .044$). (B) Kaplan-Meier curves indicating PSA progression-free survival according to CTC status and AR-V7 status. Median PSA progression-free survival in CTC– patients, CTC+/AR-V7– patients, and CTC+/AR-V7+ patients was 6.4 months (95% CI, 5.1 to not reached), 4.4 months (95% CI, 3.2 to 6.0), and 1.1 months (95% CI, 1.0 to 3.1), respectively ($P < .001$). (C) Kaplan-Meier curves indicating clinical and radiographic progression-free survival according to CTC status and AR-V7 status. Median progression-free survival in CTC– patients, CTC+/AR-V7– patients, and CTC +/AR-V7+ patients was 6.2 months (95% CI 5.4–not reached), 5.3 months (95% CI 4.1–7.7), and 2.8 months (95% CI 2.1–3.4), respectively ($P < .001$). (D) Kaplan-Meier curves indicating overall survival according to CTC status and AR-V7 status. Median overall survival in CTC– patients, CTC+/AR-V7– patients, and CTC+/AR-V7+ patients was 18.8 months (95% CI 12.5–not reached), 13.0 months (95% CI 10.0–22.6), and 8.5 months (95% CI 4.9–15.6), respectively ($P < .001$).

Conversions

Fifty-nine patients had evaluable paired blood samples from baseline and progression. Of the 14 patients with baseline CTC– samples, six (43%) remained CTC–, and the remainder converted to CTC+/AR-V7– (n = 6, 43%) or CTC+/AR-V7+ (n = 2, 14%). Of the 35 men with baseline CTC+/AR-V7– samples, 20 (57%) remained CTC+/AR-V7–, and the remainder converted to CTC– (n = 5, 14%) or CTC+/AR-V7+ (n = 10, 29%). Of the 10 men with baseline CTC+/AR-V7+ samples, nine (90%) remained CTC+/AR-V7+, and one patient (10%) converted to CTC+/AR-V7– (none of these patients converted to CTC–).

DISCUSSION

We report, to the best of our knowledge, the largest prospective study to date examining the prognostic significance of CTC-based AR-V7 testing in patients with CRPC receiving NHT with abiraterone or enzalutamide. The current results confirm our previous pilot data that CTC+/AR-V7+ patients have inferior clinical outcomes compared with CTC+/AR-V7– individuals, with respect to PSA responses, PSA-PFS, PFS, and OS. Furthermore, we report, we believe for the first time, the prognostic value of CTC– results using the AdnaTest platform. As expected, CTC– patients demonstrated clinical outcomes that were superior even to CTC+/AR-V7– individuals. To this end, CTC– patients seem to have the best outcomes with NHT, CTC +/AR-V7– patients have intermediate outcomes, and CTC+/AR-V7+ patients have the worst outcomes. Furthermore, because of the increased sample size of this study, we were able to evaluate the clinical significance of biomarker status separately in patients receiving first-line NHT and second-line NHT. To this end, biomarker status remained prognostic for all clinical outcomes in both the first-line and the second-line NHT settings, although there was no statistical difference in survival between CTC– and CTC+/AR-V7– subgroups.

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The fact that a small proportion (13.9%) of CTC+/AR-V7+ patients achieved PSA responses with NHT was an important observation of our study (Data Supplement). Interestingly, responding patients generally had lower AR-FL transcript levels (median 21 *v* 36 copies) and lower AR-V7/AR-FL ratios (median, 8.8% *v* 21.2%), perhaps suggesting that a higher abundance of AR-FL and higher AR-V7 ratios may be associated with worse prognosis. It is also possible that some CTCs in a given blood sample expressed detectable levels of AR-V7, whereas others did not, reflecting individual tumor cell heterogeneity as demonstrated recently by RNA sequencing.[11] Moreover, our assay, which detects AR-V7 mRNA, does not document the presence or nuclear localization of AR-V7 protein,[6] and it is possible that untranslated mRNA was detected, which would not be expected to be pathogenic in the absence of nuclear-localized protein. Finally, it should be highlighted that despite the possibility of a PSA response in some CTC+/AR-V7+ patients, PSA responses in the CTC+/AR-V7– and CTC– populations were much higher (52.2% and 75.5%, respectively). Nevertheless, this observation highlights the notion that not all CTC+/AR-V7+ patients may have an absolute primary resistance to abiraterone and enzalutamide. Rare PSA responses in CTC+/AR-V7+ patients have also been observed in other series,[5,12] although PSA reductions alone do not constitute a clinical benefit.

The current prostate cancer clinical states model[13] recommends considering metastatic CRPC as a series of states defined by the number of prior systemic therapies received. Accordingly, it was important to examine our biomarker outcomes separately in the first-line NHT and second-line NHT settings, representing two distinct contexts of use. Interestingly, but not surprisingly, the prevalence of CTC– patients was lower and the prevalence of CTC+/AR-V7+ patients was higher in the second-line compared with the first-line NHT setting. Similarly, in another study using a CTC-based assay relying on immunofluorescence staining of nuclear AR-V7 protein,[6] AR-V7 prevalence in that analysis also increased with subsequent lines of CRPC therapy. Importantly, our current data show that our biomarker assay retains its prognostic value in both the first-line and the second-line NHT settings, with clinical outcomes remaining distinct for each of the three biomarker categories in each setting. Our data also underscore the value of considering CTC– patients as a distinct category from CTC+/AR-V7– patients. Other CTC-based AR-V7 platforms should also evaluate the prognostic implications of CTC– results.

This study has some limitations, the most significant of which was that there was some variability in the timing of PSA assessments and imaging assessments (which may have influenced our PSA-PFS and PFS estimates). In addition, this study as designed allowed us to draw conclusions only on the prognostic usefulness of our biomarker in the context of androgen-directed therapy, because all patients exclusively received NHT and we did not include chemotherapy-treated patients. Therefore, the predictive usefulness of this biomarker and the interaction between biomarker status and treatment type could not be evaluated and will form the basis of future work. Finally, this study was not powered to assess OS, and we have not yet observed enough death events to make conclusive statements about biomarker status and survival. In addition, because of the exploratory nature of this study, *P* values were not corrected for multiple comparisons.

In conclusion, this expanded analysis confirms the negative prognostic impact of CTC-based AR-V7 detection in patients with CRPC undergoing therapy with abiraterone and enzalutamide and suggests that this biomarker panel may be useful in the prediction of response to AR-targeted treatment applied in the first- and second-line NHT settings. Furthermore, we believe our study is the first to suggest that the modified-AdnaTest CTC-based AR-V7 mRNA assay should be interpreted using three separate prognostic categories: CTC–, CTC+/AR-V7–, and CTC+/AR-V7+. Prospective studies (ClinicalTrials.gov identifier NCT02269982) are currently underway to validate these findings.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

Disclosures provided by the authors are available with this article at jco.org.

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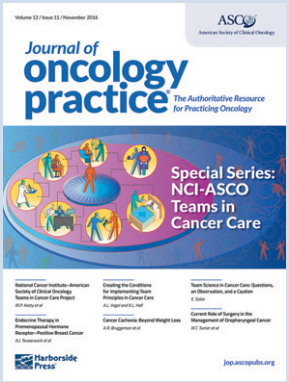
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AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

Clinical Significance of Androgen Receptor Splice Variant-7 mRNA Detection in Circulating Tumor Cells of Men With Metastatic Castration-Resistant Prostate Cancer Treated With First- and Second-Line Abiraterone and Enzalutamide

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Analytical Validation of Androgen Receptor Splice Variant 7 Detection in a Clinical Laboratory Improvement Amendments (CLIA) Laboratory Setting



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Patients with castration-resistant prostate cancer (CRPC) often are treated with drugs that target the androgen receptor (AR) ligand-binding domain. Constitutively active AR splice variant 7 (AR-V7) lacks the ligand-binding domain and, if detected in circulating tumor cells, may be associated with resistance to these agents. We validated an AR-V7 assay in a Clinical Laboratory Improvement Amendments (CLIA)–certified laboratory. Circulating tumor cells were isolated, and mRNA was reverse-transcribed into cDNA. Real-time quantitative PCR amplification of reference transcripts (beta-actin and glyceraldehyde-3-phosphate dehydrogenase), prostate-specific transcripts (prostate-specific membrane antigen, prostate-specific antigen, and AR-full length), and AR-V7 was performed. Specimens for validation included an AR-V7 expressing prostate cancer (LNCaP95), 38 peripheral blood controls, and 21 blood samples from CRPC patients. The assay detected as few as five LNCaP95 cells spiked into peripheral blood, showing high analytical sensitivity. Multiple inter-run and intrarun replicates of LNCaP95 cell line experiments yielded similar cycle threshold values for all genes, showing high analytical precision (AR-V7 cycle threshold CV of 0.67%). All 38 healthy control samples were negative for AR-V7, showing high diagnostic specificity (100%). The diagnostic accuracy was confirmed by concurrent testing of 21 CRPC samples in the research laboratory and the clinical diagnostic laboratory: concordance in AR-V7 status was achieved in all cases (positive in 4, negative in 17) (100% accuracy). This first validated clinical assay detects the AR-V7 with high analytical sensitivity, precision, specificity, and accuracy. *(J Mol Diagn 2017, 19: 115–125; http://dx.doi.org/10.1016/j.jmoldx.2016.08.003)*

Alterations of the androgen pathway are critical to prostate cancer progression. Suppression of androgen receptor (AR) signaling is the therapeutic goal of many prostate cancer drugs. Patients with residual or recurrent disease after surgery and radiotherapy are treated with chemical castration (gonadotropin-releasing hormone agonists or antagonists). After showing an initial response, most patients develop progressive disease, referred to as castration-resistant prostate cancer (CRPC). Intriguingly, CRPC is not androgen-independent and several drugs designed to further suppress the AR pathway have shown improved survival, including abiraterone and enzalutamide.[1–4] Abiraterone is a CYP17A1 inhibitor that impairs synthesis of dihydrotestosterone (AR ligand) precursors in the adrenal glands

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and in the prostate. Alternatively, enzalutamide suppresses signaling by antagonistic binding to the ligand-binding domain of AR.

However, not all patients respond equally to these newer AR-targeting drugs. Approximately 20% to 40% of CRPC patients have poor clinical response to these agents, and nearly all patients who initially respond acquire secondary resistance. Several mechanisms of resistance to these agents have been proposed.[5] One mechanism of resistance is alternate splicing of the AR transcript that results in the truncated AR splice variant 7 (AR-V7) protein.[6,7]

The AR protein has four domains—the N-terminal domain, the DNA binding domain, the hinge-region, and the androgen-binding C-terminal ligand-binding domain. Binding of the ligand (dihydrotestosterone) to the AR ligand-binding domain results in nuclear localization of the AR. In the nucleus, the AR N-terminal domain acts as a transcriptional activator of proliferative signals and androgen-regulated genes (eg, *PSA*). Discovered in 2008, the splice variants of the AR arise through splicing of the intronic cryptic exons to the upstream exons encoding the DNA-binding domain.[6,8] Of the 22 known variants, AR-V7 is the most widely studied variant and carries the most prognostic information. In addition, AR-V7 is the only variant that produces a protein that can be detected in clinical samples.[9] AR-V7 contains only the first three of the eight exons seen in AR—full length (FL), followed by cryptic exon 3 encoding a novel, variant-specific peptide of 16 amino acids. AR-V7 lacks the ligand-binding domain of the protein but retains transcriptional activity. Therefore, AR-V7 acts as a constitutively active AR protein, independently of its binding by dihydrotestosterone. AR-targeting drugs disrupt the dihydrotestosterone-dependent AR signaling. Therefore, these drugs can inhibit signaling by AR-FL protein, but should not inhibit signaling by AR-V7. In CRPC patients, multiple preclinical studies have correlated the disease progression and mortality to the presence and abundance of AR splice variants.[10–14]

Clinical detection of AR-V7 in circulating tumor cells (CTCs) of CRPC patients has been shown by four independent groups to be associated with resistance to AR-targeting agents (*Discussion*).[15–18] However, expression of AR-V7 does not appear to affect the response to taxane chemotherapy adversely.[18–21] Taxanes affect the microtubule network resulting in cytoplasmic sequestration of AR, thereby disrupting the AR nuclear signaling. AR-V7 detection in the CTCs of CRPC patients therefore could be incorporated into therapeutic decision making if a clinically validated assay were available.[22,23]

Despite the many published studies supporting the clinical significance of AR-V7 to date, the analytical performance of the test has not been reported. AR-V7 testing now is being used for several ongoing clinical trials. Seven registered clinical trials (1 phase III trial, 5 phase II trials, and 1 pilot trial) were identified from ClinicalTrials.gov (*https://clinicaltrials.gov*, last accessed January 3, 2016) using the terms "AR-V7" or "ARV7" (summarized by Maughan and Antonarakis[24]). Analytical validation of the AR-V7 test is critically important given the collective data supporting AR-V7 as a predictive marker and the need for further clinical validation through clinical trials. In this report, we describe the analytical validation and the performance characteristics of the first laboratory-developed AR-V7 test in a Clinical Laboratory Improvement Amendments (CLIA)—certified laboratory (live date: August 13, 2015).

Materials and Methods

This study was performed under institutional review board approval, with informed consent.

Cell Lines and Specimens

The prostate cancer cell lines LNCaP and LNCaP95, generously provided by Dr. Luo's laboratory (Baltimore, Maryland), were cultured as described.[15] Validation included DNA fingerprinting, RT-PCR, and Sanger sequencing of the AR-FL transcript from LNCaP cells, and the AR-V7 transcript from LNCaP95 cells. After confirming that LNCaP95 expressed AR-V7, RNA was used as a positive control,[9] and human spleen mRNA (category number: 636525; Clontech, Mountain View, CA) was used as a negative control. As a positive control for prostate cancer cell isolation, LNCaP95 cells were expanded, subcultured, counted, and cryopreserved in single-use aliquots (10,000 cells) in liquid nitrogen. Empirically, larger numbers of cells were required in contrast to the analyte spike in experiments. Cells were diluted into 5 mL of RPMI and processed in parallel to whole blood samples.

Deidentified discarded peripheral blood specimens obtained from 38 healthy bone marrow donors were used to characterize the expression of AR and AR-V7 (14 males, age, 20 to 40 years; 24 females, age, 20 to 69 years).

Blood samples (minimum, 10 mL) from men with metastatic CRPC were collected in two or more acid citrate dextrose (yellow-top) vacutainer tubes (BD, Franklin Lakes, NJ) and sent to the clinical laboratory cold. Two tubes of blood were used for duplicate routine clinical testing. When a third tube was available, it was analyzed independently in the research laboratory. The CTCs were enriched and RNA was harvested within 24 hours of sample collection (generally within 4 hours).

CTC Enrichment

CTCs were enriched using the AdnaTest ProstateCancerSelect kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. Briefly, this method uses immunomagnetic enrichment of tumor cells via epithelial and tumor-associated antigens. Magnetic beads with proprietary antibodies were used to bind to the tumor cells in peripheral blood, and captured using a magnet (LSKMAGS15;

MilliPore, Darnsted, Germany). mRNA from cell lysates was isolated using the AdnaTest ProstateCancerDetect kit (Qiagen), which contains oligo-$(dT)_{25}$-coated beads, according to the manufacturer's protocol. All mRNA isolated was reverse-transcribed to cDNA by the SensiScript RT kit (catalog number: 205213; Qiagen) in a 20-μL reaction using the following thermocycler settings: 37°C for 60 minutes, 93°C for 5 minutes, and 4°C hold. Negative and positive control cDNA were produced in single master lots and stored as single-use aliquots at −20°C.

cDNA was used as a template in a multiplex PCR reaction using HotStar Taq Master Mix (Qiagen) and a pool of primers (provided with the kit) that amplify three tumor-associated antigen mRNAs [epidermal growth factor receptor, prostate-specific antigen (PSA), and prostate-specific membrane antigen (PSMA)] and one reference mRNA beta-actin (ACTB). The following PCR conditions were used: 95°C for 15 minutes; 94°C for 30 seconds, 61°C for 30 seconds, and 72°C for 30 seconds × 42 cycles; 72°C for 10 minutes; and 4°C hold. The amplified PCR products were detected using an Agilent TapeStation 2200 (Agilent, Santa Clara, CA) to confirm cDNA synthesis.

qPCR

Each of the two cDNA syntheses were tested independently as replicates. The cDNA (1/10 of each cell lysate yield) was used as a template for six independent real-time quantitative PCRs (qPCRs) (20 μL reactions) using TaqMan Universal MasterMix II without UNG (Life Technologies, Carlsbad, CA) and primer-probe sets (Table 1) to amplify three prostate-associated antigen mRNAs (PSA, PSMA, and AR-FL), two reference transcripts [glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and ACTB], and the AR-V7 transcript.

Table 1 Custom-Ordered qPCR and Sequencing Oligonucleotides

AR-FL qPCR	
Forward primer	5′-CCTGCTCAAGACGCTTCTAC-3′
Reverse primer	5′-GAACTGATGCAGCTCTCTCG-3′
Hydrolysis probe	5′-CTCCTGGACTCCGTGCAGCCTA-3′
AR-V7 qPCR	
Forward primer	5′-TGAAGCAGGGATGACTCTGG-3′
Reverse primer	5′-TCAGCCTTTCTTCAGGGTCTG-3′
Hydrolysis probe	5′-CCGGGTTGGCAATTGCAAGCA-TCT-3′
Sequencing	
PSMA forward primer	5′-CCACCTTTCAGTGCTTTCTC-3′
PSMA reverse primer	5′-TTTTCATGTCCCGTTCCAAT-3′
PSA forward primer	5′-GTCCCGGTTGTCTTCCTCAC-3′
PSA reverse primer	5′-GGGAATGCTTCTCGCACT-3′
AR-FL forward primer	5′-CCTGCTCAAGACGCTTCTAC-3′
AR-FL reverse primer	5′-GAACTGATGCAGCTCTCTCG-3′
AR-V7 reverse primer	5′-TTTGAATGAGGCAAGTCAGCCT-TTCT-3′

AR, androgen receptor; FL, full length; PSA, prostate-specific antigen; PSMA, prostate specific membrane antigen; qPCR, real-time quantitative PCR; V7, splice variant 7.

PCR conditions were as follows: 95°C for 10 minutes; and 95°C for 15 seconds and 60°C for 1 minute × 50 cycles.

The qPCR assay was performed using hydrolysis probes containing fluorescein amidite and a minor groove binding nonfluorescent quencher and the ABI 7900 HT qPCR instrument (Applied Biosystems, Waltham, MA). The cycle threshold (Ct) value was determined visually as the cycle number at which the fluorescence signal crosses 50% of maximal intensity. Commercially available sets of primers and probes (ThermoFisher Scientific, Waltham, MA) were used for GAPDH (Hs02758991_g1), ACTB (Hs01060665_g1), PSMA (Hs00379515_m1), and PSA (Hs02576345_m1) transcripts. Custom-designed primers and probes (Table 1) spanning the exon 7−exon 8 junction for AR-FL (NM_000044.3) and the exon 3−cryptic exon 3 junction for AR-V7 (transcript described by Hu et al,[11]) were used to amplify 77 and 84 base pair amplicons, respectively. Primer specificity was confirmed by the Basic Local Alignment Search Tool (BLAST; NCBI, *https://blast.ncbi.nlm.nih.gov/Blast.cgi*, last accessed November 3, 2016) against the human transcriptome. Primers and probes were from single master lots in single-use aliquots.

Sanger Sequencing

qPCR amplification products from PSMA, PSA, AR-FL, and AR-V7 were sequenced using primers in Table 1. Products were cycle-sequenced using BigDye v3.1 (Applied Biosystems) per the manufacturer's instructions on an Applied Biosystems 9700 thermocycler and products were resolved on an Applied Biosystems 3730XL DNA sequencer.

Results

We developed an assay to detect AR-V7 mRNA from the CTCs from metastatic CRPC patients (Figure 1). Briefly, CTCs were isolated, and their presence or absence initially was assessed by using cDNA in a multiplex PCR (Adna ProstateCancerDetect kit) for amplification of three tumor-associated antigens (epidermal growth factor receptor, PSA, and PSMA) using the Agilent TapeStation 2200. The mRNA from enriched cells was isolated using Oligo-$d(T)_{25}$−coated beads and reverse-transcribed into cDNA. Separate qPCR reactions for six target mRNAs were performed in duplicate, including two reference gene transcripts (ACTB and GAPDH), three prostate-specific gene transcripts (PSMA, PSA, and AR-FL), and AR-V7 mRNA. Each analyte was interpreted qualitatively and the test results were reported as follows: i) no mRNA detected: this category is used when all RT-PCR reactions have failed; ii) no CTCs detected: used when housekeeping genes are positive and prostate-specific markers are negative; iii) CTCs detected/AR-V7 not detected: used when housekeeping and prostate-specific markers both are positive; and iv) CTCs detected/AR-V7 detected: used when

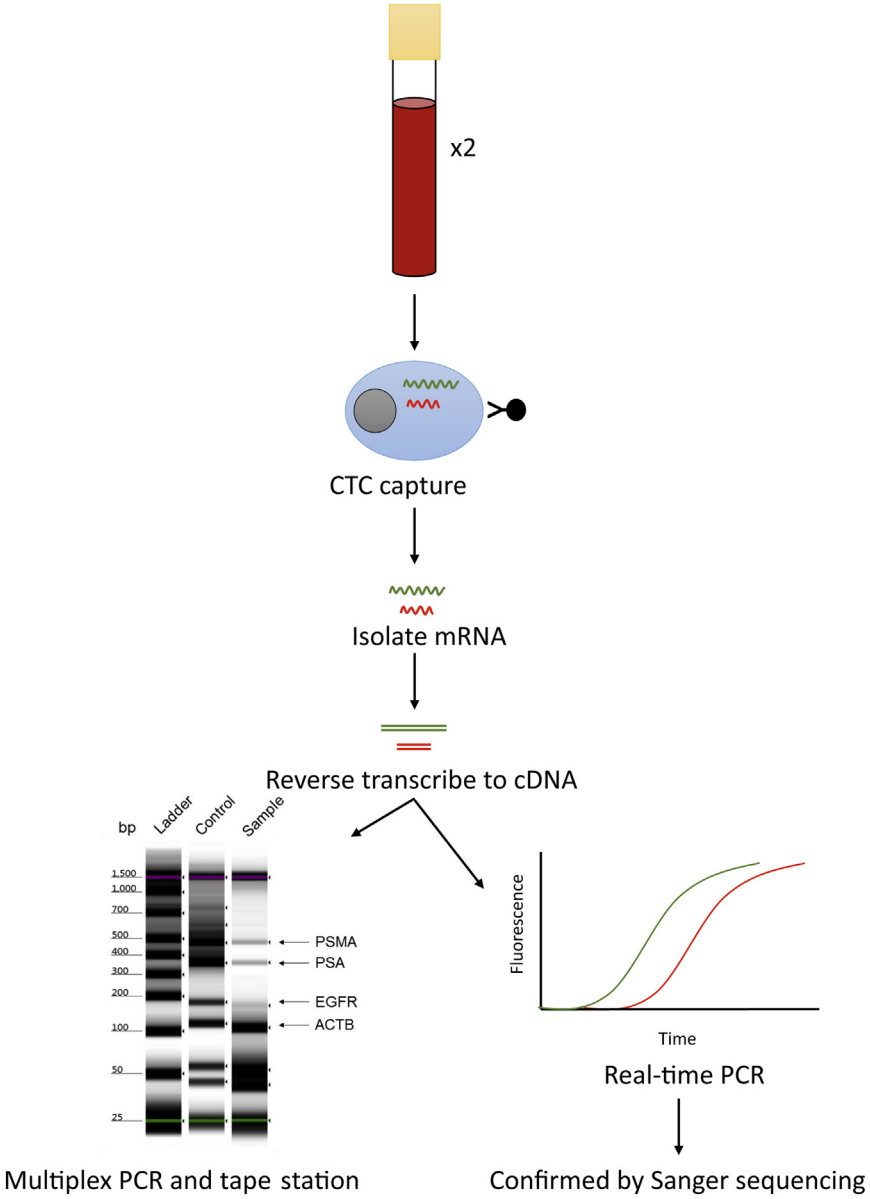


Figure 1 Androgen receptor splice variant 7 (AR-V7) test workflow. Patient blood samples in a minimum of two (preferably three) yellow-top tubes are obtained. Two tubes are used to test the patient in duplicate using our assay as follows. Circulating tumor cells (CTCs) are enriched from the blood samples and mRNA is isolated. **Green lines** and **red lines** designate AR-FL and AR-V7 mRNA, respectively. The RNA is reverse-transcribed to cDNA. cDNA is used in a multiplexed PCR reaction followed by electrophoresis on TapeStation to detect the presence of CTCs. cDNA also is used as a template for real-time quantitative PCR to amplify six target regions to investigate the presence of CTCs and AR-V7. Any amplified prostate-specific antigen (PSA), prostate-specific membrane antigen (PSMA), AR-FL, or AR-V7 products were confirmed by Sanger sequencing. **Purple horizontal bar** shows 1500-bp size standard; **green horizontal bar**, 25-bp size standard. ACTB, beta-actin; EGFR, epidermal growth factor receptor.

prostate-specific markers and AR-V7 reactions both are positive. Amplification of either PSA or PSMA was evidence for CTC detected. Text from the reports is provided in Supplemental Appendix S1. Positive control (LNCaP95 mRNA), negative control (human splenic mRNA), and no-template control (water) were included in each qPCR run.

Validation Approach

The approach to assay validation included confirming the identity of the cell line and the presence of the AR-V7 transcript, validation of the individual steps of the assay including CTC isolation, confirming the identity of qPCR amplification products by Sanger sequencing, and testing the performance characteristics of the assay.

Characterization of LNCaP95 Cell Lines

The LNCaP95 cell line is a derivative of LNCaP, and was characterized by amplifying DNA with the Identifiler kit (Figure 2B). The microsatellite profile of LNCaP95 matched the parental LNCaP (not shown), and the profile listed by ATCC (Manassas, VA). We confirmed AR-V7 expression using RT-PCR and Sanger sequencing (data not shown).

Validation of CTC Enrichment

We showed that the AdnaTest ProstateCancerSelect kit was capable of isolating CTCs from blood by spiking 10,000 and 50,000 prostate cancer (LNCaP95) cells into 4 mL of a donor blood specimen. The large number of cells was required because we used microsatellite fingerprinting as the

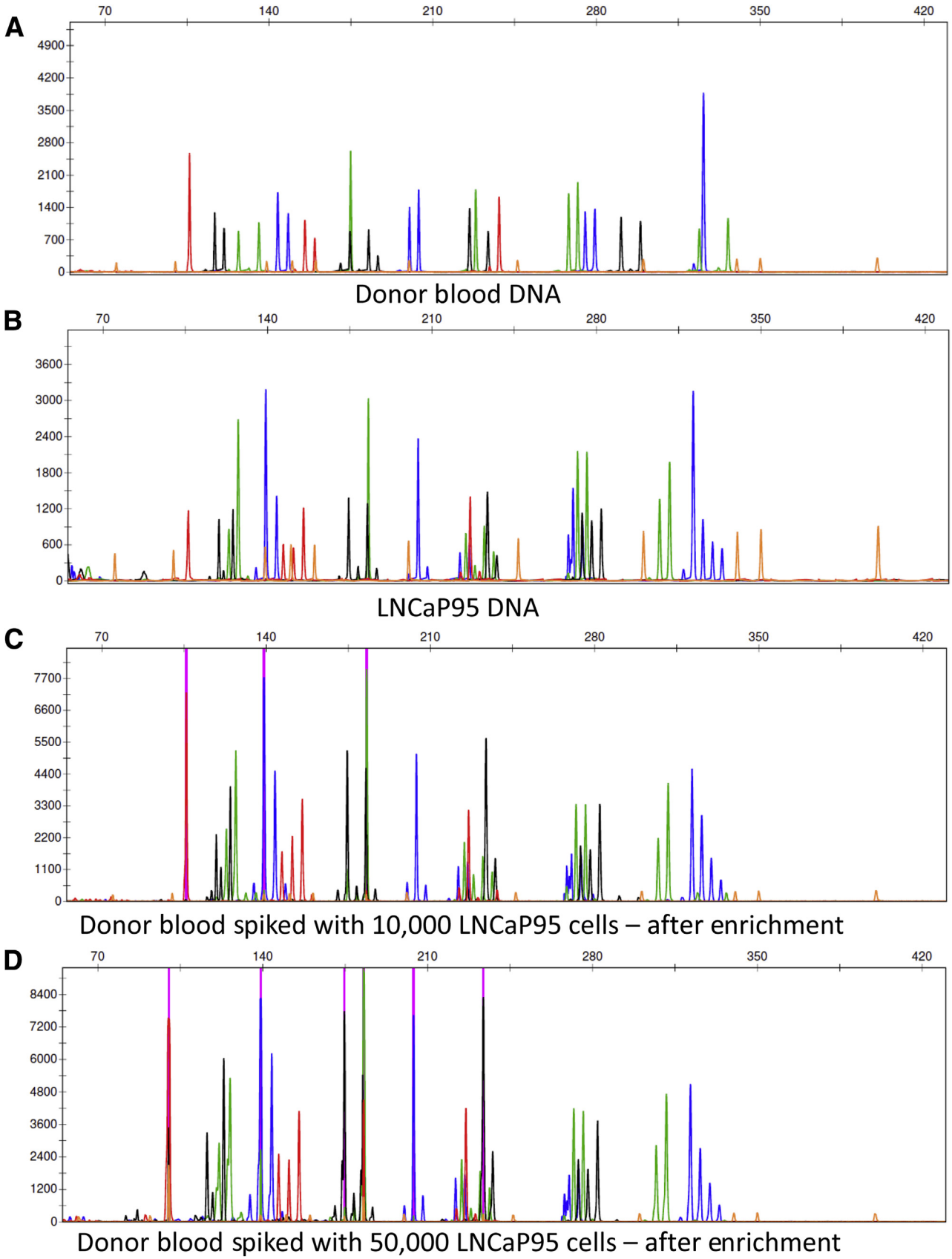


Figure 2 **A:** Microsatellite profile of unspiked donor blood DNA. **B:** Microsatellite profile of LNCaP95 DNA. **C:** Microsatellite profile of donor blood spiked with 10,000 LNCaP95 cells after enrichment. **D:** Microsatellite profile of donor blood spiked with 50,000 LNCaP95 cells after enrichment. After CTC-enrichment the majority of the DNA (≥90%) is derived from the prostate cancer cells, showing effective enrichment.

Table 2 Validation of CTC Enrichment

LNCaP95 cells spiked, *n*	Total donor cells	LNCaP95 cells in sample, %	Pre-enrichment		After enrichment		Fold enrichment
			Donor DNA, %	LNCaP95 DNA, %	Donor DNA, %	LNCaP95 DNA, %	
0	1.5×10^7	0.00	100	0			N/A
10,000	1.5×10^7	0.07	100	0	10	90	1350
50,000	1.5×10^7	0.33	100	0	2	98	294

CTC, circulating tumor cell; N/A, not applicable.

end point. The percentages of the LNCaP95 DNA and donor DNA were quantified (Figure 2, A and B). No LNCaP95 DNA was detected in any of the pre-enriched specimens (Table 2). After enrichment, the samples with 10,000 (Figure 2C) and 50,000 (Figure 2D) spiked cells consisted of approximately 90% and 98% of LNCaP95, respectively. This represents an approximate 1350-fold and 294-fold enrichment, respectively (Table 2).

Validation of qPCR

We tested the cDNA that was reverse-transcribed from 1 ng of harvested LNCaP95 mRNA for the presence of six-target transcripts (AR-V7, AR-FL, PSA, PSMA, GAPDH, and ACTB) using qPCR. As expected LNCaP95 showed expression of all six-target regions, including AR-V7 (Supplemental Figure S1). The identity of the amplified PCR products from the four nonreference targets (PSMA, PSA, AF-FL, and AR-V7) was confirmed using Sanger sequencing (Supplemental Figure S2). In contrast, by using human splenic mRNA, GAPDH, ACTB, PSMA, and AR-FL were detected, consistent with the documented expression of some prostate-specific antigens in other organs (*http://www.ncbi.nlm.nih.gov/UniGene/ESTProfileViewer.cgi?uglist=Hs.654487*, last accessed May 9, 2016), but did not show expression of PSA or AR-V7 (Supplemental Figure S1).

Limit of Detection of the CTC

We spiked 5 mL of healthy blood samples with different numbers of freshly harvested LNCaP95 cells, in duplicate. After enrichment, we consistently detected as few as five LNCaP95 cells using both the CTC detection multiplex kit (Figure 3A), as well as by qPCR of the four prostate-specific transcripts: AR-V7, AR-FL, PSA, and PSMA (Supplemental Table S1). The average Ct value for qPCR correlated inversely with the number of spiked LNCaP95 cells as expected (Figure 3B). The average RNA concentration obtained after CTC enrichment correlated with the number of spiked LNCaP95 cells (Supplemental Figure S3). We were unable to detect CTCs reliably from samples spiked with fewer than 5 cells (data not shown). This compares favorably with data from Bitting et al[25] who showed a median number of 16 CTCs per 7.5 mL of blood.

Analytical Sensitivity

To determine the analytical sensitivity, we generated serial dilutions of harvested LNCaP95 RNA, converted to cDNA, and tested for the presence of all six target transcripts. Our assay consistently detected all analytes, except PSA, from an estimated 1 pg of input LNCaP95 RNA (Figure 4 and Supplemental Table S2). The limit of detection for PSA transcripts was 10 pg of input RNA.

Analytical Precision

We compared the intrarun and inter-run Ct values for each of the amplified targets when using 1 ng input RNA from LNCaP95 to measure the analytical precision of our assay. There was good intrarun reproducibility of the Ct values (means $\pm$ SD) for each of the amplified targets from 12 separate reactions (Table 3 and Supplemental Table S3). The average ΔCt (Ct of AR-V7 $-$ Ct of AR-FL) for LNCaP95 was 3.69 ± 0.23 (CV, 6.2%), implying an AR-FL to AR-V7 transcript ratio of between 8- and 16-fold (Supplemental Table S3). Similar reproducibility was seen in two replicates from an independent run. The Ct (and ΔCt) values of the positive control (LNCaP95 RNA) were determined for each run and plotted on a Levey–Jennings chart as quality control.[26]

Diagnostic Specificity

We de-identified and tested the peripheral blood from 38 healthy controls (14 males, age, 20 to 40 years; 24 females, age, 20 to 69 years) for the six transcripts. All control samples were negative for AR-V7, but expressed both reference genes, showing high (100%) diagnostic specificity (Supplemental Table S4). All healthy control samples were negative for PSA and PSMA; however, AR-FL transcripts were detected at very low levels (Ct value, >39) in 3 healthy donors: 2 older women (age, >50 years) and 1 man (age, <40 years) (Supplemental Table S4), consistent with the documented AR-FL expression from nonprostatic tissue (*http://www.ncbi.nlm.nih.gov/UniGene/ESTProfileViewer.cgi?uglist=Hs.76704*, last accessed May 9, 2016).

Diagnostic Accuracy

Before clinical implementation, we tested 21 CRPC patient blood specimens concurrently with Dr. Luo's laboratory.[15]

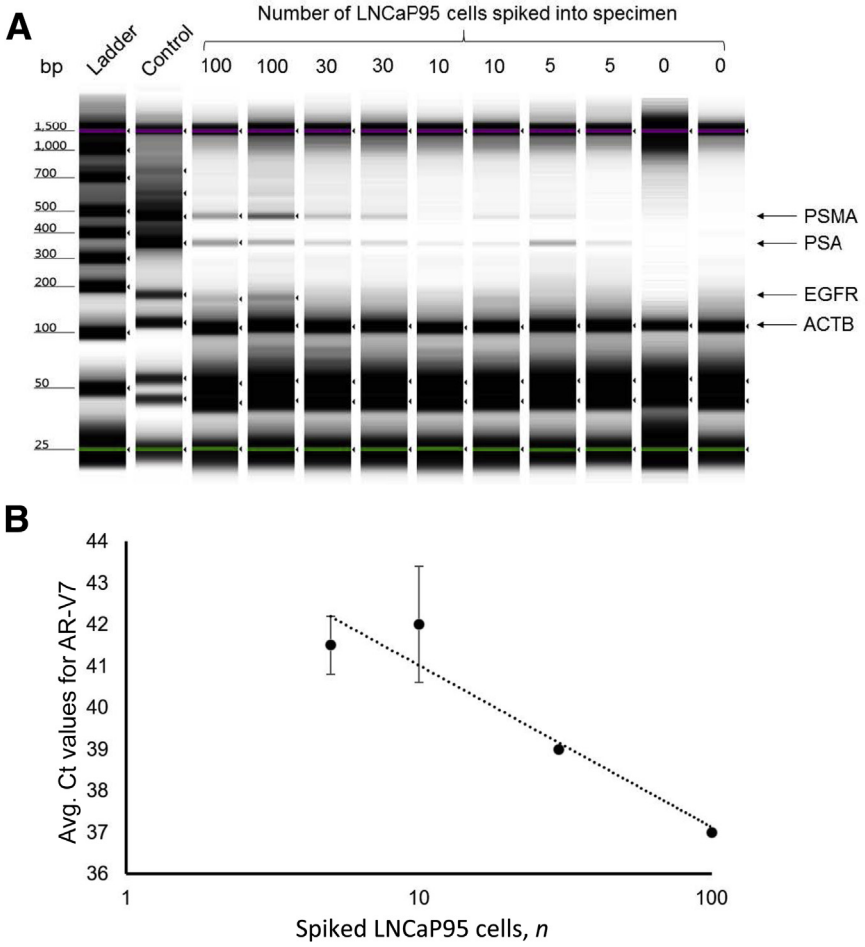


Figure 3 Different estimated numbers of LNCaP95 cells in duplicate were spiked into a healthy donor blood sample and detected using the Adna ProstateCancerDetect kit, as well as our assay. **A:** TapeStation image after ProstateCancerDetect kit showing amplification of prostate-associated antigens [prostate-specific antigen (PSA), prostate-specific membrane antigen (PSMA), epidermal growth factor receptor (EGFR), beta-actin (ACTB)] from samples spiked with 5 or more cells. **B:** The average cycle threshold (Ct) value of androgen receptor splice variant 7 (AR-V7) real-time quantitative PCR amplification correlates inversely with the number of spiked LNCaP95 cells. **Purple horizontal bar** shows 1500-bp size standard; **green horizontal bar**, 25-bp size standard. Experiments were performed in duplicate and error bars are ± 1 SD. Avg, average.

Results were concordant for AR-V7 for all 21 cases (positive in 4 cases, negative in 17 cases) (Table 4 and Supplemental Table S5). In addition, the ratio of AR-FL to AR-V7 transcripts, as estimated by ΔCt (Ct of AR-FL − Ct of AR-V7), was comparable for all four positive cases between the laboratories (Supplemental Table S5). The difference in ΔCt between the laboratories averaged 0.725 (range, −0.77 to 1.825). There were occasional discrepancies between the laboratories regarding the presence or absence of individual prostate-specific transcripts (PSA, PSMA, and AR-FL). However, overall, 19 of the 21 CRPC cases were categorized identically by both laboratories (Table 4). The laboratories differed in two cases regarding the presence of CTCs. Our assay detected low levels of PSMA transcripts in these two patients at high Ct values of 38.82 and 38.86, respectively, but not PSA or AR-FL (Supplemental Table S5). The research laboratory categorized these cases as no CTCs detected. In this regard, the presence or absence of CTCs was not clinically actionable because the indication for testing was limited to men with known metastatic disease.

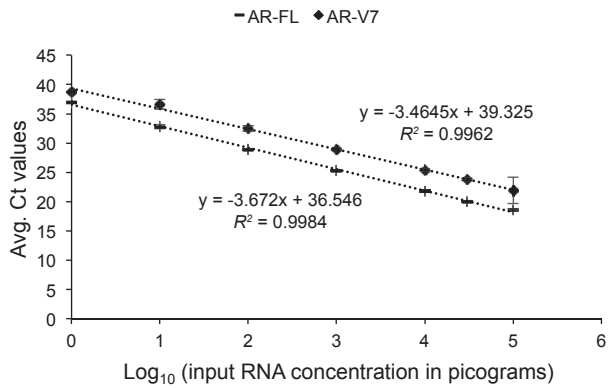


Figure 4 Real-time quantitative PCR amplification using diluting concentrations of input LNCaP95 RNA was performed. The graph of average cycle threshold (Ct) value versus $\log_{10}$ (input RNA concentration) shows amplification of both AR-FL and AR-V7 from up to 1 pg (0 on $\log_{10}$ scale) of input LNCaP95 RNA. Experiments were performed in duplicate and error bars are ± 1 SD. AR, androgen receptor; Avg, average; FL, full length; V7, splice variant 7.

Proposed Indications for Testing and Clinical Implementation

AR-V7 testing may be indicated in men with metastatic CRPC, to be integrated into therapeutic decision making about the potential benefit of AR-directed agents (enzalutamide and abiraterone) versus taxanes (docetaxel and

Table 3 Precision

mRNA	Mean Ct	SD	CV
GAPDH	25.0	0.06	0.24%
ACTB	23.55	0.11	0.47%
PSA	31.76	0.13	0.41%
PSMA	27.92	0.08	0.29%
AR-FL	27.64	0.17	0.62%
AR-V7	31.34	0.21	0.67%

ACTB, beta-actin; AR, androgen receptor; Ct, cycle threshold; FL, full length; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PSA, prostate-specific antigen; PSMA, prostate-specific membrane antigen; V7, splice variant 7.

cabazitaxel) based on the correlation between the presence of AR-V7 transcripts and the lack of therapeutic response to AR-targeting agents.[15–17]

Clinical testing was implemented as follows. Each clinical sample was tested in duplicate. The parental LNCaP cell line spiked into RPMI was used as a positive control with each CTC isolation. The cDNA was tested in duplicate in a multiplex PCR using the proprietary kit and detected using electrophoresis (TapeStation) to initially confirm the presence/absence of CTCs. Stored cDNA was tested by qPCR, with a positive control (LNCaP95 RNA), a negative control (human splenic RNA), and a no-template control in each run. The identity of amplified prostate-specific targets (PSA, PSMA, AR-FL, and AR-V7) was confirmed by Sanger sequencing. Residual blood samples subsequently were tested by the research laboratory independently to determine concordance as quality control.

Subsequent Testing After Validation

After launching the clinical test, patient results were released based on testing in the molecular diagnostic laboratory only, and without knowledge of the research testing laboratory results. Seventeen patient samples subsequently were tested by the research laboratory. Overall, 16 of 17 patients were categorized identically between the two laboratories (94.11% concurrency): 4 as no CTCs detected; 6 as CTCs detected, no AR-V7 detected; and 6 as CTCs detected, AR-V7 detected (Table 4 and Supplemental Table S6). All prostate-associated targets amplified by our laboratory from the first 13 clinical samples (PSMA, PSA, AR-FL, and AR-V7) were confirmed by sequencing. From sample 14 onward, only AR-FL− and AR-V7−positive amplicons were sequenced. There was one discrepancy (sample 17) between the laboratories, the clinical laboratory detected the AR-V7 transcript at a very low level (Ct, 38.10) in one of the two duplicates, but this was not detected by the research laboratory. The residual cDNA for this sample was tested and confirmed the presence of AR-V7, and was confirmed further by Sanger sequencing. To date, no sample has failed testing (ie, no mRNA detected). Since implementation, all AR-V7−positive samples have been confirmed by Sanger sequencing.

Discussion

Detection of AR-V7 in patients with metastatic CRPC has been shown to correlate with a lack of response to AR-targeting agents by four independent groups.[15–18] The first report by Antonarakis et al[15] from Johns Hopkins reported 62 patients treated with either enzalutamide or abiraterone. Eighteen (29%) patients expressed AR-V7 at baseline, showed a lack of PSA response, and showed significantly reduced progression-free and overall survival compared with patients who lacked AR-V7 expression. The second study from Houston, TX, followed up 60 patients with bone metastatic CRPC treated with enzalutamide. The presence of AR-V7 in bone marrow specimens (detected at the protein level using immunohistochemistry) in 26% of patients was associated with primary resistance to enzalutamide treatment.[17] The third study from Germany looked at the clinical response of 37 patients treated with hormonal therapy (enzalutamide or abiraterone). Only 1 of 15 (7%) AR-V7−positive patients showed a PSA response to therapy in

Table 4 Diagnostic Accuracy

	Dr. Luo's Laboratory			
Results	No mRNA detected	No CTCs detected	CTCs (+), AR-V7 (−)	CTCs (+), AR-V7 (+)
Results of concurrent testing during validation				
CLIA laboratory	0	0	0	0
No mRNA detected				
No CTCs detected	0	6	0	0
CTCs (+), AR-V7 (−)	0	2	9	0
CTCs (+), AR-V7 (+)	0	0	0	4
Results of subsequent testing during live testing				
CLIA laboratory				
No mRNA detected	0	0	0	0
No CTCs detected	0	4	0	0
CTCs (+), AR-V7 (−)	0	0	6	0
CTCs (+), AR-V7 (+)	0	0	1	6

AR, androgen receptor; CLIA, Clinical Laboratory Improvement Amendments; CTC, circulating tumor cell; V7, splice variant 7.

contrast to 71% of AR-V7−negative patients.[16] Most recently, a fourth study by Scher et al[18] from Memorial Sloan Kettering Cancer Center, using independent methodology, confirmed that the expression of AR-V7 predicted a lack of response to AR targeting agents. Although additional confirmation is desirable, we consider the AR-V7 test to be sufficiently clinically validated.

In this study, we present analytical validation of the first qualitative AR-V7 test with high analytical sensitivity, precision, diagnostic specificity, and accuracy. The clinical assay is similar to that described previously by Dr. Luo's research laboratory[15] with the following differences. We used sequence-specific hydrolysis probes instead of the intercalating dye (SYBR green), and we Sanger sequenced the amplified products to confirm their identity.

In our assay, detection of the prostate-associated antigen transcripts (PSA or PSMA) from the patient's blood was interpreted as CTCs detected. Because the AR-FL transcript was detected in 3 healthy control patients' blood, we did not use AR-FL by itself as evidence of CTC positivity. AR-FL positivity is used as additional evidence of the presence of CTCs when another transcript (PSA or PSMA) is positive. Furthermore, none of the patients had detectable AR-FL transcript in the absence of PSA and PSMA.

Our current assay is qualitative. Low levels of expression of AR-V7 are reported as AR-V7 positive. Whether quantitative AR-V7 differences are associated with a differential response to therapy remains to be determined. In theory, low-level AR-V7 positivity by our assay may reflect either capture of scant CTCs that are all AR-V7 positive, or may reflect a small subset of captured CTCs that express AR-V7. Whether tumor heterogeneity exists in the CTCs, and if that is representative of the *in situ* tumor, and how that affects response to therapy, is not currently known.

Because our laboratory is the first CLIA−certified laboratory performing AR-V7 testing, interlaboratory proficiency currently is not possible. In the interim, we plan to perform repeat testing of stored cDNA. Currently, residual samples subsequently are tested by Dr. Luo's laboratory and results are compared. Detection of AR-V7 and other transcripts could be adapted to next-generation sequencing−based testing, possibly using RNA annealing, selection, and ligation sequencing technology.[27]

CRPC patients resistant to the novel hormonal therapy often are treated with taxane chemotherapy. Taxanes disrupt the intracellular microtubule trafficking and thus disrupt androgen signaling by sequestering the AR protein in the cytoplasm. Thus, they should be effective against both the AR-FL and AR-V7 CRPCs. However, data from *in vitro* and xenograft model systems have shown that when treated with taxanes (docetaxel), AR-V7 can traffic into the nucleus in a microtubule-independent mechanism, and the AR-V7−expressing cells can escape growth inhibition.[28−30] In contrast, clinical data has documented better outcomes for AR-V7−expressing CRPC patients when treated with taxanes in comparison with novel hormonal therapy.[19] Another group has shown that response to cabazitaxel in CRPC patients is independent of the AR-V7 status of the patient.[21] Prospective clinical trials (eg, NCT02379390, *https://clinicaltrials.gov/ct2/show/NCT02379390*, last accessed November 18, 2016) are ongoing to confirm the clinical response to taxanes in AR-V7−expressing patients.

In addition to taxanes, several newer therapeutic agents have shown promising results in preclinical studies against AR splice variants. These include galeterone (Tokai Pharmaceuticals, Boston, MA), an AR-targeting agent that potentially can inhibit both AR-FL− and AR-V7−mediated signaling[31−33]; the ESSA Pharmaceuticals Inc. (EPI, Vancouver, CA) family of small molecules (including EPI-506) that target the AR N-terminal domain[34−36]; agents targeting the bromodomain extraterminal family of proteins that bind with AR to facilitate its transcriptional activation[37,38]; and niclosamide, which can inhibit AR-FL− and AR-V7−mediated transcriptional activity in preclinical models.[39,40]

In addition to the AR splice variants, several less common possible mechanisms of resistance to androgen-deprivation therapy have been documented in CRPC patients.[41] These include AR gene amplification and overexpression, conversion of weak androgens to potent androgens, intratumoral androgen production, somatic point mutations in the N-terminal domain of AR, and stimulation of AR via androgen-independent pathways (reviewed by Maughan and Antonarakis[22]). Our assay does not detect these possible alternative mechanisms of resistance to androgen-deprivation therapy. Data that AR-V7−positive CRPCs respond to taxanes potentially allows AR-V7 to serve as a clinically actionable treatment-selection biomarker in this setting.

In summary, AR-V7 testing in metastatic CRPC patients may serve as a clinically relevant biomarker to guide prognosis as well as therapy decisions. Several ongoing clinical trials are likely to provide information regarding the best therapeutic choice in this high-risk population. The development of a robust AR-V7 assay performed in a CLIA−certified laboratory documented in this report is the crucial first step in its further clinical validation.

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Supplemental Data

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Prostate Cancer

Clinical Utility of Circulating Tumour Cell Androgen Receptor Splice Variant-7 Status in Metastatic Castration-resistant Prostate Cancer

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Abstract

Background: Detection of androgen receptor splice variant-7 (AR-V7) mRNA in circulating tumour cells (CTCs) is associated with worse outcome in metastatic castration-resistant prostate cancer (mCRPC). However, studies rarely report comparisons with CTC counts and biopsy AR-V7 protein expression.
Objective: To determine the reproducibility of AdnaTest CTC AR-V7 testing, and associations with clinical characteristics, CellSearch CTC counts, tumour biopsy AR-V7 protein expression and overall survival (OS).
Design, setting, and participants: CTC AR-V7 status was determined for 227 peripheral blood samples, from 181 mCRPC patients with CTC counts (202 samples; 136 patients) and matched mCRPC biopsies (65 samples; 58 patients).
Outcome measurements and statistical analysis: CTC AR-V7 status was associated with clinical characteristics, CTC counts, and tissue biopsy AR-V7 protein expression. The association of CTC AR-V7 status and other baseline variables with OS was determined.
Results and limitations: Of the samples, 35% were CTC+/AR-V7+. CTC+/AR-V7+ samples had higher CellSearch CTC counts (median CTC; interquartile range [IQR]: 60, 19–184 vs 9, 2–64; Mann-Whitney test $p < 0.001$) and biopsy AR-V7 protein expression (median H-score, IQR: 100, 63–148 vs 15, 0–113; Mann-Whitney test $p = 0.004$) than CTC+/AR-V7− samples. However, both CTC− (63%) and CTC+/AR-V7− (62%) patients had detectable AR-V7 protein in contemporaneous biopsies. After accounting for baseline characteristics,

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there was shorter OS in CTC+/AR-V7+ patients than in CTC− patients (hazard ratio [HR] 2.13; 95% confidence interval [CI] 1.23–3.71; $p = 0.02$); surprisingly, there was no evidence that CTC+/AR-V7+ patients had worse OS than CTC+/AR-V7− patients (HR 1.26; 95% CI 0.73–2.17; $p = 0.4$). A limitation of this study was the heterogeneity of treatment received.

Conclusions: Studies reporting the prognostic relevance of CTC AR-V7 status must account for CTC counts. Discordant CTC AR-V7 results and AR-V7 protein expression in matched, same-patient biopsies are reported.

Patient summary: Liquid biopsies that determine circulating tumour cell androgen receptor splice variant-7 status have the potential to impact treatment decisions in metastatic castration-resistant prostate cancer patients. Robust clinical qualification of these assays is required before their routine use.

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1. Introduction

Prostate cancer (PC) is the most common malignancy and the second leading cause of cancer-related death in men in the Western world [1]. While most patients with advanced PC initially respond to androgen deprivation therapy, inevitably the majority progress to lethal metastatic castration-resistant PC (mCRPC) with maintained androgen receptor (AR) activity. This has led to the development of new therapies targeting AR signalling such as abiraterone acetate (AA) and enzalutamide (E) that have further improved outcome for patients with mCRPC and de novo metastatic castration-sensitive PC [2–9]. However, resistance to AA and E remains common, and the development of predictive biomarkers that identify patients who benefit from these therapies is critically important.

One promising biomarker is AR splice variant-7 (AR-V7), which has been implicated in the emergence and progression of mCRPC, and the development of resistance to current AR-targeting therapies [10–20]. A plethora of studies using platforms that quantify both AR-V7 mRNA and protein from isolated circulating tumour cells (CTCs) or whole blood have demonstrated AR-V7 to be associated with worse outcomes from AR-targeting therapies [10–15,17,20,21]. As such, CTC AR-V7 tests have undergone analytical and clinical validation [22]. However, studies have rarely reported direct comparisons with CTC enumeration and AR-V7 protein expression in matched mCRPC tissue biopsies.

We aimed to evaluate the reproducibility of AdnaTest CTC AR-V7 status, and compare CTC AR-V7 status with clinical characteristics and CellSearch CTC counts to determine whether these impact clinical outcome analyses. Finally, we evaluated whether CTC AR-V7 status accurately predicted AR-V7 protein expression in matched, same-patient, contemporaneous mCRPC biopsies.

2. Patients and methods

2.1. Patients

All patients and study cohorts are detailed in the Supplementary material and summarised in Supplementary Fig. 1.

2.2. AdnaTest mRNA extraction, cDNA conversion, and AdnaTest CTC call

Isolation and enrichment of CTCs from mCRPC patient peripheral blood (PB) draws were carried out using the AdnaTest ProstateCancerSelect (Qiagen, Hilden, Germany), and mRNA purification was performed using the AdnaTest ProstateCancerDetect (Qiagen) as per the manufacturer's instructions (Supplementary material and Supplementary Fig. 2). CTC calls for each sample are presented as CTC call positive (actin positive, and prostate-specific membrane antigen [PSMA] and/or prostate-specific antigen [PSA] and/or epidermal growth factor receptor[EGFR] positive; CTC+), CTC call negative (actin positive and all other markers negative; CTC−), or failed cDNA conversion (actin negative; failed; Supplementary Figs. 2 and 3).

2.3. mRNA quantification of AR-FL and AR-V7

CTC+ samples were used for the measurement and quantification of AR-FL and AR-V7 transcripts. Quantitative real-time polymerase chain reaction (PCR) was carried out with primers for AR-FL (forward: 5′-CAGCCTATTGCGAGAGAGCTG-3′, reverse: 5′-GAAAGGATCTTGGGCACTTGC-3′) and AR-V7 (forward: 5′-CCATCTTGTCGTCTTCGGAAATGTTA-3′, reverse: 5′-TTTGAATGAGGCAAGTCAGCCTTTCT-3′) along with IQ SYBR Green supermix (Bio-rad, California, USA) and run on a Rotor-Gene Q MDx 2Plex HRM (Qiagen). Ct values were converted to absolute copy numbers using a standard curve using a *gBlock* gene fragment (Integrated DNA Technologies, Iowa, USA) containing the primer targets and internal amplified sequences for AR-FL and AR-V7 (Supplementary Figs. 2 and 3). AR-V7 status is presented as continuous (copies/ml) and binary (present ≥1 copy/ml and absent <1 copy/ml) outcomes; AR-FL status is presented as binary (present ≥1 copy/ml and absent <1 copy/ml) outcomes. Blinded control samples were run at the Institute of Cancer Research (ICR) and Johns Hopkins University (JHU) to confirm optimisation of the AdnaTest platform within both laboratories (Supplementary Table 1).

2.4. CellSearch CTC enumeration

CTC counts were determined from mCRPC patient PB draws using the CellSearch CTC kit (Menarini; Silicon Biosystems, Pennsylvania, USA) according to the Food and Drug Administration-cleared manufacturer's method, as previously described (Supplementary material) [23,24].

2.5. Immunohistochemistry staining for AR-FL and AR-V7 protein expression

Immunohistochemistry (IHC) for AR-FL (AR N terminus, AR441; Dako, Agilent Technologies, California, USA or ab133273; Abcam, Cambridge,

UK) and AR-V7 (Clone RM7, GTX33604; GeneTex, California, USA) was performed on mCRPC patient biopsies as previously described (Supplementary material) [19].

2.6. AR copy number and mutational status

Next-generation sequencing was used to determine AR copy numbers and mutational status for mCRPC patient biopsies as previously prescribed [25,26].

2.7. Statistical analysis

All statistical analyses were performed using Stata v15.1 or GraphPad Prism v6, and are indicated within all figures and tables. Detailed methods for all statistical analyses can be found in the Supplementary material.

3. Results

3.1. CTC AR-V7 positivity is associated with more advanced disease

Between January 2015 and January 2018, the AdnaTest was performed on 277 PB draws from 181 patients with mCRPC (whole PB cohort; Supplementary Fig. 1 and Supplementary Table 2). Overall, 95/277 samples (34%) were CTC−, 86/277 samples (31%) were CTC+/AR-V7−, and 96/277 samples (35%) were CTC+/AR-V7+ (Fig. 1). There was evidence of differences in CellSearch CTC count ($p < 0.001$), Eastern Cooperative Oncology Group Performance Status (ECOG PS; $p = 0.03$), the number of taxane therapies received (-$p < 0.001$), haemoglobin ($p = 0.009$), alkaline phosphatase ($p = 0.0006$), lactate dehydrogenase ($p = 0.001$), and PSA ($p = 0.0002$) by CTC/AR-V7 status (Table 1). Taken together, these data suggest that CTC+/AR-V7+ samples were taken from patients with a higher disease burden at the time of PB draw.

3.2. Intralaboratory analysis identifies reduced concordance of CTC AR-V7 status at low mRNA expression levels

We next determined whether intralaboratory (technical replicates) reproducibility was impacted by CTC AR-V7 mRNA expression. CTC+/AR-V7+ patients with high ($n = 48$; >median; >7.00 copies/ml) AR-V7 mRNA expression demonstrated high agreement (pairwise agreement 92%) between replicates (Fig. 1). In contrast, CTC+/AR-V7+ patients with low ($n = 48$; ≤median; 7.00 copies/ml) AR-V7 mRNA expression demonstrated lower agreement (pairwise agreement 43%) between replicates (Fig. 1). Nearly all (170/182; 93%) the CTC+ samples expressed AR-FL mRNA with high agreement (pairwise agreement 90%) between replicates (Fig. 1). These data demonstrate that binary reporting of CTC AR-V7 status has reduced agreement at lower levels of CTC AR-V7 mRNA expression.

3.3. Interlaboratory analysis confirms reduced concordance of CTC AR-V7 status at low mRNA expression levels

To compare the AdnaTest between institutions, 56 samples (two sequential PB draws; biological replicates) from 49 patients were analysed at the ICR in London, UK, and at JHU in Baltimore, USA (interlaboratory cohort; Supplementary Figs. 1 and 4). Thirty-eight (68%) samples at the ICR and 46 (82%) samples at JHU were called CTC+, with 10 (18%) samples being CTC− at both institutions, demonstrating good overall interlaboratory agreement (48/56; 86%;

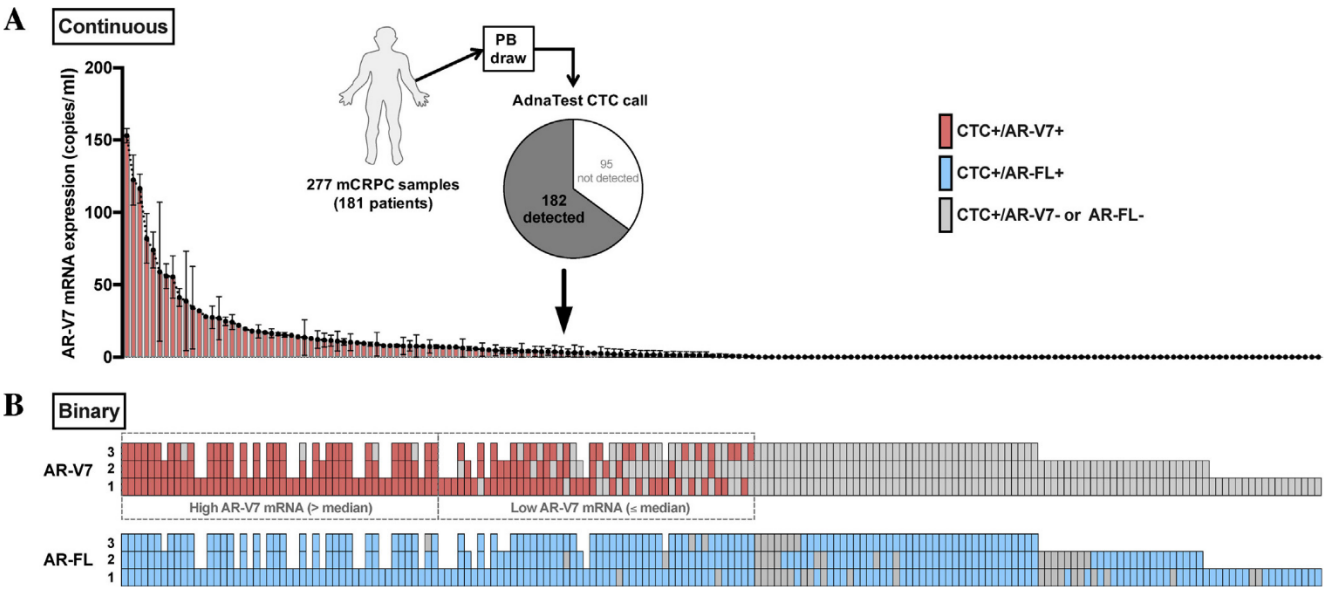


Fig. 1 – CTC AR-V7 mRNA as a continuous and binary variable: decreased assay concordance at lower mRNA levels. A total of 277 peripheral blood (PB) draws from 181 mCRPC patients were identified for AdnaTest analysis between January 2015 and January 2018. CTCs were detected in 182 samples and CTC AR-V7 and AR-FL status was determined. (A) Continuous (mean ± SD) AR-V7 mRNA expression (copies/ml; red bars) from technical replicates is shown. (B) Binary CTC AR-V7 (red boxes positive; grey boxes negative) and AR-FL (blue boxes positive; grey boxes negative) results shown for each technical replicate (numbered 1–3). AR-V7 = androgen receptor splice variant-7; CTC = circulating tumour cell; mCRPC = metastatic castration-resistant prostate cancer; SD = standard deviation.

Table 1 – Patient baseline characteristics at time of peripheral blood draw (whole PB cohort)

Characteristic	CTC− (*N* = 95)		CTC+/AR-V7− (*N* = 86)		CTC+/AR-V7+ (*N* = 96)		*p* value[a]
	N	%	*N*	%	*N*	%	
CellSearch CTC count							
<5	38	40.0	27	31.4	6	6.3	<0.001
≥5	28	29.5	38	44.2	65	67.6	–
Missing analysis	29	30.5	21	23.1	25	26.0	–
ECOG PS at draw							
0	12	12.6	13	15.1	6	6.3	0.03
1	66	69.5	57	66.3	60	62.5	–
≥2	7	7.4	11	12.8	23	24.0	–
NR	10	10.5	5	5.8	7	7.3	–
Metastatic sites							
Lymph node only	12	12.6	8	9.3	9	9.4	0.8
Visceral	17	17.9	21	24.4	23	24.0	0.5
Bone	71	74.7	74	86.1	81	84.4	0.1
AR-targeting therapies							
0	4	4.2	7	8.1	0	0.0	0.08
1	51	53.7	50	58.1	54	56.3	–
2	30	31.6	25	29.1	36	37.5	–
NR	10	10.5	4	4.7	6	6.3	–
Taxane therapies							
0	23	24.2	20	23.3	12	12.5	<0.001
1	43	45.3	33	38.4	27	28.1	–
2	19	20.0	29	33.7	51	53.1	–
NR	10	10.5	4	4.7	6	6.3	–

Characteristic	CTC− (*N* = 95)		CTC+/AR-V7− (*N* = 86)		CTC+/AR-V7+ (*N* = 96)		*p* value[b]
	Median	IQR	Median	IQR	Median	IQR	
Age (yr)	71.0	66.8–75.6	69.6	64.9–72.3	70.4	65.3–74.6	0.07
Hb (g/dl)	11.7	10.4–12.8	11.4	10.3–12.8	10.7	9.7–12.3	0.009
ALT (U/l)	16.0	12.5–23.0	14.0	10.0–19.0	15.0	11.0–19.0	0.03
ALP (U/l)	83.0	66.0–163.0	111.5	76.3–200.5	180.0	93.8–346.0	0.0006
Albumin (g/l)	35.0	32.5–38.0	35.0	31.8–39.0	34.0	31.0–37.0	0.1
LDH (U/l)	179.5	146.3–241.3	184.5	151.3–266.0	230.0	175.5–433.5	0.001
PSA (μg/l)	110.0	29.0–300.5	147.0	51.0–345.0	244.5	109.3–746.8	0.0002

ALP = alkaline phosphatase; ALT = alkaline transferase; AR = androgen receptor; AR-V7 = androgen receptor splice variant-7; CTC = circulating tumour cell; ECOG PS = European Cooperative Oncology Group performance status; Hb = haemoglobin; IQR = interquartile range; LDH = lactate dehydrogenase; *N* = number; NR = no result; PB = peripheral blood; PSA = prostate-specific antigen.

[a] χ^2 test.

[b] Kruskal-Wallis equality-of-populations rank test.

Supplementary Fig. 4). For samples analysed at the ICR, 18/56 samples (32%) were CTC−, 11/56 samples (20%) were CTC+/AR-V7−, and 27/56 samples (48%) were CTC+/AR-V7+ when using a binary assay readout (Supplementary Fig. 4). Notably, analyses at JHU confirmed negativity in all 11 (100%) CTC+/AR-V7− samples deemed negative at the ICR, but only 15/27 (56%) CTC+/AR-V7+ of ICR-positive samples were deemed positive. However, as with the intralaboratory comparisons, samples with higher AR-V7 mRNA expression (>median; >7.16 copies/ml) had high agreement (12/13; 92%) and samples with lower AR-V7 mRNA expression (≤median; 7.16 copies/ml) had low agreement (3/14; 21%) between sites (Supplementary Fig. 4). Finally, CTC+/AR-FL+ cases were more common at the ICR (37/38; 97%) than at JHU (33/46; 72%). These data confirm that although the AdnaTest is comparable between independent international laboratories when all samples are considered, there is some variability for this assay at lower AR-V7 mRNA expression.

3.4. *CTC AR-V7 positivity is associated with higher CellSearch CTC enumeration*

Having demonstrated that CTC+/AR-V7+ patients have more advanced disease, we next investigated the association between CTC AR-V7 status and contemporaneous CellSearch CTC enumeration in 202 samples from 136 mCRPC patients (CTC cohort; Fig. 2A and Supplementary Fig. 1). The median (interquartile range [IQR]) number of days between contemporaneous samples was 0 (0.0–0.0); 174 samples were taken on the same day and 28 samples within 1 month. The CellSearch CTC count (median; IQR) was significantly lower ($p < 0.001$) in CTC− (4; 0–23) compared with CTC+ (26; 5–99) samples by AdnaTest (Fig. 2B and Supplementary Fig. 5). Furthermore, samples that were CTC+/AR-V7− had significantly lower ($p < 0.001$) CTC counts (9; 2–64) than those that were CTC+/AR-V7+ (60; 19–184; Fig. 2C). Finally, there was a statistically significant

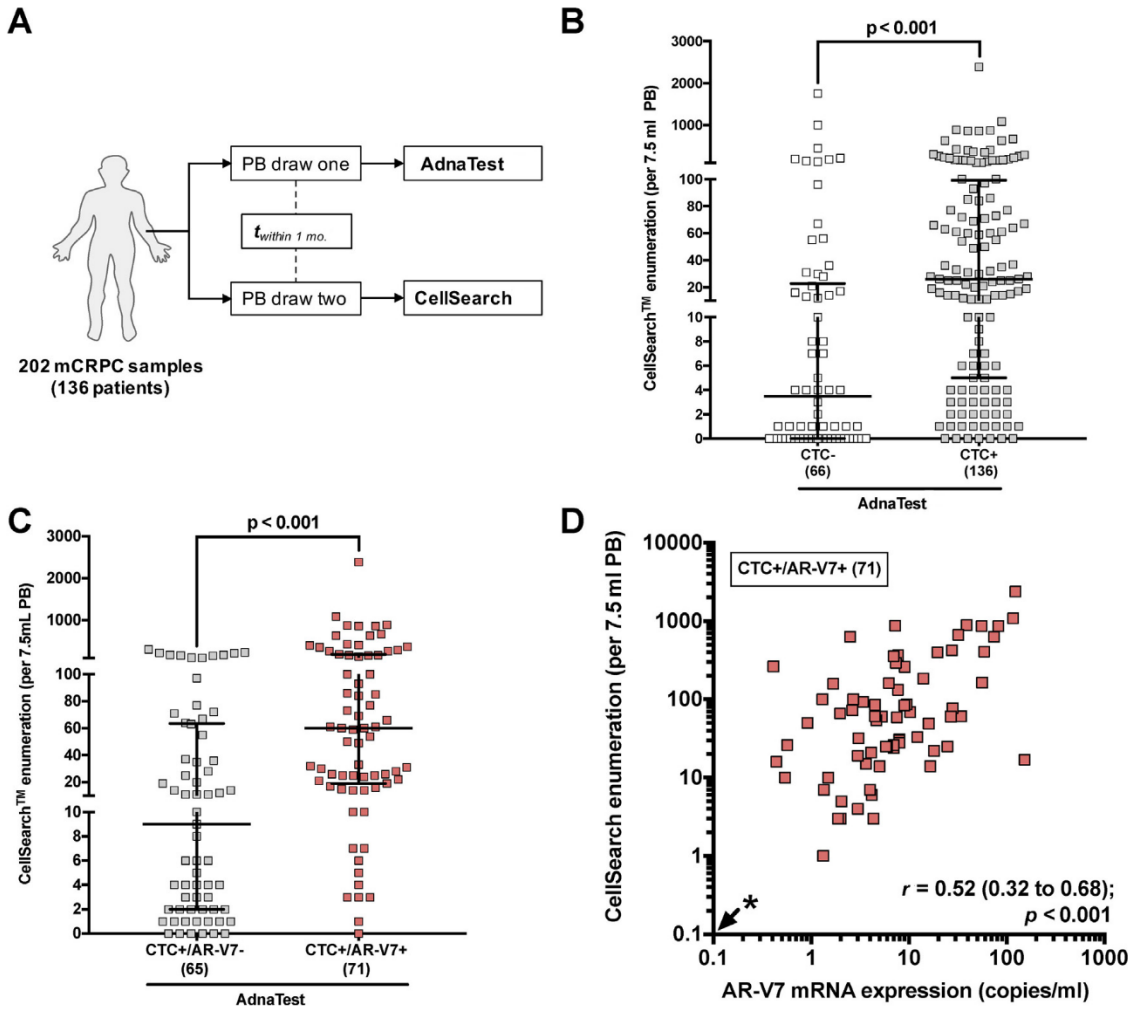


Fig. 2 – CTC AR-V7 positivity is associated with CellSearch CTC enumeration in mCRPC. (A) A total of 202 peripheral blood (PB) draws from 136 mCRPC patients for AdnaTest analysis with contemporaneous (all within a month) PB draws for CellSearch CTC enumeration. (B) CellSearch enumeration for contemporaneous samples with (grey boxes) and without (white boxes) CTCs detected by AdnaTest are shown. Median CTCs/7.5 ml PB and interquartile range are shown. The *p* value was calculated using Mann-Whitney test. (C) For 136 AdnaTest CTC-positive samples (B, grey boxes), CellSearch counts for contemporaneous AR-V7– positive (red boxes) and AR-V7– negative (grey boxes) samples detected by AdnaTest are shown. Median (and interquartile range) CTCs/7.5 ml in PB are shown. The *p* value was calculated using the Mann-Whitney test. (D) For 71 AdnaTest CTC AR-V7– positive samples (C, red boxes), CellSearch enumeration is compared with continuous (mean of technical replicates) AR-V7 mRNA expression (copies/ml). Spearman's rank correlation is shown. * One sample had no CTC by CellSearch enumeration and was not plotted. AR-V7 = androgen receptor splice variant-7; CTC = circulating tumour cell; mCRPC = metastatic castration-resistant prostate cancer.

correlation between mean CTC AR-V7 mRNA expression (copies/ml) and CellSearch CTC count in CTC+/AR-V7+ patients ($r = 0.52$ [95% confidence interval {CI} 0.32–0.68]; $p < 0.001$; Fig. 2D). These data demonstrate the limitation of CTC detection by AdnaTest. Furthermore, AdnaTest CTC+/AR-V7+ status is associated with higher CellSearch CTC counts.

3.5. *Differences are observed between CTC AR-V7 status and matched tumour biopsy AR-V7 protein expression in patients with mCRPC*

Next, we determined AR-V7 status in 65 contemporaneous, same-patient PB samples and metastatic biopsies from 58 mCRPC patients (IHC cohort; Fig. 3A and Supplementary Fig. 1) to determine whether the CTC AR-V7 AdnaTest gave a precise estimate of tumour AR-V7 protein expression. The median (IQR) number of days between contemporaneous samples was 5 (0–9). H-scores (HSs) were determined by IHC for nuclear AR-V7 and AR-FL (Supplementary Fig. 6). Nuclear AR-V7, but not AR-FL, protein expression (median HS; IQR) was significantly ($p = 0.004$) higher for CTC+/AR-V7+ (100; 63–148; $n = 28$) patients than for CTC+/AR-V7– (15; 0–113; $n = 21$) patients (Fig. 3B and C). However, "false positive" (2/28; 7%) and "false negative" (13/21; 62%) blood results were identified when comparing CTC mRNA and tissue protein analyses (Fig. 3B and D). In addition, 63% (10/16) AdnaTest CTC– patients had detectable AR-V7 protein expression in their matched mCRPC biopsy (Fig. 3B and 3D). All mCRPC biopsies were positive for AR-FL protein expression and all CTC+ samples expressed AR-FL mRNA (Fig. 3C and 3D). Finally, for mCRPC biopsies with next-generation sequencing available, AR amplification was more common in CTC+/AR-V7+ (19/26; 73%) patients compared

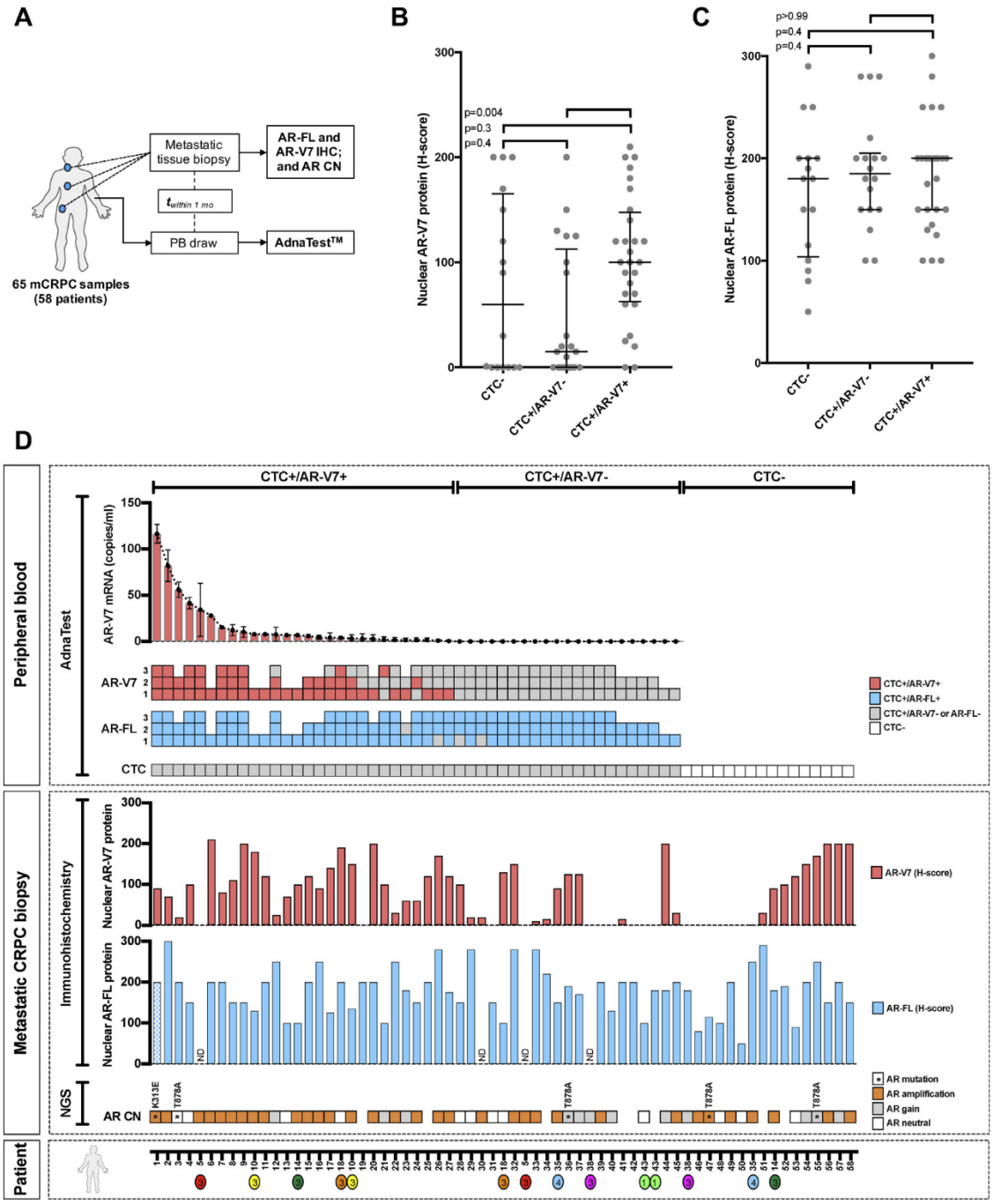


Fig. 3 – Nuclear AR-V7 protein expression and AR-V7 mRNA expression in contemporaneous mCRPC tissue and liquid biopsies. (A) Sixty-five peripheral blood (PB) draws for AdnaTest analysis with contemporaneous (all within a month) metastatic tissue biopsy for nuclear AR-V7 and AR-FL IHC from 58 mCRPC patients were identified. (B) Median nuclear AR-V7 H-score (and interquartile range) is shown for CTC−, CTC+/AR-V7−, and CTC+/AR-V7+ patients. The *p* values were calculated using the Mann-Whitney test. (C) Median nuclear AR-FL H-score (and interquartile range) is shown for CTC−, CTC+/AR-V7−, and CTC+/AR-V7+ patients. The *p* values were calculated using the Mann-Whitney test. AR-FL IHC was not performed on four biopsies. (D; top panel; peripheral blood) For AdnaTest analysis, continuous (mean ± SD) AR-V7 mRNA expression (copies/ml; red bars) from technical replicates is shown. Technical replicates for binary AR-V7 (red boxes positive; grey boxes negative) and AR-FL (blue boxes positive; grey boxes negative) mRNA status from liquid biopsies with CTCs detected (grey boxes) are shown. Each box represents a technical replicate (numbered 1–3). (D; bottom panel; mCRPC biopsy) For mCRPC tissue biopsies, AR-V7 protein expression (H-score; red bars) and AR-FL protein expression (H-score; blue bars) are shown. AR-FL protein expression (H-score; blue/white hatched bar) in a single patient was determined using alternative AR-N terminus antibody as the presence of AR^{K313E} mutation (binding epitope of study antibody). AR-FL IHC was not performed on four biopsies (ND). AR copy number and mutational status were determined by next-generation sequencing (NGS). AR neutral (white box), AR gain (grey box), AR amplified (orange box), and AR mutant (*) cases are shown. Seven patients with duplicate samples (coloured dots) and months between samples is indicated. AR = androgen receptor; AR-V7 = androgen receptor splice variant-7; CTC = circulating tumour cell; IHC = immunohistochemistry; mCRPC = metastatic castration-resistant prostate cancer; SD = standard deviation.

with CTC+/AR-V7− (7/17; 41%) and CTC− (7/14; 50%) patients (chi-square $p = 0.09$; Fig. 3D). Overall, these data demonstrate that CTC AR-V7 positivity is associated with higher AR-V7 protein expression in contemporaneous mCRPC tissue biopsies, although both false positives and negatives were identified.

3.6. CTC AR-V7 status identifies mCRPC patients with more advanced disease and therefore poorer prognosis

Having demonstrated CTC AR-V7 positivity to be associated with more advanced disease, we next determined whether this impacted survival analyses in an unselected cohort of 162 mCRPC patients (prognostication cohort; Supplementary Fig. 1). Overall, 56/162 (35%) samples were CTC−, 53/162 (33%) CTC+/AR-V7−, and 53/162 (33%) CTC+/AR-V7+. Baseline characteristics for each patient at the time of PB draw demonstrated differences by CTC/AR-V7 status (Supplementary Table 3). The median (95% CI) survival was 12.5 (9.8–14.6) months and the median (IQR) follow-up among patients who did not die was 19 (11–31) months. In univariable analysis, CTC AR-V7 status ($p < 0.001$), CellSearch CTC count ≥5 ($p < 0.001$), higher ECOG PS ($p < 0.001$), receiving more taxane therapies ($p < 0.001$), lower haemoglobin ($p < 0.001$), higher alkaline phosphatase ($p < 0.001$), lower albumin ($p < 0.001$), higher lactate dehydrogenase ($p < 0.001$), and higher PSA ($p < 0.001$) were associated with worse overall survival (OS; Fig. 4A and Table 2). In light of CTC+/AR-V7+ being associated with more advanced disease, we performed a multivariable analysis to adjust for imbalances in baseline characteristics. There remained a significant association with CTC AR-V7 status ($p = 0.02$), CellSearch CTC count ≥5 ($p < 0.001$), ECOG PS ($p = 0.01$), and ALP ($p = 0.05$; Table 2). The bootstrapped (number of replications = 1000) C-index for the multivariable model was 0.789, and the C-index values were 0.777, 0.773, 0.771, and 0.781 for multivariable models with CTC AR-V7 status, CellSearch CTC count, ECOG PS, and ALP removed, respectively. The bootstrapped C-index for the multivariable model without CTC AR-V7 status and CellSearch CTC count was 0.752.

However, differences in OS by CTC AR-V7 status appeared to be due to worse survival in CTC+/AR-V7+ patients compared with CTC− patients (hazard ratio [HR] 2.13; 95% CI 1.23–3.71; $p = 0.02$). There was no evidence of significantly inferior OS in CTC+/AR-V7+ patients compared with CTC+/AR-V7− patients (HR 1.26; 95% CI 0.73–2.17; $p = 0.4$; Fig. 4B). These data suggest that CTC AR-V7 positivity identifies mCRPC patients with more advanced disease and subsequent worse prognosis.

4. Discussion

In this study, we present data on CTC AR-V7 mRNA detection using the AdnaTest alongside contemporaneous CellSearch CTC counts and AR-V7 protein expression from matched mCRPC biopsies. Our analyses indicate three key findings: (1) the agreement of CTC AR-V7 binary reporting is decreased at lower CTC AR-V7 mRNA expression levels; (2) CTC AR-V7 positivity is associated with more advanced disease and higher CTC counts confounding clinical outcome analyses; and (3) overall CTC AR-V7 status is associated with AR-V7 protein expression in matched mCRPC biopsies, but false positive and negative correlations between blood and biopsy results were identified. The most significant limitation of this study was the variable treatments received by this cohort. Therefore, we were unable to evaluate AR-V7 expression as a predictive biomarker of response to treatment [13,14,17,20,27].

Consistent with previous studies utilising CTC AR-V7 assays, we demonstrate that 33% of mCRPC patients are CTC+/AR-V7+ [10,11,13–15,17,20,21,27]. Previous studies utilising the AdnaTest platform have reported data without replicates and as binary outcomes [10,20,27]. We demonstrate through intra- and inter-laboratory analyses that CTC AR-V7 detection is most challenging at low AR-V7 mRNA levels with increasing variability. This is an important finding as studies have shown that some CTC+/AR-V7+

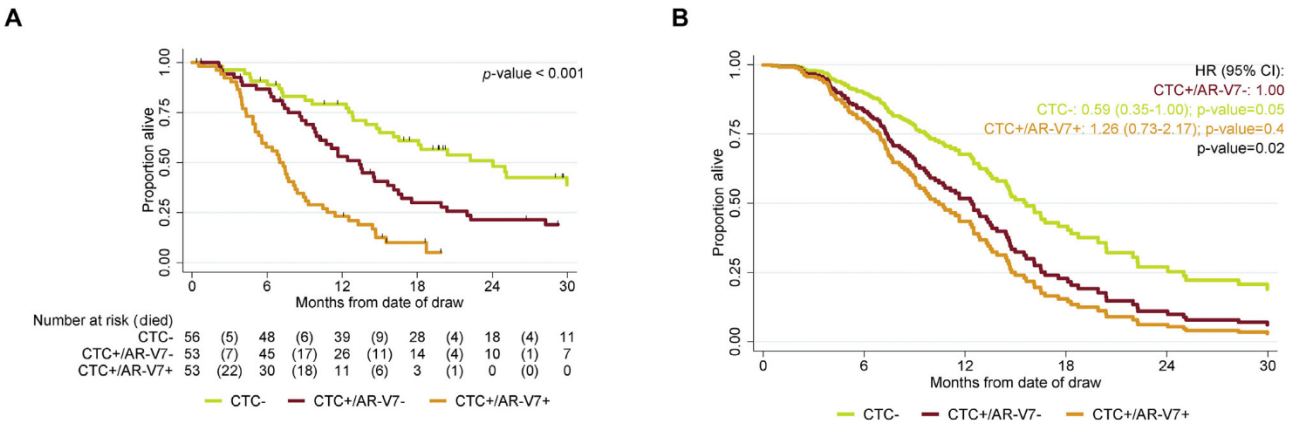


Fig. 4 – Association between CTC AR-V7 and overall survival in mCRPC. A total of 162 mCRPC patients with CTC AR-V7 status were determined. (A) Kaplan-Meier curve shows overall survival (OS) from peripheral blood draw for mCRPC patients divided by CTC−, CTC+/AR-V7−, and CTC+/AR-V7+ status. The *p* value was calculated using the log-rank test. (B) Estimated survivor function for CTC−, CTC+/AR-V7−, and CTC+/AR-V7+ groups with other covariates at their mean from the multivariable Cox model. Multivariable hazard ratios (HRs) with 95% confidence intervals (CIs), and pairwise and global *p* values are shown. AR-V7 = androgen receptor splice variant-7; CTC = circulating tumour cell; IHC = immunohistochemistry; mCRPC = metastatic castration-resistant prostate cancer.

Table 2 – Univariable and multivariable Cox models of overall survival from date of draw (prognostic cohort)

Characteristic	Univariable			Multivariable		
	HR	95% CI	*p* value	HR	95% CI	*p* value
AR-V7						
CTC+/AR-V7−	1.00	–	<0.001	1.00	–	0.02
CTC+/ARV7+	2.62	1.68–4.07	–	1.26	0.73–2.17	–
CTC−	0.59	0.37–0.93	–	0.59	0.35–1.00	–
CellSearch CTC count						
<5	1.00	–	<0.001	1.00	–	<0.001
≥5	5.45	3.23–9.18	–	3.23	1.65–6.31	–
Missing CTC	1.32	0.77–2.28	–	1.19	0.62–2.29	–
ECOG PS at draw						
0	1.00	–	<0.001	1.00	–	0.01
1	2.12	1.16–3.88	–	2.07	0.97–4.42	–
≥2	4.69	2.31–9.54	–	3.42	1.38–8.47	–
NR	121.8	11.82–1254.16	–	26.6	2.03–349.5	–
Metastatic sites						
Lymph node only	0.64	0.34–1.23	0.18	0.17	0.02–1.76	0.14
Visceral	1.39	0.93–2.07	0.11	0.89	0.56–1.44	0.6
Bone	1.54	0.83–2.87	0.17	0.17	0.02–1.69	0.13
AR-targeting therapies	1.37	0.99–1.89	0.06	1.07	0.73–1.56	0.8
Taxane therapies	1.52	1.21–1.91	<0.001	1.02	0.76–1.39	0.9
Age (per 10 yr)	0.88	0.70–1.11	0.28	0.79	0.59–1.06	0.12
Hb (per dg/l)	0.70	0.63–0.79	<0.001	0.96	0.35–2.61	0.9
ALT (log_{10} U/l)	0.58	0.25–1.34	0.20	0.75	0.40–1.41	0.4
ALP (log_{10} U/l)	2.73	1.77–4.21	<0.001	0.94	0.89–1.00	0.05
Albumin (g/l)	0.91	0.87–0.95	<0.001	2.58	0.89–7.44	0.08
LDH (log_{10} U/l)	3.10	1.75–5.48	<0.001	1.33	0.96–1.84	0.08
PSA (log_{10} µg/l)	1.80	1.36–2.37	<0.001	0.79	0.59–1.06	0.12

ALP = alkaline phosphatase; ALT = alkaline transferase; AR = androgen receptor; AR-V7 = androgen receptor splice variant-7; 95% CI = 95% confidence interval; CTC = circulating tumour cell; ECOG PS = European Cooperative Oncology Group performance status; Hb = haemoglobin; HR = hazard ratio; LDH = lactate dehydrogenase; NR = no result; PSA = prostate-specific antigen.

patients respond to AR-targeting therapies, and it remains important to identify whether patients with lower levels of AR-V7 mRNA still benefit from novel endocrine agents [20].

One potential limitation of CTC AR-V7 testing is its dependence on the presence of CTCs. This is important when reporting survival outcomes, as we demonstrate CTC AR-V7 positivity to be highly associated with higher CTC counts. Consistent with this, when baseline characteristics are accounted for, there is surprisingly no difference in OS between CTC+/AR-V7+ and CTC+/AR-V7− patients. This is in contrast with previous studies and may be the consequence of stratification by CTC counts [10–15,17,20]. Therefore, studies reporting on CTC AR-V7 assays and survival outcomes must incorporate CTC quantitation.

A further important consideration is how accurately CTC AR-V7 assays predict AR-V7 protein expression in matched mCRPC tissue biopsies. We demonstrate that patients with CTC+/AR-V7+ tests have significantly higher AR-V7 protein expression in matched mCRPC biopsies than CTC+/AR-V7− patients. However, false positives and false negatives were common, consistent with reported intrapatient tumour sampling variability [19]. In addition, tumour and CTC microenvironments are different and may drive differences in AR-V7 expression. The clinical significance of these observations remains unknown, but it is important to consider whether CTC AR-V7−positive patients with AR-V7−negative biopsies derive clinical benefit from novel endocrine therapies.

Consistent with previous studies, we demonstrate differences in CTC detection between the AdnaTest and CellSearch platforms [28,29]. It is therefore important to consider the different detection methods utilised; once CTCs are enriched using EpCAM (plus HER2 for AdnaTest) antibodies, the presence of CTCs is defined by morphology that is DAPI and cytokeratin protein positive, and CD45 protein negative (CellSearch), or by actin with EGFR and/or PSA and/or PSMA mRNA positivity (AdnaTest) [23,28]. Taken together, the different molecular markers utilised by individual platforms, and the technical challenges of isolating and maintaining mRNA integrity compared with DNA and protein may give rise to the differences seen in CTC enumeration.

A final consideration is whether individual CTC AR-V7 assays are limited by their ability to detect CTCs and therefore AR-V7; technologies that greater sample a patient's disease are likely to demonstrate increased AR-V7 positivity in mCRPC. Consistent with this, recent studies utilising droplet digital PCR of PB and direct metastatic tissue biopsies demonstrated AR-V7 positivity in CRPC to be 67% and 75%, respectively [19,21]. Going forward, this will be important to understand, as current CTC-based assays have reported AR-V7 as an important biomarker for the outcome from endocrine therapies [10–15,17,20,21]. However, this may not be entirely a consequence of AR-V7 positivity, and such assays may provide little information regarding the role of AR-V7 in CRPC biology.

5. Conclusions

Liquid biopsies that identify AR-V7 positivity have the potential to inform treatment decisions in mCRPC. Our study highlights a number of considerations to optimally interpret these assays. Firstly, binary reporting of CTC AR-V7 status has limited concordance at low AR-V7 mRNA expression levels. Secondly, AdnaTest CTC AR-V7 positivity is associated with higher CTC counts, which may confound outcome analyses. Finally, patients who have no detectable CTCs by AdnaTest frequently have CTCs by CellSearch and express AR-V7 protein in matched tumour tissue. Future studies reporting CTC AR-V7 assay results in mCRPC should consider these findings and these limitations, before suggesting clinical utilisation of this assay.

Author contributions: Johann S. de Bono had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study concept and design: Sharp, Welti, Lambros, Dolling, Rodrigues, Pope, Aversa, Figueiredo, Fraser, Ahmad, Lu, Rescigno, Kolinsky, Bertan, Seed, Riisnaes, Miranda, Crespo, Pereira, Ferreira, Fowler, Ebbs, Flohr, Neeb, Bianchini, Petremolo, Sumanasuriya, Paschalis, Mateo, Tunariu, Yuan, Carreira, Plymate, Luo, de Bono.
Acquisition of data: Sharp, Welti, Lambros, Dolling, Rodrigues, Pope, Aversa, Figueiredo, Fraser, Ahmad, Lu, Rescigno, Kolinsky, Bertan, Seed, Riisnaes, Miranda, Crespo, Pereira, Ferreira, Fowler, Ebbs, Flohr, Neeb, Bianchini, Petremolo, Yuan, Carreira.
Analysis and interpretation of data: Sharp, Welti, Lambros, Dolling, Rodrigues, Pope, Aversa, Figueiredo, Fraser, Ahmad, Lu, Rescigno, Kolinsky, Bertan, Seed, Riisnaes, Miranda, Crespo, Pereira, Ferreira, Fowler, Ebbs, Flohr, Neeb, Bianchini, Petremolo, Sumanasuriya, Paschalis, Mateo, Tunariu, Yuan, Carreira, Plymate, Luo, de Bono.
Drafting of the manuscript: Sharp, Welti, Lambros, Dolling, Plymate, Luo, de Bono.
Critical revision of the manuscript for important intellectual content: Sharp, Welti, Lambros, Dolling, Rodrigues, Pope, Aversa, Figueiredo, Fraser, Ahmad, Lu, Rescigno, Kolinsky, Bertan, Seed, Riisnaes, Miranda, Crespo, Pereira, Ferreira, Fowler, Ebbs, Flohr, Neeb, Bianchini, Petremolo, Sumanasuriya, Paschalis, Mateo, Tunariu, Yuan, Carreira, Plymate, Luo, de Bono.
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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.eururo.2019.04.006.

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Prostate Cancer

Androgen Receptor Modulation Optimized for Response—Splice Variant: A Phase 3, Randomized Trial of Galeterone Versus Enzalutamide in Androgen Receptor Splice Variant-7–expressing Metastatic Castration-resistant Prostate Cancer

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Abstract

Background: : Detection of androgen receptor (AR) splice variant-7 (AR-V7) messenger RNA (mRNA) in circulating tumor cells (CTCs) is associated with a suboptimal response to abiraterone and enzalutamide in metastatic castration-resistant prostate cancer (mCRPC). Galeterone inhibits CYP17 and AR, and induces AR protein degradation. We hypothesized that galeterone would be clinically superior to enzalutamide in AR-V7–positive (AR-V7+) mCRPC.
Objective: : To screen and characterize AR-V7+ mCRPC, and evaluate galeterone compared with enzalutamide.
Design, setting, and participants: : This was a multicenter randomized phase 3 trial; enzalutamide-, abiraterone-, and chemotherapy-naïve mCRPC patients had AR-V7 prescreening using a CTC-based mRNA assay.
Intervention: : AR-V7+ patients were randomized (1:1) to open-label galeterone or enzalutamide; planned sample size was 148.
Outcome measurements and statistical analysis: : The primary endpoint was radiographic progression-free survival (rPFS). Baseline AR-V7 status was correlated with patient characteristics.
Results and limitations: : Overall, 953 men were prescreened for AR-V7; 323 (34%) had detectable CTCs, and 73/323 had AR-V7 mRNA. The AR-V7+ prevalence was 8% (73/953; 95% confidence interval [CI] 6–10%). AR-V7 was associated with indicators of advanced and high-volume disease at baseline, including higher prostate-specific antigen (PSA) level ($p < 0.001$), more bone metastases ($p < 0.001$), docetaxel for hormone-sensitive disease ($p < 0.001$), prior first-generation androgen deprivation therapy ($p < 0.001$), and shorter time from diagnosis to enrollment ($p < 0.001$). Of 73 eligible patients, 38 were randomized to galeterone ($n = 19$) or enzalutamide ($n = 19$); 35 dropped out before randomization. Owing to high censorship for the rPFS events, the data monitoring committee recommended early closure based on interim evidence that the primary endpoint would not be met. The PSA50 values were 2/16 (13%) and 8/19 (42%) for galeterone and enzalutamide respectively (proportion difference = −0.278, 95% CI −0.490 to 0.097).
Conclusions: : The prevalence of CTC mRNA AR-V7 in first-line mCRPC was 8% (95% CI 6–10%). AR-V7+ was associated with the characteristics of aggressive and advanced disease. These men had rapid disease progression. Development of galeterone will not be pursued.
Patient summary: : Of men with metastatic castration-resistant prostate cancer, 8% had the androgen receptor splice variant-7 (AR-V7) blood biomarker. The AR-V7+ patients had features of aggressive disease. Thirty-eight men were treated with either galeterone or enzalutamide; the trial was stopped early prior to determining efficacy because too many patients transitioned off the trial due to advancing cancer before having required radiographs.
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1. Introduction

Lethal prostate cancer evolves from hormone-sensitive to metastatic castration-resistant prostate cancer (mCRPC) [1]. The androgen receptor (AR) is central to tumor growth, and androgen deprivation therapy (ADT) is the primary therapy [2]. The AR pathway remains a driver of tumor growth in most mCRPC patients and thus remains a valid therapeutic target. Tumor addiction to AR together with selective pressure from ADT leads to AR pathway alterations including AR gene amplification, overexpression, mutation, splice variants, and increases in adrenal and intratumoral androgens [3]. Each of these alterations is a possible therapeutic target, and the challenge remains designing and aligning therapy to match tumor vulnerabilities.

Second-generation ADT is currently in use for both hormone-sensitive prostate cancer and mCRPC [4]. Abiraterone acetate (hereafter abiraterone) is a potent inhibitor of cytochrome P450 (CYP)17A1 that suppresses extragonadal androgen synthesis; enzalutamide is a next-generation AR antagonist. Abiraterone and enzalutamide have improved survival in mCRPC patients [5–8]. In the prechemotherapy setting, approximately 70–80% of patients respond favorably to abiraterone; however, the response rate and duration of response is much lower in second line [9–11]. An AR-directed therapy effective in the context of primary or secondary resistance to abiraterone/enzalutamide would greatly benefit patients.

Constitutive activation of AR from AR variants with ligand-binding domain truncation is implicated in AR-targeted therapy resistance [12]. The AR isoform encoded by splice variant 7 (AR-V7) lacks the ligand-binding domain; yet, it is constitutively active. AR-V7 can be detected in circulating tumor cells (CTCs) in some cases of mCRPC [13] by quantitative reverse-transcriptase polymerase chain reaction (RT-PCR). Detection of AR-V7 in CTCs (distinguished from tumor detection) has been associated with resistance to enzalutamide and abiraterone in mCRPC [13]. An expanded analysis confirmed that AR-V7 detection was prognostic of poor outcomes [14,15]. Patients with AR-V7 can respond to taxanes [16,17] or theoretically to AR-directed therapy not dependent on AR ligand-binding domain; further analysis is needed to understand the role of AR-V7 in mCRPC.

Galeterone has been reported to inhibit AR signaling through multiple mechanisms: CYP17 inhibition, competitive antagonism of AR, and induction of AR and AR-V7 protein degradation [18]. In phase 1/2 trials, galeterone was tolerable at 2550 mg/d and resulted in reduction in serum androgens and prostate-specific antigen (PSA), as would be expected from CYP17 inhibition even in AR-V7–positive (henceforth AR-V7+) patients [19]. The Androgen Receptor Modulation Optimized for Response—Splice Variant (ARMOR3-SV) program, an open-label, multicenter, phase 3 trial designed to test the hypothesis that galeterone is superior to enzalutamide in CTC AR-V7+ mCRPC, included large-scale screening for CTC-transcript AR-V7 and codevelopment of the AR-V7 assay as a companion diagnostic with galeterone.

2. Patients and methods

2.1. Study population

Informed consent was obtained for AR-V7 prescreening; study consent was subsequently obtained for AR-V7+ patients. Participants had biopsy-confirmed prostatic adenocarcinoma, maintained primary castration, and had asymptomatic or mildly symptomatic progressive mCRPC according to the Prostate Cancer Working Group 2 (PCWG2) [20]. They were Eastern Cooperative Oncology Group (ECOG) $\leq$1 and naïve to abiraterone, enzalutamide, chemotherapy, and investigational agents; docetaxel for hormone-sensitive cancer was allowed. Eligible AR-V7+ patients were randomized (1:1, nonblinded) to daily oral galeterone (2550 mg) or enzalutamide (160 mg). Patients continued study therapy until confirmed radiological progression per Response Evaluation Criteria in Solid Tumors (RECIST 1.1), PCWG2 unequivocal clinical progression, or consent withdrawal. All aspects of the clinical trial and AR-V7 prescreening were funded by Tokai Pharmaceuticals.

2.2. AR-V7 assay

The CTC-based AR-V7 clinical trial assay utilized modification of the methods reported by Antonarakis et al [13]. The ARMOR3-SV program included the codevelopment of an AR-V7 assay as a companion diagnostic.

2.3. Efficacy assessments

The primary objective was to compare radiographic progression between arms, based on assessments by central, blinded, and independent radiologists. Computer tomography (CT) scans and bone scans were performed every 8 wk. Determinations of response or progression were made by two independent radiologists. Radiographic progression-free survival (rPFS) was measured from randomization and based on the occurrence of one of the following: radiographic progression (PCWG2) [20] for bone disease and/or RECIST 1.1 for soft tissue/visceral disease, or death from any cause. Secondary endpoints included time on treatment, PSA50 ($\geq$50% decrease within the first 8 wk) or PSA30 response (PCWG2), time to ECOG deterioration by $\geq$1 point, best overall soft tissue response (RECIST 1.1), time to initiation of next prostate cancer therapy, and galeterone pharmacokinetics (PK).

2.4. Safety assessments

The frequency and intensity of adverse events, and changes in hematologic and chemistry parameters were recorded and compared in cohorts.

2.5. Statistical analysis

Eligibility for randomization required patients to be CTC positive and AR-V7+; AR-V7 status could be ascertained

only in CTC-positive patients. The relationship between baseline characteristics and CTCs (regardless of AR-V7 status), and AR-V7 detection are characterized using descriptive statistics. The p values test the homogeneity of prevalence across the subsets defined by the factor being analyzed. All p values below 0.10 are considered consistent with evidence of differences across subsets.

The primary endpoint was rPFS. The primary statistical analysis was specified as a log-rank test comparing arms (intention to treat). It was hypothesized that the estimated median rPFS for enzalutamide would be approximately 4 mo, while the median rPFS for the galeterone cohort would be 82% longer than that of the enzalutamide cohort (corresponding, under exponential assumptions, to a hazard ratio for rPFS of $\leq$0.55). A one-sided type I error probability of 0.025 and a target power of 90% required 120 rPFS events among 148 total patients. It was anticipated that 2000 patients would be prescreened, based on the assumptions of percentages of patients who would have both CTCs and AR-V7+, to enroll 148 patients [15].

The trial was monitored by an independent data monitoring committee (DMC) governed by a charter based on regulatory guidance for DMCs. The rate of censored events (patient coming off study for reasons other than rPFS) was monitored in a pooled manner by the sponsor because of concerns that clinical events could drive therapy change, resulting in a reduction in the required events.

3. Results

3.1. AR-V7 prescreening

The trial was conducted at 120 sites in nine countries. From December 2015 to July 2016, 953 patients were prescreened; CTCs were detected in 323 (34%, 95% confidence interval [CI] 31–37%) and AR-V7 was detected in 73 of these 323 (23%, 95% CI 18–27%; Fig. 1). Among all the 953 patients who were screened, 73 (8%, 95% CI 6–10%) tested positive for CTC-transcript AR-V7. This detection rate is consistent with an estimated rate of 8–10% for AR-V7+ in mCRPC patients naïve to abiraterone and enzalutamide [15].

AR-V7 status could be ascertained only in patients with CTCs; thus, the following baseline factors were considered for prevalence estimates: Gleason score, years from diagnosis, screening PSA, ECOG performance status (PS), metastases at diagnosis, prior docetaxel (for hormone-sensitive disease), prior ADT (eg, bicalutamide), and number of bone lesions. Figs. 2 and 3 show the prevalence estimates (status probability estimates) defined by these factors for all patients screened for CTCs and AR-V7, respectively. Fig. 4 shows the prevalence of AR-V7 for CTC-positive patients.

CTC detection (Fig. 2) was correlated with Gleason score (31% in Gleason 7 vs 64% in Gleason 10; $p = 0.006$), years from diagnosis (28% in >4.2 yr vs 45% in <4.2 yr; $p < 0.001$, 4.2 yr being median), prior docetaxel for hormone-sensitive disease (32% in docetaxel nonrecipients vs 49% in docetaxel recipients; $p = 0.001$), metastatic disease at diagnosis (30% without vs 41% with metastases at diagnosis; $p = 0.001$), number of bone metastases (21% without bone metastases vs 66% with >21 metastases; $p < 0.001$), ECOG PS (29% in ECOG = 0 vs 43% in ECOG $\geq$ 1; $p < 0.001$), and screening PSA (21% with PSA < 15.5 ng/ml vs 47% with PSA > 15.5 ng/ml; $p < 0.001$). AR-V7 detection in total screened population (Fig. 3) correlated with years from diagnosis (5% in >4.2 yr vs 13% in <4.2 yr; $p < 0.001$), prior docetaxel for hormone-sensitive disease (6% docetaxel nonrecipients vs 22% docetaxel recipients; $p < 0.001$), prior first-generation ADT (4% ADT nonrecipients vs 11% ADT recipients; $p < 0.001$), presence of metastases at diagnosis (6% without vs 10% with metastases; $p = 0.046$), number of bone metastases (4% without any bone metastases vs 30% with >21 bone metastases; $p < 0.001$), ECOG PS (6% ECOG = 0 vs 11% ECOG $\geq$ 1; $p = 0.023$), and screening PSA (3% with PSA < 15.5 ng/m vs 13% with PSA > 15.5 ng/m; $p < 0.001$). Similar to the total screened population, the majority of these factors were associated with AR-V7+ in the CTC-positive cohort (Fig. 4).

3.2. Patients and treatment

Overall, 38 patients were randomized to galeterone (19) or enzalutamide (19); the remaining AR-V7+ patients ($n = 35$) dropped out before randomization due to rapid disease progression or screen failure. Patient characteristics are shown in Table 1.

After 38 patients enrolled, 24 had sufficient data for DMC meeting. After analysis, the DMC recommended trial termination because the trial was unlikely to meet its primary endpoint of rPFS due to rapid progression resulting in a censoring rate of 58%. Twelve patients had come off therapy and seven discontinued without a qualified rPFS event. Most patients coming off the study rapidly transitioned to chemotherapy or palliative care due to unequivocal clinical progression. Five patients had qualified events that could be included in endpoint analysis (one death and four rPFS).

Formal efficacy analysis was not possible due to premature termination of trial and data collection. Of the 38 randomized patients, 23 had CT/magnetic resonance imaging scans reviewed by RECIST 1.1 (11 enzalutamide and 12 galeterone). Three of 23 (13%) patients had declared progression during treatment (all galeterone). Eleven patients had bone scans evaluated centrally, and seven men (six enzalutamide and one galeterone) had a bone scan outside the flare window (16 wk after C1D1); progressive disease was confirmed in two of seven patients (one enzalutamide and one galeterone).

Of the 38 randomized patients, 35 were evaluable for PSA response at 8 wk or the end of treatment whichever came first. PSA50 response was 8/19 (42%) on enzalutamide and 2/16 (13%) on galeterone (proportion difference = −0.278, 95% CI −0.490 to 0.097). PSA response was not confirmed due to study termination. PSA progression according to PCWG1 (not PCWG2 that requires confirmation) is illustrated in Supplementary Fig. 1.

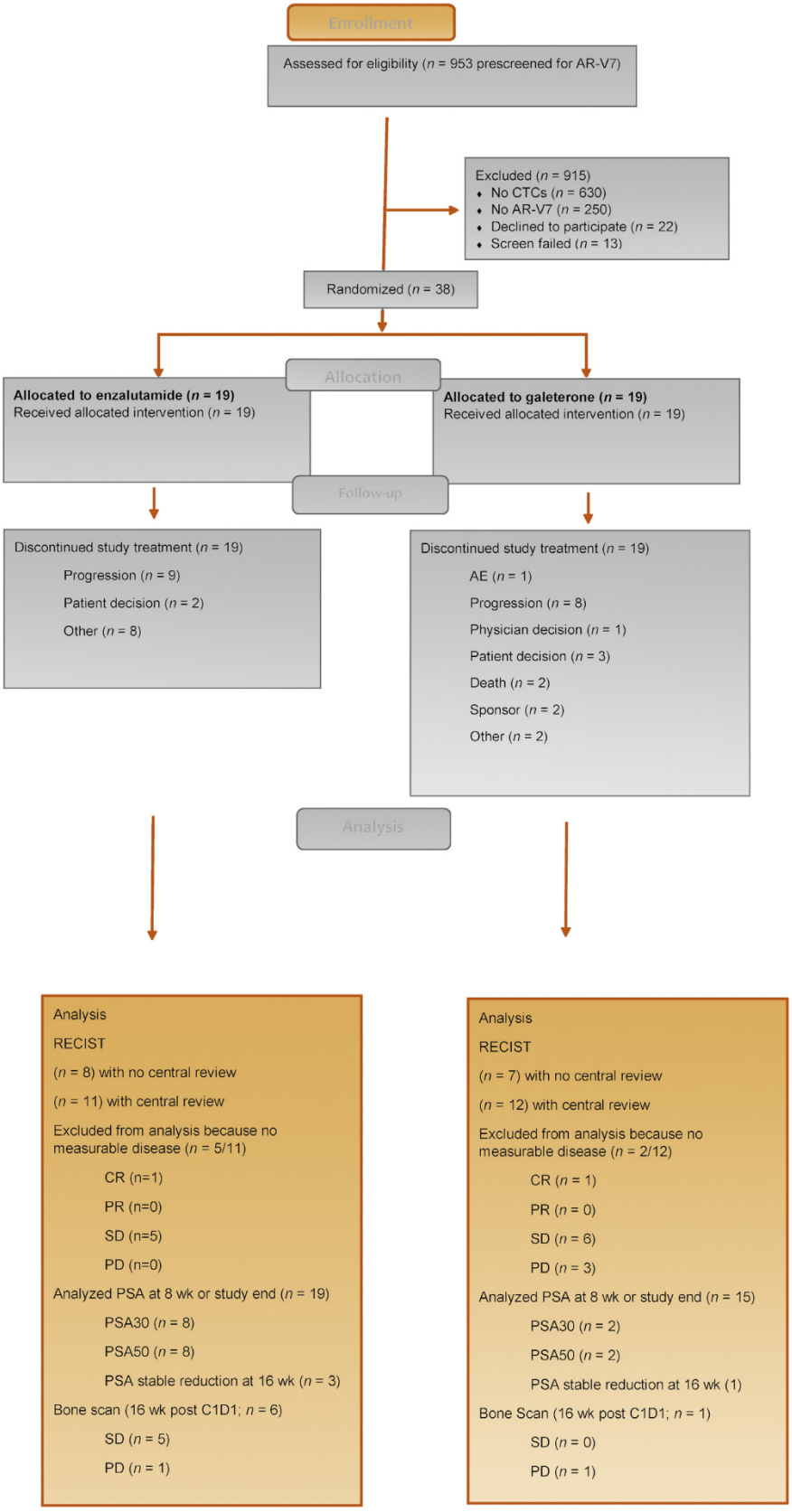


Fig. 1 – CONSORT diagram of patients prescreened for AR-V7 demonstrating those with detectable CTCs and AR-V7. In addition to detectable CTCs, full-length AR was required to detect AR-V7. A total of 38 AR-V7+ patients were randomized to receive either enzalutamide (19) or galeterone (19). AE = adverse event; AR = androgen receptor; AR-V7 = androgen receptor splice variant-7; CR = complete response; CTC = circulating tumor cell; PD = progressive disease; PR = partial response; PSA = prostate-specific antigen; RECIST = Response Evaluation Criteria in Solid Tumors; SD = stable disease.

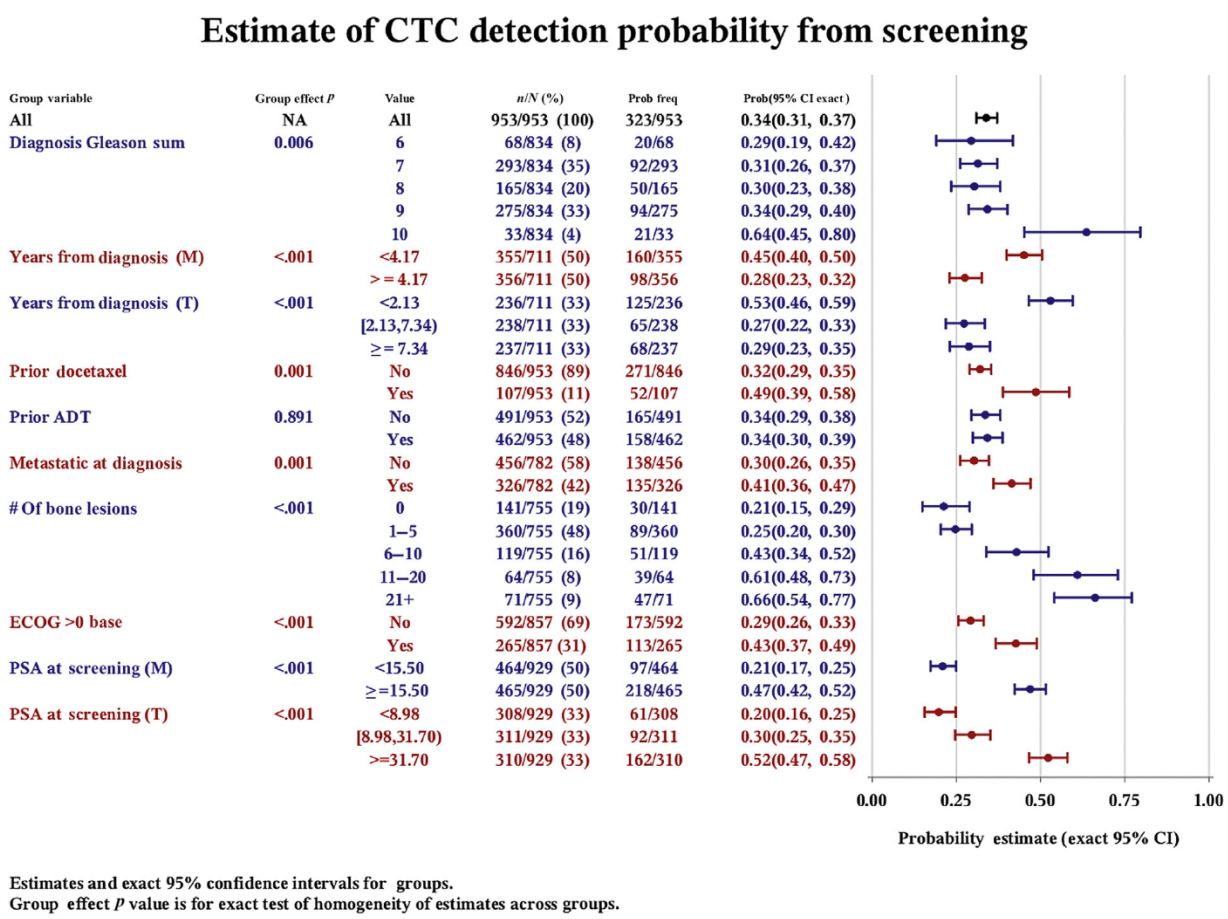


Fig. 2 – Baseline patient characteristics and the probability of circulating tumor cell (CTC) detection ($n = 953$). The p values test the homogeneity of prevalence across the subsets defined by the factor being analyzed. Small p values (below 0.10) are considered consistent with evidence of differences across subsets. ADT = first-generation androgen deprivation therapy (bicalutamide); CI = confidence interval; ECOG = Eastern Cooperative Oncology Group; Freq = frequency; M = median; NA = not available; Prob = probability; PSA = prostate-specific antigen; T = tertile.

Treatment emergent adverse events (TEAEs) for galeterone included fatigue (46%), anorexia (31%), asthenia (31%), nausea (31%), anemia (26%), anxiety (21%), constipation (16%), dizziness (16%), insomnia (16%), and rash (16%). All other TEAEs were reported in <15%. There were four cases of serious adverse reactions: two anemia, one fatigue, and one hepatic failure (possibly related in context of rapidly progressive disease).

PK evaluation was completed on 13 galeterone-treated patients; 10 achieved steady-state levels with galeterone 2550 mg daily (three with dose reductions precluding assessment). The mean Cmax ($n = 10$) was 2.73 μM, consistent with steady-state Cmax estimates of 2.5 μM. A steady-state plasma level of >2 μM was predicted to translate to >10 μM in tumor, a concentration required for AR degradation in vitro [18].

4. Discussion

There remains an urgent need for more durable therapies in mCRPC and for biomarkers to direct the most impactful therapy to appropriate patients. In 2014 when ARMOR3-SV was designed, AR-V7 had been reported as a putative biomarker predicting resistance to abiraterone and enzalutamide. The activity of abiraterone and enzalutamide depends on an intact AR ligand-binding domain; galeterone has AR degrading properties mediated via the proteasome that can impact both AR-FL and AR-Vs [18]. The role of AR-Vs as drivers of tumor growth opposed to a predictive biomarker without a driver role remains unclear [21]; the rationale supporting the efficacy of galeterone in AR-V7+ CRPC remains in either case.

The abrupt early termination of ARMOR3-SV after 38 patients had been enrolled was due to censoring of seven of 12 patients (58%) without confirmation of the primary endpoint (rPFS). Most of these patients had rapid cancer-related deterioration, and treating physicians appropriately determined that they urgently needed non-protocol interventions. When the trial was designed, there were no data on the clinical profiles of CTC + AR-V7+ patients naïve to abiraterone or enzalutamide; thus, the aggressive disease of the targeted population was not anticipated. Radiographic PFS, although a valid endpoint in mCRPC, is not achievable in this select high-risk population,

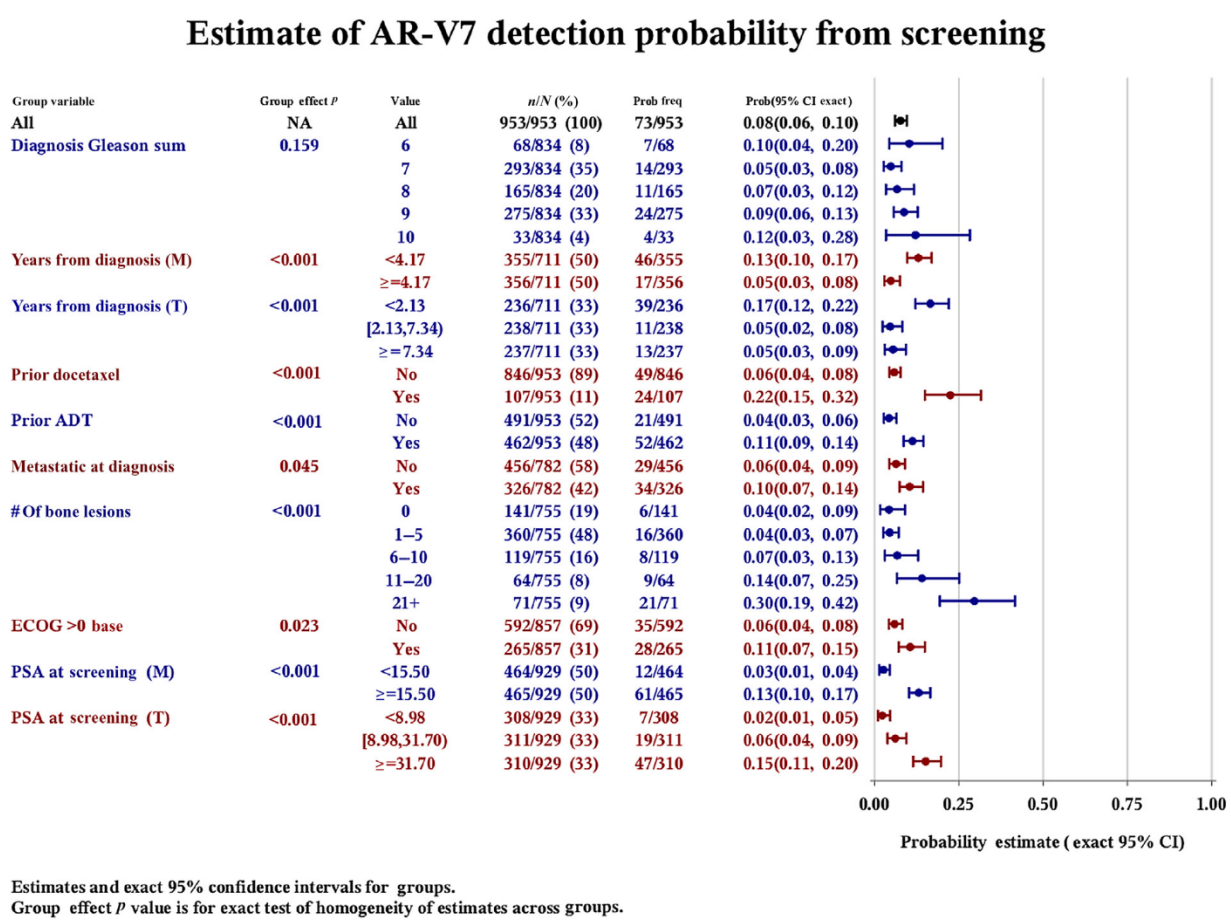


Fig. 3 – Baseline patient characteristics and the probability of AR-V7 detection for the total cohort screened (*n* = 953). AR-V7 positivity required both detectable CTCs and full-length AR. The *p* values test the homogeneity of prevalence across the subsets defined by the factor being analyzed. Small *p* values (below 0.10) are considered consistent with evidence of differences across subsets. ADT = first-generation androgen deprivation therapy (bicalutamide); AR-V7 = androgen receptor splice variant-7; CI = confidence interval; CTC = circulating tumor cell; ECOG = Eastern Cooperative Oncology Group; Freq = frequency; M = median; NA = not available; Prob = probability; PSA = prostate-specific antigen; T = tertile.

and this experience warrants caution for future biomarker-directed trials.

This ARMOR3-SV screening experience is the largest global, multicenter cohort evaluating CTC-transcript AR-V7 and provides valuable insights. A total of 953 patients were prescreened with CTC-transcript RT-PCR assay. As expected, the incidence of AR-V7+ in pre-abiraterone/enzalutamide patients was low (8%) and consistent with a recent report [15]. Other assays including a CTC-based immunohistochemical assay (Epic Sciences) were considered [17], and the RT-PCR assay was chosen because it had the largest published data and could be scaled up to a large international cohort. Recent data demonstrate that the PCR-based AR-V7 assay correlates with CTC counts, AR-V7+ correlates with other poor-prognosis biomarkers, and prognostication related to AR-V7+ is due in part to higher CTC counts [25,26].

CTC AR-V7 messenger RNA (mRNA) positivity (Fig. 3 and 4) was associated with features of biologically aggressive disease: shorter time from diagnosis to study entry, increased bone lesions, higher PSA, metastases at diagnosis, prior docetaxel, and ADT. CTC enumeration was not done; thus, an analysis of AR-V7+ as a reflection of CTC burden is not possible. Consistent with the biological AR-V hypothesis, the prevalence of AR-V7 (but not CTC positivity) increased with prior ADT (bicalutamide) exposure (Fig. 2–4) due to AR-V7 induction by prior therapy targeting the AR ligand-binding domain [22,23]. What was less expected was the positive correlation between higher AR-V7 and exposure to docetaxel for hormone-sensitive disease. Previous studies in mCRPC suggested a reduction in AR-V7 upon docetaxel treatment [16]; the current findings imply that men selected to receive docetaxel for hormone-sensitive disease may have been skewed toward more aggressive features or a higher disease burden, resulting in a higher AR-V7 prevalence especially at the time of disease progression. However, even in the context of aggressive features, AR-V7 was detected in the minority in any subset. For example, AR-V7+ prevalence was 15% with PSA > 31 ng/ml and 30% with >21 bone lesions. The AR-V7 detection rate for men with PSA between 9 and 32 ng/ml was only 6%. These metrics should be considered when considering the cost and clinical utility of AR-V7 testing in early mCRPC.

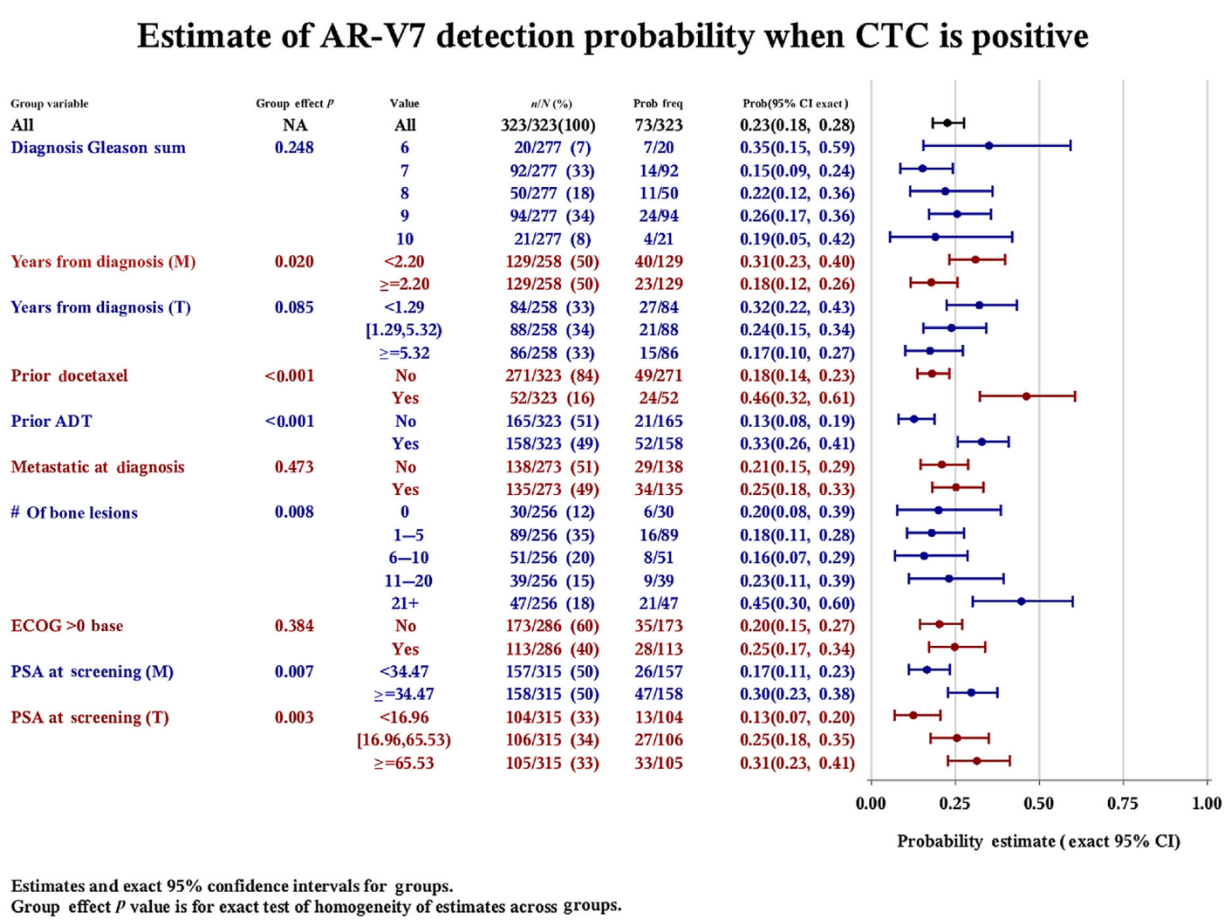


Fig. 4 – Baseline patient characteristics and the probably of AR-V7 detection for the cohort of CTC-positive patients (n = 323). The p values test the homogeneity of prevalence across the subsets defined by the factor being analyzed. Small p values (below 0.10), are considered consistent with evidence of differences across subsets. ADT = first-generation androgen deprivation therapy (bicalutamide); AR-V7 = androgen receptor splice variant-7; CI = confidence interval; CTC = circulating tumor cell; ECOG = Eastern Cooperative Oncology Group; Freq = frequency; M = median; NA = not available; Prob = probability; PSA = prostate-specific antigen; T = tertile.

Table 1 – Characteristics of patients assigned to enzalutamide or galeterone treatment.

Arm		Enzalutamide	Galeterone
Number randomized		19	19
ECOG = 1 (%)		57.9	52.6
Percent having number of bone scan lesions	0	6.7	12.5
	1–5	20.0	43.8
	6–10	20.0	0.0
	11–20	33.3	6.3
	21+	20.0	37.5
Age (SD)	Median (Q1, Q3)	72.0 (62.0, 77.0)	72.0 (62.0, 77.0)
PSA	Median (Q1, Q3)	50.82 (12.830, 144.60)	96.15 (53.600, 313.40)
LDH	Median (Q1, Q3)	264.00 (213.00, 322.00)	294.00 (200.00, 328.00)
HGB	Median (Q1, Q3)	11.90 (10.80, 12.40)	12.35 (11.40, 13.60)
Albumin	Median (Q1, Q3)	4.20 (3.90, 4.50)	4.10 (3.90, 4.20)
Alkaline phosphatase	Median (Q1, Q3)	222.00 (120.00, 280.00)	256.50 (107.00, 675.00)

ECOG = Eastern Cooperative Oncology Group; HGB = hemoglobin; LDH = lactate dehydrogenase; PSA = prostate-specific antigen; Q1 and Q3 = first and third quartiles; SD = standard deviation.
The factors for which means are presented met the assumptions of the Central Limit Theorem.

Little can be concluded about the efficacy of galeterone or enzalutamide. Once the DMC recommended closure, ongoing efficacy assessments were stopped. Galeterone appeared well tolerated: PK assessments suggested drug levels at which AR degradation was expected. The PSA50 response (unconfirmed) was 8/19 (42%) for enzalutamide and 2/16 (13%) for galeterone. One can speculate that AR-V7 detection in first-line mCRPC does not preclude responses to enzalutamide and that galeterone may be a weak AR antagonist/degrader. Future treatment options for these

patients include more potent AR degraders, inhibitors of AR N-terminal or DNA-binding domains, inhibitors of AR complementary pathways (eg, EZH2, PI3K inhibitors) [21], epigenetic modulators of AR (eg, bromodomain/BET inhibitors) [24], or chemotherapy [16,17].

5. Conclusions

In conclusion, 8% of mCRPC patients naïve to abiraterone or enzalutamide had CTC-transcript AR-V7 mRNA detected using an RT-PCR assay. Patients presenting with de novo metastasis, short duration of response to ADT, higher PSA, and high-volume bone disease were more likely to be AR-V7 +. The majority of AR-V7+ patients had clinically aggressive disease that was not controlled by AR-targeting therapies including galeterone or enzalutamide. Novel study designs and alternative treatment approaches are urgently needed for CTCs expressing AR-V7 mCRPC.

Author contributions: Mary-Ellen Taplin had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Acquisition of data: Taplin, Antonarakis, Ferrante, Horgan, Blumenstein, Saad, Luo, de Bono.
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Supervision: Taplin, Antonarakis, Ferrante, Blumenstein, Saad, Luo, de Bono.
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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.eururo.2019.08.034.

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