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Original Research

Associations between *AR-V7* status in circulating tumour cells, circulating tumour cell count and survival in men with metastatic castration-resistant prostate cancer

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Abstract ***Background:*** The interpretation of the presence of *AR-V7* in circulating tumour cells (CTCs) in men with metastatic castration-resistant prostate cancer (mCRPC) remains to be elucidated. *AR-V7* may hold promise as a predictive biomarker, but there may be prognostic impact of *AR-V7* positivity as well. To investigate the clinical value of *AR-V7*, we determined whether *AR-V7* detection in CTCs in patients with mCRPC is associated with CTC counts and survival.

Methods: Between December 2011 and January 2019, three prospective clinical trials collected clinical data of patients with mCRPC, who progressed after docetaxel and/or enzalutamide or abiraterone. Baseline (and follow-up) blood samples were withdrawn determining CTC count and *AR-V7* expression. The majority of patients started cabazitaxel as the next line of treatment after *AR-V7* characterisation.

Results: A total of 127 samples were evaluable for the analysis of CTC count versus *AR-V7* status. Although an association was observed between *AR-V7* and CTC count in all patients with mCRPC ($p = 0.017$), no such association was found in the prognostic unfavourable subgroup of patients with ≥ 5 CTCs. After adjusting for clinical prognostic factors, *AR-V7* expression in CTCs was not associated with overall survival (hazard ratio = 1.33, 95% confidence

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interval = 0.81–2.15, p = 0.25).
Conclusion: We found that *AR-V7* expression in CTCs had no additional prognostic value in patients with mCRPC, mostly treated with cabazitaxel. In patients with mCRPC with a predefined worse prognosis of a higher CTC count (≥5), a predictive biomarker is an important unmet medical need. Prospective trials should investigate whether *AR-V7* detection in CTCs may guide treatment selection for these adverse prognosis patients.

1. Introduction

AR-V7 is an mRNA splice variant of the androgen receptor (AR) translating to a constitutively active receptor that lacks the ligand-binding domain [1]. Several studies investigating the correlation between the presence of *AR-V7* in circulating tumour cells (CTCs) of patients with metastatic castration-resistant prostate cancer (mCRPC) and treatment response found that *AR-V7*–positive patients had decreased sensitivity to AR-targeted therapy (ART), such as enzalutamide and abiraterone, but not to taxanes [2,3]. Therefore, *AR-V7* expression is being explored as a potential predictive biomarker for ART. However, *AR-V7* positivity has also been related to unfavourable baseline characteristics; therefore, it may merely reflect a higher tumour burden [4–6]. This could be of influence on how to interpret the presence of AR-V7 in CTCs as it suggests a prognostic value of *AR-V7*. To improve our understanding of the clinical value of *AR-V7*, we performed a post hoc analysis evaluating the relationship between *AR-V7* status in CTCs, CTC count and overall survival (OS).

2. Methods

2.1. Patients and trials

Patients with mCRPC after docetaxel therapy were included in one of three different trials performed in the Netherlands between December 2011 and January 2019: the PRELUDE trial (MEC-2015-353), the CABARESC trial [7] or the CABA-V7 trial (EudraCT number: 2016-002993-11). The PRELUDE and CABA-V7 trial communicated the *AR-V7* test results to the treating physician before new treatment was started. All patients in the CABARESC trial received cabazitaxel. The design of each study is described in the Supplementary Methods / Supplementary Table 1. These trials were performed according to the Declaration of Helsinki, and all participants gave written informed consent.

2.2. Outcomes

The primary objective was to investigate associations between *AR-V7* status in CTCs and CTC count in all patients with mCRPC and in patients with an unfavourable prognosis based on CTC count ≥5. In addition, OS was compared between *AR-V7*-positive and *AR-V7*-negative patients for all patients with mCRPC and the patients with ≥5 CTCs to investigate its potential prognostic value. OS was calculated from the date when blood samples were withdrawn until the date of death from any cause or end of the study, whichever came first.

2.3. Sample processing and quality control

Two blood samples (one CellSave tube for CTC count and one ethylene diamine tetra-acetic acid [EDTA] tube for mRNA profiling) were withdrawn from patients with mCRPC and processed within 96 h (CTC count) or 24 h (for RNA profile) at the laboratory of Translational Cancer Genomics and Proteomics of the Department of Medical Oncology, Erasmus MC Cancer Institute. CTC enrichment occurred from 7.5 mL of peripheral blood using the CellSearch System. After CTC enrichment from the EDTA tube, cells were lysed, and RNA isolation was carried out, followed by reverse transcription - quantitative polymerase chain reaction (RT-qPCR). Processing of the samples occurred via standard operation procedures in a pre-PCR environment and was approved by an independent external audit. The steps of our quality control are described by Sieuwerts et al. [8]. In short, the first checkpoint was blood collection of at least 7.5 mL. To ensure we could compare the experiments of different trials and different processing sessions with each other, a calibrator of cultured VCaP RNA with known expression of our gene expression markers was included in all RT-qPCR sessions for evaluation of these samples. If samples were analysed without a VCaP calibrator, these samples were excluded from the analysis. By the usage of the average Cq value of three references genes (*GUSB*, *HMBS* and *HPRT1*), the quantity and quality of RNA was checked. Only samples with an average reference gene Cq value < 26.5 were considered to be of sufficient quality and quantity for a meaningful *AR-V7* analysis. The Cq values measured for *AR-V7* were normalised by the mean Cq value of two epithelial genes (*EPCAM* and *KRT19*) to correct for the number of epithelial CTCs present in the sample. Only samples with the Cq

value < 26.0 for the average of the epithelial genes were considered to have enough epithelial load for a meaningful *AR-V7* analysis. If a sample had sufficient Cq values for the reference and epithelial targets, but did not produce a quantitative PCR signal for *AR-V7* within 8.5 cycles after the average Cq of the epithelial markers, the sample was considered *AR-V7* negative. The rationale for this cut-off has been explained in detail before [8].

2.4. Statistical analysis

AR-V7 status was tested for associations with CTC count, using a Mann–Whitney U test. Log-rank testing was used to perform OS analysis, and Kaplan–Meier curves were drafted to visualise OS differences. Prognostic factors for OS were identified by univariate and multivariate Cox regression analysis. All factors with a P-value <0.05 detected in univariate analyses were included in multivariate analyses together with *AR-V7* as the variable of main interest. A backward selection method was used for the multivariate model, where a threshold of P < 0.05 was applied. All tests were two-sided, and a P-value of <0.05 was considered statistically significant. Statistical analyses were performed using STATA, SPSS version 24 or GraphPad Prism 5.

3. Results

In this post hoc analysis, 212 patient samples of three trials were included: the CABARESC trial (n = 109), the PRELUDE trial (n = 31) and the CABA-v7 trial (n = 72). Eighty-five samples had an insufficient epithelial signal to reliably give a result on *AR-V7* status. A total of 127 samples were evaluable and included in the current analysis. For the survival analysis, data of 94 patients were available (Fig. 1). Baseline characteristics and prior and subsequent life-prolonging therapies are shown in Table 1.

3.1. AR-V7 status and CTC count

Of the 127 evaluable samples, the presence of *AR-V7* was detected in 45 patients (35%). Despite the correction for the epithelial signal, a significantly higher median CTC count of 25 CTCs/7.5 mL of blood was detected in *AR-V7*–positive patients compared with a median CTC count of 10 CTCs/7.5 mL of blood in *AR-V7*–negative patients (p = 0.017, Fig. 2a). In patients with ≥5 CTCs, no significant difference in median CTC count could be detected between *AR-V7*–positive (n = 36) and *AR-V7*–negative (n = 50) patients (34 and 33, respectively, p = 0.24, Fig. 2b). In the 0–4 CTC group, no

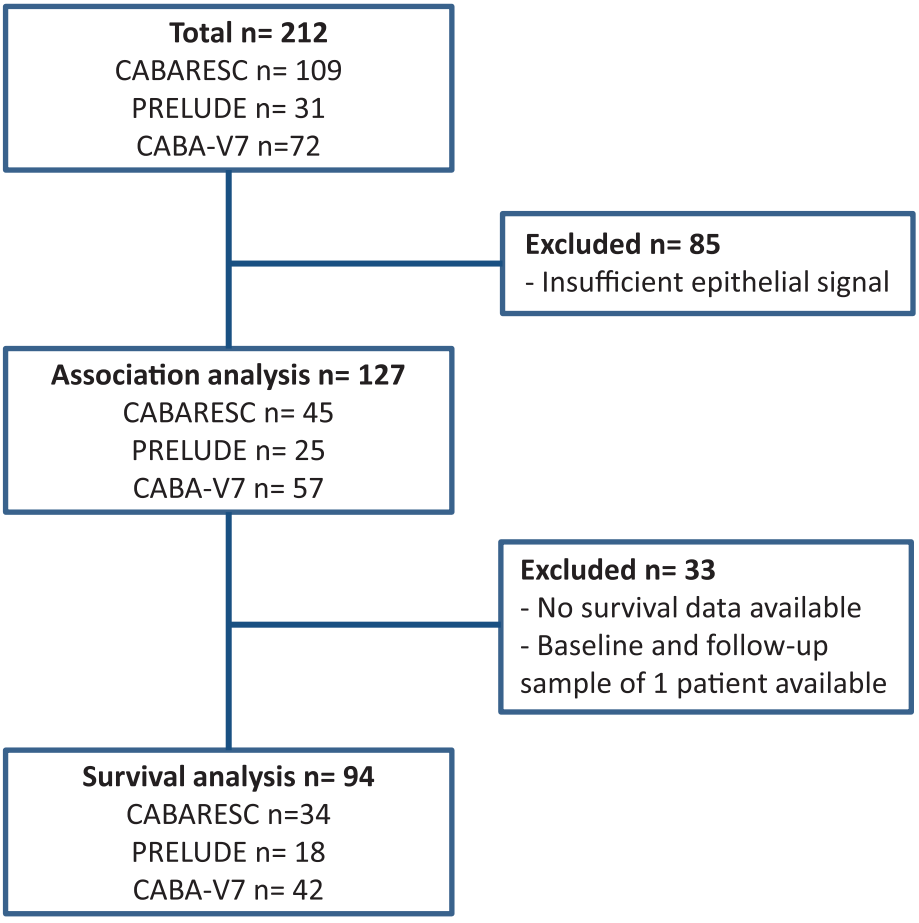

Fig. 1. Consort diagram.

Table 1
Baseline characteristics.

Patient characteristics	Total (n = 94) (median, IQR)	*AR-V7* pos (n = 34) (median, IQR)	*AR-V7* neg (n = 60) (median, IQR)
Age (years)	69 (65–75)	68 (64–73)	73 (66–76)
WHO PS, n (%)			
- 0	34 (36%)	13 (38%)	21 (35%)
- 1	43 (46%)	15 (44%)	28 (47%)
- 2	1 (1%)	0 (0%)	1 (2%)
- Missing	16 (17%)	6 (18%)	10 (17%)
Prior therapies, n (%)			
- Docetaxel	94 (100%)	34 (100%)	60 (100%)
- Enzalutamide	27 (29%)	14 (41%)	13 (22%)
- Abiraterone	13 (14%)	5 (15%)	8 (13%)
Hb, mmol/L	7.7 (6.9–8.2)	7.3 (6.8–8.2)	7.8 (7.1–8.2)
- Missing	15 (16%)	4 (12%)	12 (20%)
PSA, µg/L	186 (67–356)	171 (68–358)	198 (61–373)
- Missing	39 (41%)	9 (26%)	30 (50%)
Subsequent Treatment after AR-V7 determination			
- Cabazitaxel	73 (78%)	32 (94%)	41 (68%)
- Enzalutamide	11 (12%)	2 (6%)	9 (15%)
- Abiraterone	4 (4%)		4 (7%)
- Apalutamide	1 (1%)		1 (1.5%)
- Radium-223	1 (1%)		1 (1.5%)
- None	4 (4%)		4 (7%)

pos = positive, neg = negative, IQR = interquartile range, WHO PS = World Health Organization Performance Score, Hb = haemoglobin, PSA = prostate-specific antigen.

association between *AR-V7* positivity and CTC count was detected (Supplementary Fig. 1).

3.2. *AR-V7* status and OS

After adjusting for CTC count and clinical prognostic factors, no difference in survival between *AR-V7*–positive and *AR-V7*–negative patients was observed (Fig. 3a/Supplementary Table 2). As expected, patients with ≥5 CTCs had a significantly worse prognosis, with a median survival of 6.9 months (interquartile range [IQR]: 4.3–13.8) compared with patients with <5 CTCs (median: 22.3 months, IQR: 19.2–34.6, Fig. 3b). In this subgroup also, no difference in survival was observed between *AR-V7*–positive and *AR-V7*–negative patients (hazard ratio of 1.1, 95% confidence interval: 0.6–1.9, p = 0.78, Fig. 3c).

4. Discussion

In this post hoc analysis of patients with mCRPC after docetaxel therapy enrolled in 3 clinical trials, we evaluated associations between the *AR-V7* expression in CTCs, CTC count and OS. A significantly higher median CTC count was observed in *AR-V7*–positive patients than in *AR-V7*–negative patients. However, this association was not found in the unfavourable prognosis patient group with ≥5 CTCs. Moreover, *AR-V7* positivity in CTCs was not associated with a worse

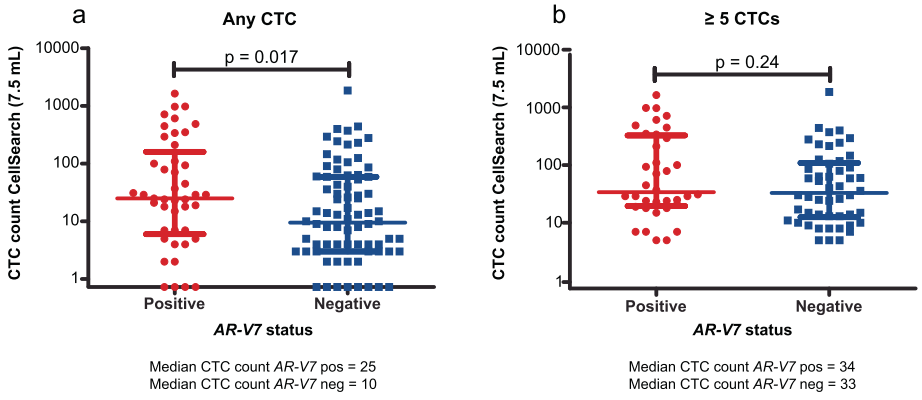

Fig. 2. **Relation between *AR-V7* status and CTC count.** All samples are checked by quality control. Blue dots are *AR-V7* negative, and red dots are *AR-V7* positive. (a) Samples with all CTC counts are included, p = 0.017. (b) Samples with at least 5 CTCs are included, p = 0.24. CTC, circulating tumour cell.

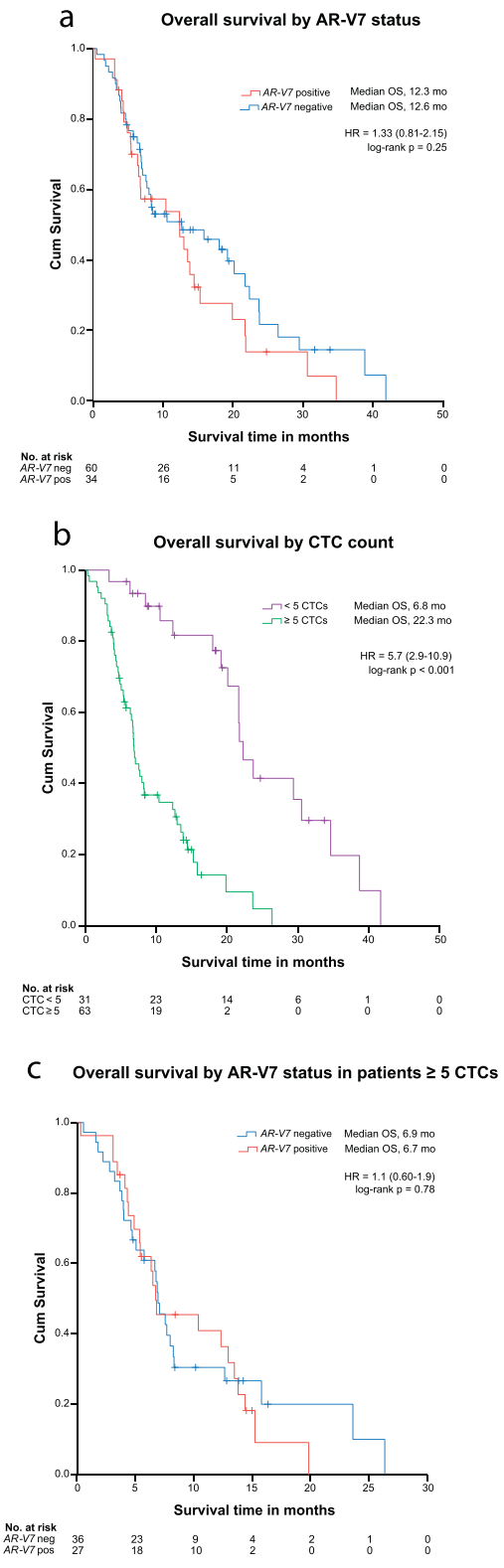

Fig. 3. **Survival curves.** (a) All patients stratified by AR-V7 status. (b) All patients stratified by CTC count. (c) Patients with ≥5 CTCs stratified by *AR-V7* status. CTC, circulating tumour cell.

prognosis in men with mCRPC mostly treated with cabazitaxel.

We observed an association between *AR-V7* status and median CTC count in all patients with mCRPC, which was probably driven by a lack of *AR-V7* positivity in patients with 4 or less CTCs. Lower incidence of *AR-V7* positivity in patients with a low CTC count could potentially be explained by intrapatient heterogeneity of *AR-V7* expression in CTCs and the lack of specificity of the test in samples with a low CTC count (or no CTCs at all) [9,10]. Other platforms for *AR-V7* determination such as nuclear *AR-V7* protein expression in tissue/CTCs, mRNA expression in extracellular vesicles such as exosomes/peripheral blood mononuclear cells/whole blood face the same issue of low specificity to a lesser or greater extent [10–13]. The availability of all these different testing methods indicates that *AR-V7* testing is far from standardised. Consensus on the analytical method of testing is needed before clinical implementation is possible.

AR-V7 may also be a biomarker that identifies a subgroup of patients with advanced disease and thus may be underrepresented in the good prognosis group. Therefore, *AR-V7* determination in the low (<5) CTC population may neither be reliable nor be relevant for clinical decision-making. The ADNA test, often used to determine *AR-V7* expression in CTCs, has no ability to count the CTCs. The CTC count could potentially be relevant to discriminate in which patients *AR-V7* determination is clinically relevant; therefore, it is suggested to incorporate the CTC count in the *AR-V7* testing. The recent study by Sharp *et al.* [14] did incorporate the CTC count by CellSearch and combined this with the *AR-V7* expression. We showed similar results (Fig. 2 of both studies) as a significant difference in CTC count between *AR-V7*–positive and *AR-V7*–negative patients was found in both studies. In addition, similar to our results, most patients with a low to zero CTCs are found to be *AR-V7* negative. It remains to be determined if these patients are truly *AR-V7* negative, which seems debatable as some of the CTC-based *AR-V7*–negative patients in the study by Sharp *et al.* [14] have *AR-V7* protein expression in the tumour biopsies, and these patients had limited response to ART. In addition to their work, we investigated the association in the poor prognosis group of patients with mCRPC with ≥5 CTCs.

We confirmed the prognostic value of the CTC count at the cut-off of 5 CTCs in this patient cohort [15]. The subgroup with ≥5 CTCs had a median OS of 6.9 months, in which the presence of *AR-V7* did not further impact survival. The short survival time of patients with ≥5 CTCs emphasises the need to carefully select treatment as the 'window of opportunity' to administer an effective treatment is relatively small. Our CTC-based *AR-V7* characterisation test has a turnaround time of less than 2 weeks, enabling early treatment advice in this specific subgroup [8]. Therefore, we propose that the value of *AR-V7* should be further assessed in patients with ≥5 CTCs as they have the highest need for rapidly available accurate predictive biomarkers.

The predictive value of *AR-V7* could not be further addressed in this study because no control group was available. However, *AR-V7* positivity in CTCs of patients with mCRPC, mostly treated with cabazitaxel, was not associated with worse outcomes. Recently, the negative prognostic value of *AR-V7* has been validated prospectively in abiraterone- and enzalutamide-treated patients, indicating that *AR-V7* positivity was associated with worse outcomes with these novel ARTs [16]. A limitation of the study by Armstrong *et al.* [16] was the heterogeneity of the patient cohort and treatment selection. In our study, most patients were started on cabazitaxel, sometimes based on the *AR-V7* result. This may confound the potential prognostic value of *AR-V7* as it may correct for an otherwise poor prognosis and potentially influence our results. However, the majority—of *AR-V7*–negative patients—also received cabazitaxel.

5. Conclusion

In conclusion, detection of *AR-V7* in CTCs has no additional prognostic value in patients with mCRPC, who were mostly treated with cabazitaxel, and prospective validation is needed to investigate if *AR-V7* could fulfil the criteria for a useful predictive biomarker.

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Conflict of interest statement

B.P.S.B. has received travel support from Astellas. R.d.W. has received advisory fees from Sanofi, Merck, Lilly, Roche, Bayer, Janssen and Clovis, speaker fees from Sanofi and Merck and research funding (institutional) from Sanofi and Bayer. M.P.L. has received research funding (institutional) from Sanofi, JnJ, Merck and Astellas, and speakers fee from incyte, amgen, Janssen, Bayer, Servier, Roche, Inca, Pfizer, Sanofi, Astellas. R.H.J.M. has received research funding (institutional) from Sanofi and Astellas. R.J.v.S. has received speaker fees from Sanofi and Astellas and advisory fees from Janssen and Bayer. A.M.S. is financially sponsored by Cancer Genomics Netherlands. B.M. has received consulting fee from Eli Lilly. J.M. has received research funding from Sanofi, Philips, Therawis and PamGene. J.M. and S.S. received research funding from Janssen Diagnostics (formerly, Veridex LLC). P.H., E.O.-d.H., M.N.V., C.M.B. and J.K. have no financial disclosure in this project. W.O., A.M.S. and S.S. patented (#WO2016133387 A1) the described AR-V7 assay. One of the included prospective trials in this post hoc analysis was financially supported by Sanofi Genzyme. The sponsor had no role in the design and conduct of the study.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejca.2019.08.005.

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