AR-V7 in Peripheral Whole Blood of Patients with Castration-resistant Prostate Cancer: Association with Treatment-specific Outcome Under Abiraterone and Enzalutamide

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Abstract

Background

Androgen receptor splice variant 7 (AR-V7) expression in circulating tumor cells (CTCs) was demonstrated to predict poor treatment response in metastatic castration-resistant prostate cancer (mCRPC) patients treated with abiraterone or enzalutamide.

Objective

To develop a practical and robust liquid profiling approach for direct quantification of AR-V7 in peripheral whole blood without the need of CTC capturing and to determine its potential to predict treatment response in mCRPC patients.

Design, setting, and participants

Whole blood samples from a prospective biorepository of 85 mCRPC patients before treatment initiation with abiraterone (n=56) or enzalutamide (n=29) were analyzed with droplet digital PCR.

Outcome measurement and statistical analyses

The association of AR-V7 status with prostate-specific antigen (PSA) response defined by PSA decline ≥50% as well as PSA-progression-free survival (PSA-PFS), clinical PFS, and overall survival (OS) was assessed.

Results and limitations

High AR-V7 expression levels in whole blood were detectable in 18% (15/85) of patients. No patient with high AR-V7 expression achieved PSA response, and AR-V7 status was an independent predictor of PSA response in multivariable logistic regression analysis (p=0.03). High AR-V7 expression was associated with shorter PSA-PFS (median 2.4 vs. 3.7 months, p<0.001), shorter clinical PFS (median 2.7 vs. 5.5 months, p<0.001), and shorter OS (median 4.0 vs. 13.9 months, p<0.001). On multivariable Cox regression analysis, high AR-V7 expression remained an independent predictor of shorter PSA-PFS (Hazard ratio (HR) 7.0, 95% CI 2.3–20.7, p<0.001), shorter clinical PFS (HR 2.3, 95% CI 1.1–4.9, p=0.02), and shorter OS (HR 3.0, 95 %CI 1.4–6.3, p=0.005).

Conclusion

AR-V7 mRNA level testing in whole blood is a simple and promising approach to predict poor treatment outcome in mCRPC patients undergoing treatment with abiraterone or enzalutamide.

Word count Abstract: 279 (Max. 300)

Patient summary

In this report we established a method for determining AR-V7 status in whole blood. This test predicted treatment resistance in patients with metastatic castration-resistant prostate cancer undergoing treatment with abiraterone or enzalutamide. Prospective validation is needed before application to clinical practice.

Introduction

Prostate cancer (PCa) is the most common cancer and the third leading cause of cancer death in European men[1]. In metastatic PCa, progression from a hormone-sensitive state to castration-resistance under androgen-deprivation therapy marks the transition to the lethal phenotype of the disease. New insights into tumor biology have contributed to the development of novel therapeutic agents that have revolutionized the treatment landscape of metastatic castration-resistant prostate cancer (mCRPC) over the last years[2]. Based on the discovery that the androgen receptor (AR) is still active in mCRPC and responsible for disease progression[3], a new generation of AR-directed agents such as the androgen-biosynthesis inhibitor abiraterone and the AR inhibitor enzalutamide have been developed and were shown to improve overall survival[4-7].

However, treatment resistance still poses a major challenge in mCRPC. Approximately one-third of patients show primary resistance to abiraterone and enzalutamide treatment without any decline in serum prostate-specific antigen (PSA) levels, and virtually all of the initial responders develop secondary resistance over time[4-8].

A main focus has been drawn on the presence of AR-splice variants as a cause for resistance. The AR splice variant 7 transcript isoform (AR-V7) is the most abundant splice variant. It lacks the androgen-binding site and remains constitutively active as transcription factor independent of androgen-signalling[9,10]. Recently, the clinical importance of this finding has been demonstrated by showing that AR-V7 is associated with resistance to abiraterone and enzalutamide in mCRPC patients[8,11,12]. Using a circulating tumor cell (CTC)-based assay to determine AR-V7 expression, these studies required elaborate processing of blood samples and detectable CTCs.

Alternatively, quantification of AR-V7 mRNA levels directly in peripheral whole blood has been reported[13-15]. This approach bears the main advantage that it detects AR-V7 expression in all blood compartments at one time reflecting the overall AR-V7 status in blood. Previous studies suggested the presence of AR-V7 transcripts not only in CTCs[8,11], but also in exosomes[16] and as cell-free RNA in plasma[17]. Moreover, AR-V7 detection in whole blood is independent of detectable CTCs and their isolation by enrichment. Epithelial-based CTC detection methods such as the widely used AdnaTest ProstateCancer and CellSearch® system, target epithelial cell surface proteins for CTC enrichment. However, epithelial-mesenchymal transition (EMT) in

CTCs, which plays a pivotal role in metastasis formation[18], causes down-regulation of epithelial proteins. Hence, these cells will be invisible to epithelial-based CTC detection methods. Taken together, this suggests high potential for determining AR-V7 status in peripheral whole blood. However, an association of AR-V7 status in whole blood with resistance to treatment with abiraterone or enzalutamide has not been reported yet.

In the present study, we established and validated a liquid profiling approach with direct, absolute quantification of AR-V7 and AR full length (AR-FL) mRNA levels in peripheral whole blood using droplet digital polymerase chain reaction (ddPCR) and determined its ability to predict treatment resistance in mCRPC patients scheduled for abiraterone or enzalutamide.

Materials and Methods

Patient cohort and healthy donors

The study cohort included 85 patients with mCRPC who were treated at the Department of Urology, Klinikum rechts der Isar, Technical University of Munich in Germany between 2009 and 2016. These patients had progressive disease as defined by PCWG2 criteria[19] at inclusion and were scheduled for a new line of systemic treatment of either abiraterone (n=56) or enzalutamide (n=29). All patients signed institutional review board-approved consent before participation and were enrolled according to a prospective biorepository protocol.

Treatment response was determined according to the institutional standard procedure including PSA levels within 1 week before and every 4 weeks after treatment initiation as well as imaging procedures (CT and bone scan) within 4 weeks before and every 3 months after treatment initiation.

The main endpoint was PSA response defined by a PSA level decline of ≥50% as marker for treatment response versus resistance. Further study endpoints included PSA progression-free survival (PSA-PFS) according to PCWG2 criteria[19], clinical progression-free survival (PFS), and overall survival (OS). Clinical progression was defined by worsening of disease-related symptoms or new cancer-related complications, radiographic progression based on RECIST criteria[20], two or more new bone lesions on bone scan, or death, whichever occurred first[19]. Results are reported in compliance with REMARK guidelines[21].

Blood samples

Blood samples were collected in 2.7 ml PAXgene™ blood RNA tubes (Qiagen) within 1 week before treatment initiation. Total RNA was extracted from blood samples according to manufacturer's instructions using the PAXgene Blood RNA Kit (Qiagen). The NanoDrop 1000 spectrophotometer (Thermo Scientific) was used for quantification and purity assessment of RNA samples. Complementary DNA (cDNA) was synthesized from 1 µg RNA using the qScript XLT SuperMix (Quanta Biosciences, Beverly, MA, USA) according to manufacturer's instructions.

Droplet digital PCR analysis

AR-V7 and AR-FL mRNA levels were simultaneously quantified in a dual colour assay using custom primer and hydrolysis probe sets on a QX200 ddPCR system with automatic droplet generation (Bio-Rad Laboratories). Analyses were performed and are reported according to the digital MIQE guidelines[22]. For further information on the ddPCR assays see Supplementary Methods. All operators involved in the measurements were blinded to the assignment of samples to healthy control subjects or patients and their outcome.

Healthy donors

We included 28 male, healthy subjects (age <40 years) to determine background levels of AR-V7 and AR-FL transcripts in peripheral whole blood. Samples from healthy subjects were obtained and stored under the same conditions as patient samples in order to minimize any bias.

Statistical analysis

See Supplementary Methods for details.

Results

Patient characteristics

We enrolled 85 mCRPC patients who were planned to undergo a new line of therapy with either abiraterone (n=56) or enzalutamide (n=29). Table 1 provides detailed information about baseline characteristics and clinical outcomes. Bone and visceral metastases were present in 96% and 28% of patients, respectively. Prior systemic treatment regimens for mCRPC were chemotherapy with docetaxel in 79% of patients and abiraterone in 28% of patients. None had previously received enzalutamide.

AR-V7 detection in peripheral whole blood

First, we assessed the analytic validity of our ddPCR assay for AR-V7 and AR-FL isoform detection. AR-V7 mRNA of one VCaP cell in a background of 1 million leukocytes could be repeatedly detected (Supplementary Fig. 1). Next, we quantified AR-V7 and AR-FL transcript levels in peripheral whole blood samples of 85 mCRPC patients and 28 healthy men as control subjects (Fig. 1A). Notably, 18 of 28 healthy control subjects had detectable (non-zero) AR-V7 levels. In order to normalize AR-V7 expression, we calculated the fraction of AR-V7 transcript over total AR (AR-V7 plus AR-FL) transcript and used this ratio in all subsequent analyses. The fraction of AR-V7 transcript in whole blood of mCRPC patients ranged from 0 to 4.0% (mean 0.3%, Fig. 1B). Using the maximum observed AR-V7 fraction in healthy men (0.6%) as a cut-off, we dichotomized the patients into an “AR-V7 high” and an “AR-V7 low” group (Fig. 1B). Overall, 15/85 (18%) patients had high AR-V7 levels. According to prior therapy with 0, 1, 2 and 3 lines of systemic treatment, the number of AR-V7 high patients was 0/9 (0%), 9/39 (23%), 3/27 (11%) and 3/10 (30%), respectively.

AR-V7 status in whole blood predicts PSA response under abiraterone or enzalutamide

The overall proportion of patients with PSA response defined by a PSA decline of 50% or more was 41% (31 of 74 men with available PSA follow-up). In patients with high AR-V7 blood levels, the PSA response rate was 0% (0 of 12 men), and in AR-V7 low patients 50% (31 of 62 men, Fig. 2). Thus, AR-V7 status showed a significant association with PSA response in univariable analysis ($p<0.001$). In a multivariable logistic regression analysis we modeled the influence on PSA response of AR-V7 status together with clinical variables (Table 2). Herein, only the association of AR-V7

status with PSA response remained significant and high AR-V7 levels in whole blood were confirmed as an independent predictor of non-PSA response to treatment with abiraterone or enzalutamide (p=0.03). Furthermore, AR-V7 was confirmed as independent predictor of non-PSA response in a multivariable model with AR-V7 expression as continuous variable (p=0.04, Supplementary Table 1).

AR-V7 status in whole blood predicts PSA-PFS, clinical PFS, and OS

High AR-V7 levels were associated with significantly shorter PSA-PFS [2.4 (95%CI 1.8–3.0) vs. 3.7 months (95%CI 2.3–3.1), p<0.001] (Fig. 3A), with shorter clinical PFS [2.7 (95%CI 2.3–3.1) vs. 5.5 months (95%CI 4.4–6.6), p<0.001] (Fig. 3B), and with shorter OS [4.0 (95%CI 2.0–6.0) vs. 13.9 months (95%CI 9.6–18.2), p<0.001] (Fig. 3C). When analyzed in multivariable Cox regression models, AR-V7 status remained significantly associated with PSA-PFS (HR 7.0, 95%CI 2.3–20.7), clinical PFS (HR 2.3, 95%CI 1.1–4.9), and OS (HR 3.0, 95%CI 1.4–6.3) (Table 3). These findings are supported by additional multivariable models with AR-V7 expression as continuous variable. Herein, we saw some evidence of poor PSA-PFS (p=0.15) and clinical PFS (p=0.06) with increasing AR-V7 levels, although this association did not meet conventional levels of statistical significance (Supplementary Tables 2-3, Supplementary Fig. 2-3). Moreover, AR-V7 was confirmed as independent prognostic factor of poor OS (p=0.02) (Supplementary Table 4, Supplementary Fig. 4).

Discussion

According to recent publications, AR-V7 expression in CTCs is associated with primary resistance to AR-directed therapies[8,11,12]. In the present study, we established an alternative liquid profiling approach to directly determine AR-V7 status in peripheral whole blood using ddPCR for absolute quantification of AR-V7 and AR-FL mRNA concentrations. Applying this assay to blood samples of mCRPC patients from a prospective biorepository, we demonstrate that high AR-V7 levels before treatment initiation predict non-response to AR-directed therapy with abiraterone or enzalutamide. In our study, high AR-V7 levels in peripheral whole blood of mCRPC patients were associated with failure to achieve PSA response as well as shorter PSA-PFS, clinical PFS and OS on multivariable analysis. To our knowledge, this is the first standardized evaluation providing evidence for the use of whole blood AR-V7 levels as a marker of resistance to next generation AR-directed agents.

Our approach analyzing whole blood instead of CTCs is supported by a recent study from Liu et al.[13]. They compared AR-V7 detection rates using RNA isolated either from whole blood or from CTCs enriched by leukocyte depletion in 10 mCRPC patients. While both methods showed similar AR-V7 detection rates in a side-by-side comparison, AR-V7 levels were approximately 40% lower in RNA isolated from CTCs suggesting a higher sensitivity of the whole blood RNA approach compared to CTC enrichment through leukocyte depletion. Moreover in support of our data, Todenhöfer et al. reported an association of AR-V7 status in whole blood with PSA-PFS and OS in 37 mCRPC patients undergoing abiraterone treatment[14]. While none of 4 AR-V7 positive patients achieved PSA response with a decline ≥50%, statistical analysis did not reach significance potentially influenced by small sample size. Furthermore, Qu et al. quantified mRNA levels of AR-V7 using ddPCR in whole blood of mCRPC patients treated with abiraterone (n=81) or enzalutamide (n=51) and found an association with time to treatment-failure[15]. As a limitation of this study, a threshold for elevated AR-V7 levels was determined somewhat arbitrarily without the use of a control group.

In line with previous findings, we observed tumor-independent background expression of AR-V7 mRNA in whole blood of healthy men[14]. This emphasizes the necessity of determining robust and clearly defined thresholds of AR-V7 levels in whole blood for translation into clinical routine testing and standardization of clinical decision making. In contrast, solely qualitative AR-V7 detection in whole blood may lead to conflicting

data as reported in a recent study[23]. Based on the findings in our control group, we introduced a threshold to distinguish physiologically low versus pathologically high AR-V7 levels in mCRPC patients (0.6% of the ratio of AR-V7 transcripts over total AR (AR-V7 plus AR-FL) transcripts). Using this threshold, 18% of mCRPC patients exhibited high AR-V7 expression in our study.

The reported fraction of AR-V7 positive mCRPC patients shows a high variation ranging between 11–68%[8,11-15]. This is attributable to various causes, most of all the variety of applied methods including CTC-derived RNA- or protein-based assays as well as different whole blood assays. However, the optimal method to determine AR-V7 status using liquid biopsies has yet to be determined. While CTC-based methods require detectable CTCs, whole blood samples show tumor-independent AR-V7 expression, potentially masking PCa-related AR-V7 expression to a certain extent. Furthermore, the variation in AR-V7 detection rate may be attributable to a heterogeneity of patient cohorts. In our study, the AR-V7 positivity ranged from 0% to 30% corresponding to a range of 0 to 3 prior lines of systemic treatment for mCRPC, comprising taxane chemotherapy and AR-directed agents. Keeping in mind that AR-V7 positivity becomes more frequent in patients pretreated with AR-inhibitors[8] and taxane pretreatment might re-establish sensitivity to AR-directed agents by AR-V7 reversion[24-26], number and sequence of prior treatment regimens may have an important impact on AR-V7 status.

Our study results are in line with the current paradigm considering AR-V7 expression as a predictor for nonresponse to next-generation AR-directed therapy. However, this paradigm has recently been challenged[11,27]. Steinestel et al. described one patient who showed PSA response to abiraterone despite CTC positivity for AR-V7 mRNA[11]. Likewise, Bernemann et al. from the same group conducted a retrospective study where PSA response to abiraterone or enzalutamide was assessed in 21 patients with AR-V7 mRNA-positive CTCs[27]. In their cohort 4 (19%) patients achieved a PSA decline ≥50%. One potential explanation is that AR-V7 mRNA-positive patients achieving PSA response might lack AR-V7 protein expression with correct nuclear localization[12]. Moreover, CTCs might express AR-V7 mRNA at physiologically low levels in relation to AR-FL causing a positive test result without leading to treatment resistance[10].

In our cohort, we also observed three AR-V7 high patients that had close to 50% PSA decline (43%, 46%, and 48%), all of whom were treated with abiraterone. However, these patients did not show prolonged benefit from it. While 2 patients developed clinical progression within 3 months, the third patient clinically progressed after 4 months and died after 6 months.

A strength of our approach is the applicability in a clinical routine setting. PAXgene™ tubes used for blood draw allow for RNA stabilization at room temperature for about 4 days and storage over long time periods at –80°C. Furthermore, digital PCR was shown to be reproducible across laboratories[28] and revealed greater precision and improved day-to-day reproducibility with equal sensitivity compared to quantitative real-time PCR (qPCR).

Our study has the following limitations. First, retrospective design and patient enrolment at a single institution limit the generalisability of our results. Second, the number of 15 AR-V7 high patients on which our findings are based is relatively low. Third, among AR-V7 low patients 50% (31 of 62) failed to show PSA response, meaning that resistance mechanisms other than AR-V7 are contributing to therapy failure which are not captured by AR-V7 testing.

Conclusion

We established a robust liquid profiling approach for direct quantification of AR-V7 mRNA levels in peripheral whole blood. In patients undergoing treatment with abiraterone or enzalutamide, high AR-V7 levels predicted resistance with non-PSA response, shorter PSA-PFS, clinical PFS and OS. This supports AR-V7 as a predictive biomarker for non-response to next-generation AR-directed therapy. Nevertheless, the optimal method for determining AR-V7 status has yet to be determined. Moreover, a randomized controlled trial is urgently needed for determining clinical utility of AR-V7 as resistance marker and quantifying survival benefit of AR-V7-guided therapy selection.

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None

Figures

Figure 1. Quantification of androgen receptor splice variant 7 (AR-V7) in abiraterone or enzalutamide treated patients and healthy controls. AR-V7 and Full-Length androgen receptor (AR-FL) mRNA levels in whole blood were quantified by droplet digital PCR in 85 patients treated with abiraterone or enzalutamide, and 28 healthy controls to determine tumor-independent AR-V7 and AR-FL background expression (A). The dotted line indicates a fraction of 0.6% AR-V7 transcript over total AR (AR-V7 plus AR-FL) that was identified as threshold to distinguish AR-V7 high versus low patient samples (B).

Figure 2. **Waterfall plots of best prostate-specific antigen (PSA) changes and androgen receptor splice variant 7 (AR-V7) status.** The dotted line depicts the threshold for defining a PSA response (≥ 50% reduction in PSA serum level from baseline). Asterisks indicate an increase of > 100% in best PSA change. All of the patients with high AR-V7 levels (n = 12) in whole blood were non-responders, and none of the PSA responders (n = 31) exhibited high AR-V7 levels.

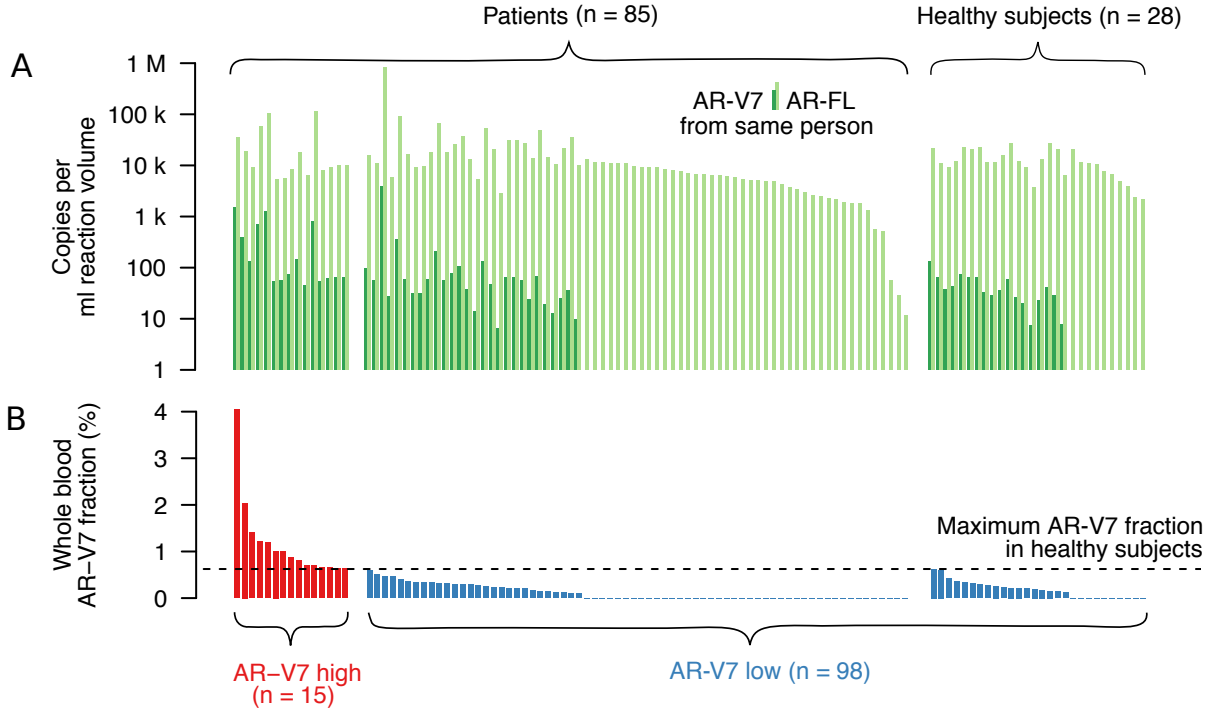
Figure 3. Kaplan-Meier analysis. Prostate-specific antigen (PSA) progression-free survival (A), clinical or radiographic progression-free survival (B), and overall survival (C) according to androgen receptor splice variant 7 (AR-V7) level in whole blood.

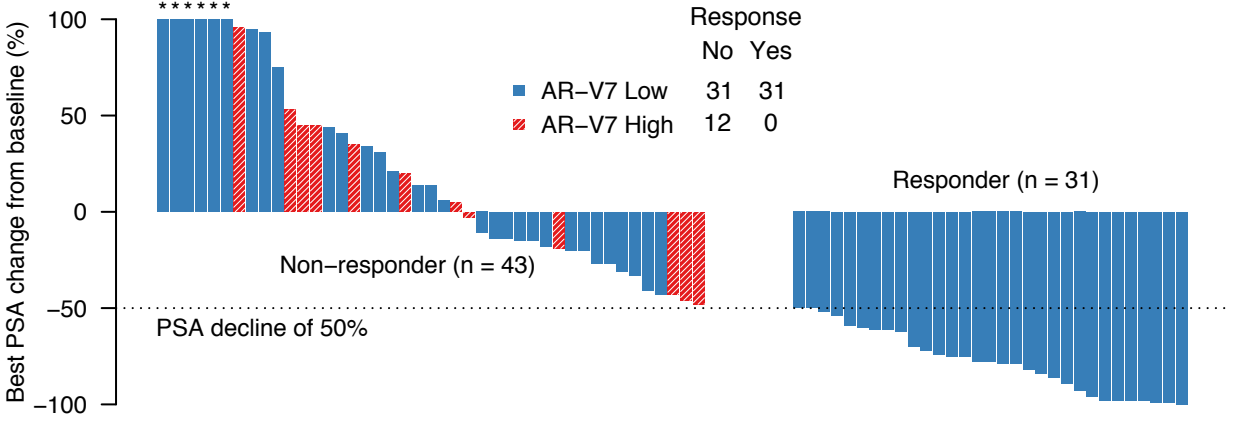
Tables

Table 1: Patient characteristics

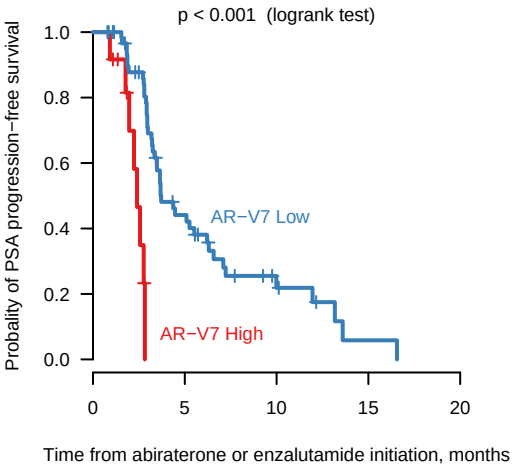
Table 2: Multivariable logistic regression analyses.

Table 3: Multivariable Cox regression analyses.


Patients (n = 85)
Healthy subjects (n = 28)
A
1 M
100 k
10 k
1 k
100
10
1
Copies per
ml reaction volume
AR-V7
AR-FL
from same person
B
4
3
2
1
0
Whole blood
AR–V7 fraction (%)
Maximum AR-V7 fraction
in healthy subjects
AR–V7 high
(n = 15)
AR-V7 low (n = 98)

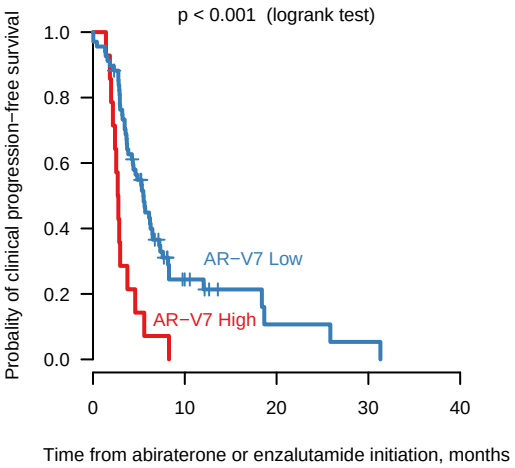

Best PSA change from baseline (%)
100
50
0
−50
−100
******
Response
No Yes
AR−V7 Low 31 31
AR−V7 High 12 0
Non−responder (n = 43)
Responder (n = 31)
PSA decline of 50%

A



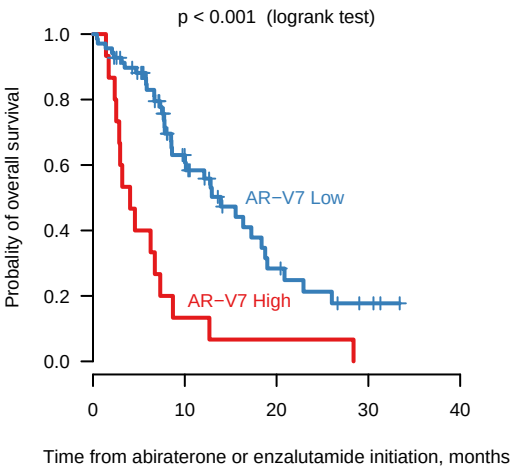
No. at risk				
AR−V7 High	12			
AR−V7 Low	62	22	6	1

B



No. at risk				
AR−V7 High	14			
AR−V7 Low	68	9	2	1

C



No. at risk				
AR−V7 High	15	2	1	
AR−V7 Low	69	27	9	3

Table 1. Patient characteristics.

No. patients	85
Age, years, median (IQR); n=85	71 (66-74)
Chemistry, median (IQR)	
PSA, ng/ml; n=84	211 (29-768)
ECOG, no. (%); n=83	
0	43 (52)
1	29 (35)
2	11 (13)
Prior systemic treatments for mCRPC, No. (%); n=85	
Docetaxel	67 (79)
Abiraterone	24 (28)
Cabazitaxel	14 (17)
Enzalutamide	0 (0)
Radium-223	6 (7)
Other	12 (14)
Prior lines of systemic treatment regimens for mCRPC; n=85	
0	9
1	39
2	27
3	10
Site of metastasis, no. (%); n=83	
Bone	80 (96)
Visceral	23 (28)
Deceased, no. (%); n=84	51 (60)
Follow-up, months, median (IQR); n=84	7.6 (4.7-12.7)
with event (death)	7.3 (3.3-12.7)
without event (death)	7.7 (5.4-12.6)
PSA-PFS, months, median (95%CI); n=74	3.6 (3.2-4.1)
Clinical PFS, months, median (95%CI); n=82	4.6 (3.1-6.2)
OS, months, median (95%CI); n=84	10.1 (5.8-14.5)

IQR, interquartile range; CI = Confidence Interval; mCRPC = metastatic Castration-Resistant Prostate Cancer; ECOG = Eastern Cooperative Oncology Group; OS = Overall Survival; PSA = Prostate Specific Antigen; PFS = Progression-Free Survival

Table 2. Multivariable logistic regression analyses. AR-V7 status, prior treatment with abiraterone or enzalutamide, ECOG performance status, presence of visceral metastases, and serum PSA levels were assessed in one multivariable model for their association with therapy response (PSA decline of 50% or more, binary variable, yes or no).

Variable	Values	Odds ratio (95% CI)	p
AR-V7	High vs Low	0.03 (0.00–0.70)	0.03
Pretreatment with abiraterone or enzalutamide	Yes vs No	0.25 (0.06–1.09)	0.06
ECOG	0, 1, or 2	0.62 (0.22–1.76)	0.37
Visceral metastases	Yes vs No	1.07 (0.29–3.94)	0.91
PSA	Continuous (units of 100 ng/ml)	1.04 (0.97–1.12)	0.26

CI, confidence interval; ECOG, Eastern Cooperative Oncology Group; PSA, prostate-specific antigen

Table 3. Multivariable Cox regression analyses. For each of the outcomes PSA-progression free survival (PSA-PFS), clinical progression-free survival (PFS), and overall survival (OS), one multivariable model for the association of the covariates AR-V7, prior treatment with abiraterone or enzalutamide, ECOG performance status, presence of visceral metastases, and serum PSA levels with the outcome variable was created.

		PSA-PFS		Clinical PFS		OS	
Variable	**Values**	**HR (95% CI)**	**p**	**HR (95% CI)**	**p**	**HR (95% CI)**	**p**
AR-V7	High vs Low	6.99 (2.36–20.7)	< 0.001	2.33 (1.12–4.86)	0.02	2.97 (1.39–6.33)	0.005
Pretreatment with abiraterone or enzalutamide	Yes vs No	1.54 (0.72–3.27)	0.26	1.27 (0.65–2.46)	0.48	1.6 (0.72–3.57)	0.25
ECOG	0, 1, or 2	1.81 (1.02–3.21)	0.04	1.73 (1.11–2.72)	0.02	2.46 (1.47–4.11)	< 0.001
Visceral metastases	Yes vs No	2.03 (1.05–3.94)	0.04	2.27 (1.28–4.05)	0.005	1.13 (0.6–2.12)	0.71
PSA	Continuous (units of 100 ng/ml)	0.99 (0.95–1.03)	0.62	0.99 (0.96–1.02)	0.47	1 (0.97–1.03)	0.79

HR, hazard ratio; CI, confidence interval; ECOG, Eastern Cooperative Oncology Group; PSA, prostate-specific antigen