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Prostate Cancer

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

Adam Sharp [a,b,1], Jon C. Welti [a,1], Maryou B.K. Lambros [a,1], David Dolling [a], Daniel Nava Rodrigues [a], Lorna Pope [a], Caterina Aversa [b], Ines Figueiredo [a], Jennifer Fraser [a], Zai Ahmad [a], Changxue Lu [g], Pasquale Rescigno [a,b,c], Michael Kolinsky [d], Claudia Bertan [a], George Seed [a], Ruth Riisnaes [a], Susana Miranda [a], Mateus Crespo [a], Rita Pereira [a], Ana Ferreira [a], Gemma Fowler [a], Berni Ebbs [a], Penny Flohr [a], Antje Neeb [a], Diletta Bianchini [b], Antonella Petremolo [b], Semini Sumanasuriya [a,b], Alec Paschalis [a,b], Joaquin Mateo [e], Nina Tunariu [b], Wei Yuan [a], Suzanne Carreira [a], Stephen R. Plymate [f,2], Jun Luo [g,2], Johann S. de Bono [a,b,2,]*

[a] *The Institute for Cancer Research, London, UK;* [b] *The Royal Marsden NHS Foundation Trust, London, UK;* [c] *University of Naples Federico II, Naples, Italy;* [d] *University of Alberta, Edmonton, Alberta, Canada;* [e] *Vall d'Hebron Institute of Oncology (VHIO), Barcelona, Spain;* [f] *University of Washington and VAPSHCS GRECC, Seattle, WA, USA;* [g] *Johns Hopkins University School of Medicine, Baltimore, MD, USA*

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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,

[1] These authors are co-first authors.
[2] These authors are co-senior authors.
* Corresponding author. The Institute of Cancer Research, The Royal Marsden NHS Foundation Trust, London SM2 5NG, UK. Tel. +44 20 8722 4028; Fax: +44 208 642 7979.
E-mail address: Johann.de-Bono@icr.ac.uk (J.S. de Bono).

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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.

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 $\geq$1 copy/ml and absent $<$1 copy/ml) outcomes; AR-FL status is presented as binary (present $\geq$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%;

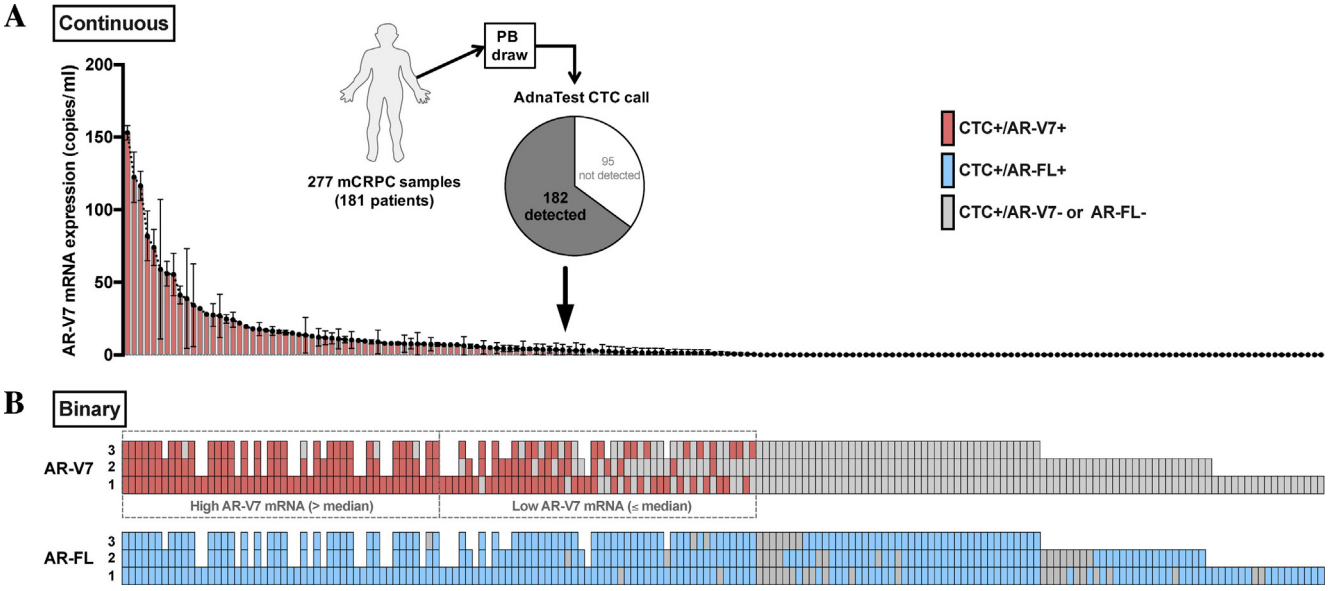

Fig. 1 – CTC AR-V7 mRNA as a continuous and binary variable: decreased assay concordance at lower mRNA levels. A total of 277 peripheral blood (PB) draws from 181 mCRPC patients were identified for AdnaTest analysis between January 2015 and January 2018. CTCs were detected in 182 samples and CTC AR-V7 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

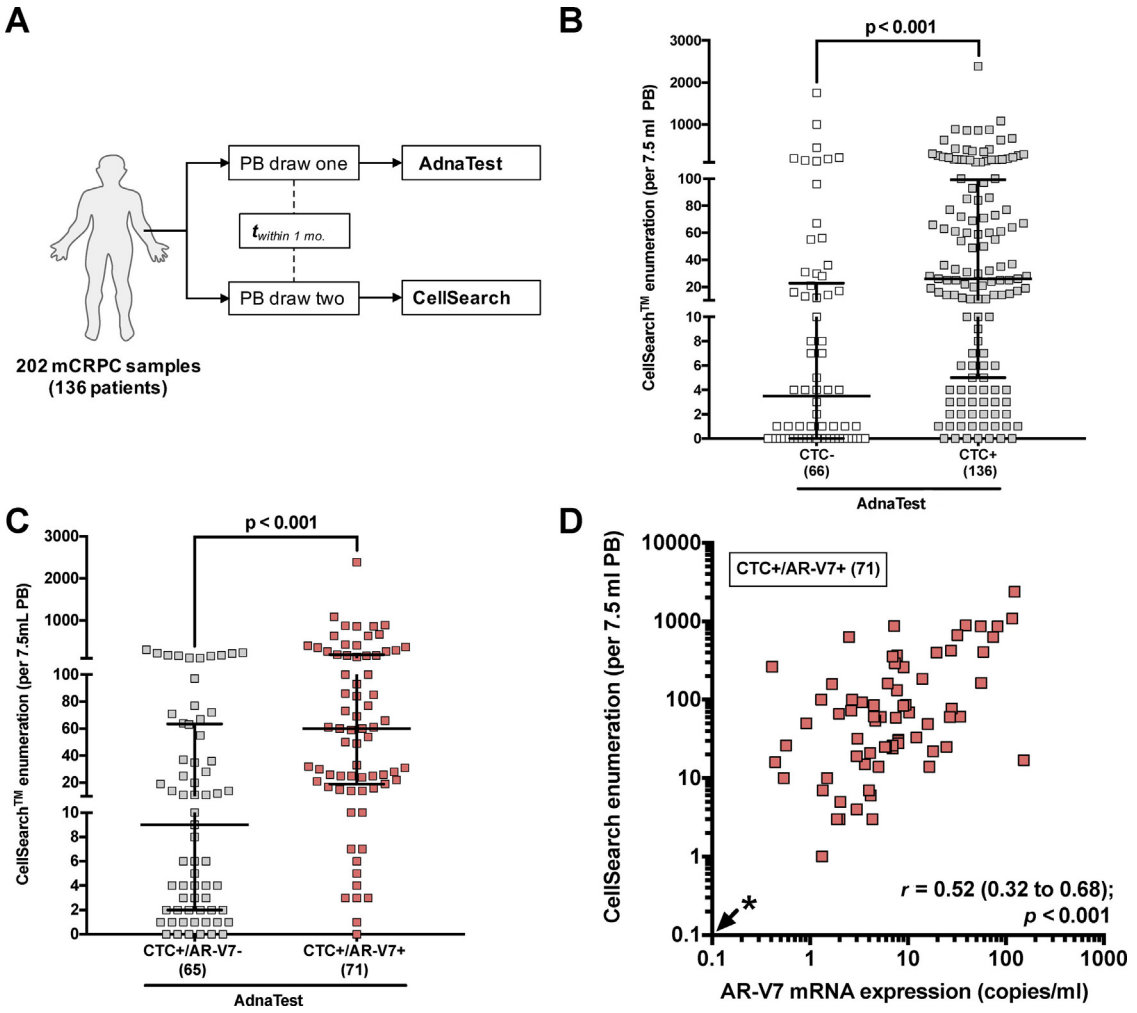

Fig. 2 – CTC AR-V7 positivity is associated with CellSearch CTC enumeration in mCRPC. (A) A total of 202 peripheral blood (PB) draws from 136 mCRPC patients for AdnaTest analysis with contemporaneous (all within a month) PB draws for CellSearch CTC enumeration. (B) CellSearch enumeration for contemporaneous 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

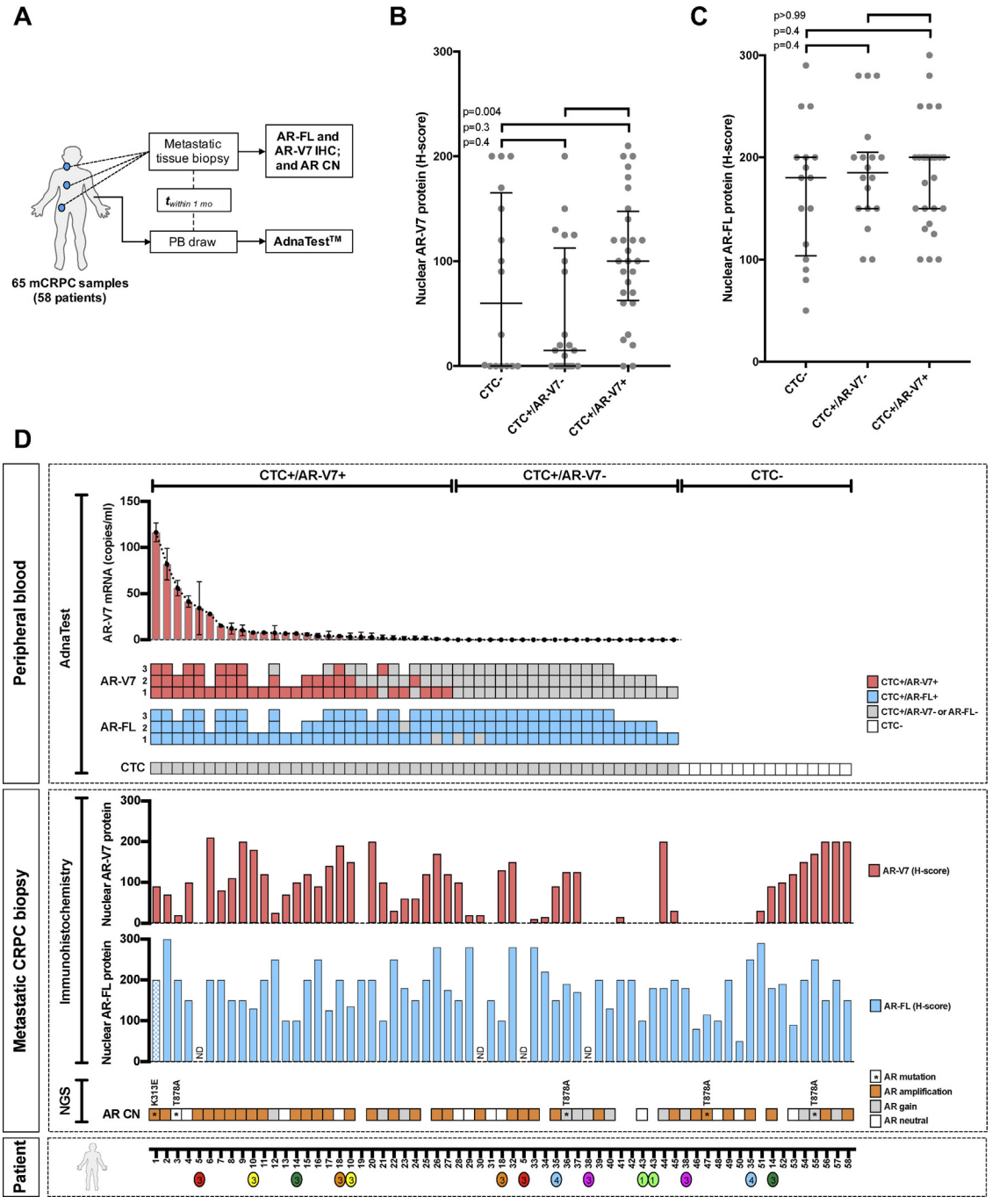

Fig. 3 – Nuclear AR-V7 protein expression and AR-V7 mRNA expression in contemporaneous mCRPC tissue and liquid biopsies. (A) Sixty-five peripheral blood (PB) draws for AdnaTest analysis with contemporaneous (all within a month) metastatic tissue biopsy for nuclear AR-V7 and AR-FL IHC from 58 mCRPC patients 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+

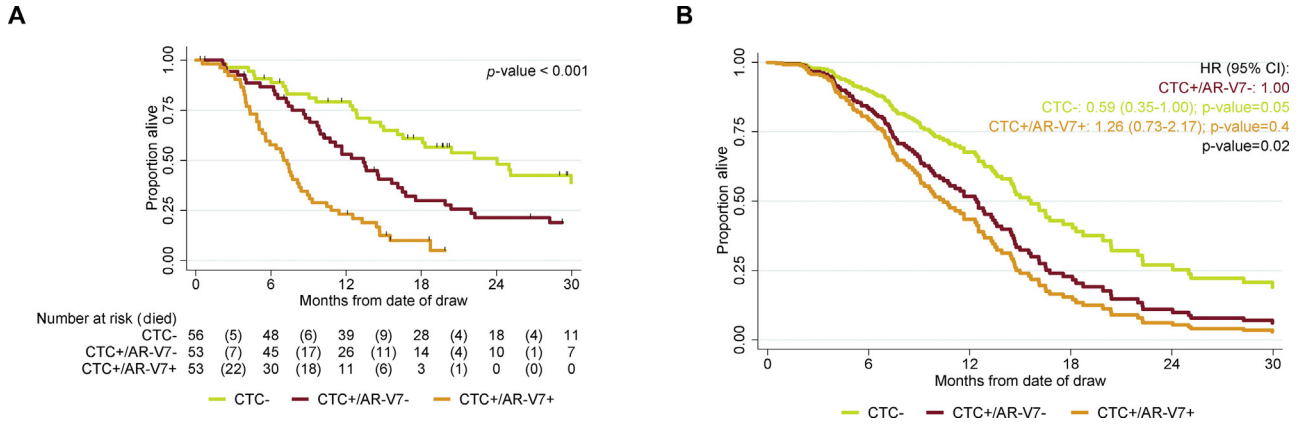

Fig. 4 – Association between CTC AR-V7 and overall survival in mCRPC. A total of 162 mCRPC patients with CTC AR-V7 status were determined. (A) Kaplan-Meier curve shows overall survival (OS) from peripheral blood draw for mCRPC patients divided by CTC−, CTC+/AR-V7−, and CTC+/AR-V7+ status. The *p* value 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

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