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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1. Introduction

Prostate cancer (PCa) is the most common cancer and the third leading cause of cancer death among 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 for metastatic castration-resistant PCa (mCRPC) over recent years [2]. On the basis of 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 have been shown to improve overall survival (OS) [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 research focus has been on the presence of AR splice variants as a cause of this resistance. AR splice variant 7 (AR-V7) is the most abundant splice variant. It lacks the androgen-binding site and remains constitutively active as a transcription factor, independent of androgen signaling [9,10]. The clinical importance of this finding was recently 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.

Alternative quantification of AR-V7 mRNA levels directly in peripheral whole blood has been reported [13–15]. The main advantage of this approach is 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 via 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 development [18], causes downregulation of epithelial proteins. Hence, these cells will be invisible to epithelial-based CTC detection methods. Taken together, these points suggest high potential for determining AR-V7 status in peripheral whole blood. However, an association between AR-V7 status in whole blood and resistance to treatment with abiraterone or enzalutamide has not been reported to date.

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 therapy.

2. Patients and methods

2.1. 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 2011 and 2016. These patients had progressive disease as defined according to Prostate Cancer Clinical Trials Working Group (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 wk before and every 4 wk after treatment initiation, as well as imaging procedures (computed tomography and bone scan) within 4 wk before and every 3 mo after treatment initiation.

The main endpoint was PSA response, defined as a PSA level decline of $\geq$50%, as a 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 OS. Clinical progression was defined as worsening of disease-related symptoms or new cancer-related complications, radiographic progression according to Response Evaluation Criteria In Solid Tumors [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].

2.2. Blood samples

Blood samples were collected in 2.7-ml PAXgene blood RNA tubes (Qiagen, Hilden, Germany) within 1 wk before treatment initiation. Total

RNA was extracted from blood samples according to the manufacturer's instructions using the PAXgene blood RNA kit (Qiagen). A NanoDrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA) was used for quantification and purity assessment of RNA samples. cDNA was synthesized from 1 μg of RNA using qScript XLT SuperMix (Quanta Biosciences, Beverly, MA, USA) according to the manufacturer's instructions.

2.3. ddPCR analysis

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

2.4. Healthy donors

We included 28 healthy male subjects (age <40 yr) 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 for patient samples to minimize any bias.

2.5. Statistical analysis

The Supplementary material contains details on the statistical analysis.

3. Results

3.1. Patient characteristics

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

Table 1 – Patient characteristics and clinical outcomes

Parameter	Value
Patients (n)	85
Median age, yr (IQR) [n = 85]	71 (66–74)
Median PSA, ng/ml (IQR) [n = 84]	211 (29–768)
Eastern Cooperative Oncology Group performance score, n (%) [n = 83]	
0	43 (52)
1	29 (35)
2	11 (13)
Prior systemic treatments for mCRPC, n (%) [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) [n = 85]	
0	9
1	39
2	27
3	10
Site of metastasis, n (%) [n = 83]	
Bone	80 (96)
Visceral	23 (28)
Deceased, n (%) [n = 84]	51 (60)
Median follow-up, mo (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)
Median PSA-PFS, mo (95% CI) [n = 74]	3.6 (3.2–4.1)
Median clinical PFS, mo (95% CI) [n = 82]	4.6 (3.1–6.2)
Median overall survival, mo (95% CI) [n = 84]	10.1 (5.8–14.5)

IQR = interquartile range; CI = confidence interval; mCRPC = metastatic castration-resistant prostate cancer; PSA = prostate-specific antigen; PFS = progression-free survival.

3.2. AR-V7 detection in peripheral whole blood

First we assessed the analytical validity of our ddPCR assay for AR-V7 and AR-FL isoform detection. AR-V7 mRNA from one VCaP cell against 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 from 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. To normalize AR-V7 expression, we calculated the fraction of AR-V7 transcripts over total AR (AR-V7 plus AR-FL) transcripts, and used this ratio in all subsequent analyses. The fraction of AR-V7 transcripts in whole blood of mCRPC patients ranged from 0% to 4.0% (mean 0.3%; Fig. 1B). Using the maximum AR-V7 fraction observed among healthy men (0.6%) as a cutoff, we dichotomized patients into "AR-V7 high" and "AR-V7 low" groups (Fig. 1B). Overall, 15/85 patients (18%) had high AR-V7 levels. According to prior therapy with zero, one, two, and three lines of systemic treatment, the number of patients with high AR-V7 levels was 0/9 (0%), 9/39 (23%), 3/27 (11%), and 3/10 (30%), respectively.

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

The overall proportion of patients with a PSA response defined as a PSA decline of ≥50% was 41% (31 of 74 men with available PSA follow-up). The PSA response rate was 0% in patients with high AR-V7 levels (0 of 12 men), and 50% in patients with low AR-V7 levels (31 of 62 men, Fig. 2). Thus, AR-V7 status was significantly associated 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). Only the association between AR-V7 status and PSA response remained significant, and high AR-V7 levels in whole blood were confirmed as an independent predictor of no PSA response to treatment with abiraterone or enzalutamide (p = 0.03). Furthermore, AR-V7 was confirmed as an independent predictor of no PSA response in a multivariable model with AR-V7 expression as a continuous variable (p = 0.04; Supplementary Table 1).

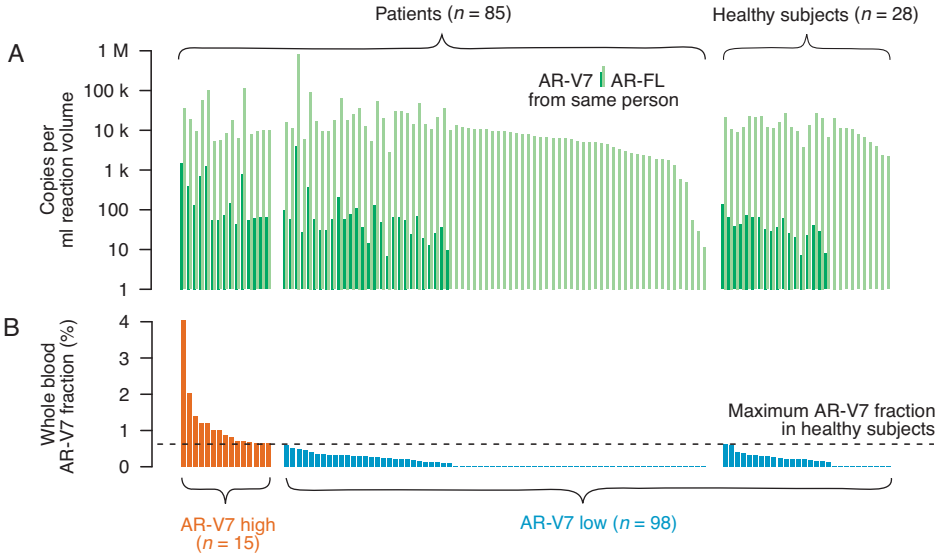

Fig. 1 – Quantification of androgen receptor splice variant 7 (AR-V7) in abiraterone- or enzalutamide-treated patients and healthy controls. (A) AR-V7 and full-length androgen receptor (AR-FL) mRNA levels in whole blood were quantified by droplet digital polymerase chain reaction for 85 patients treated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

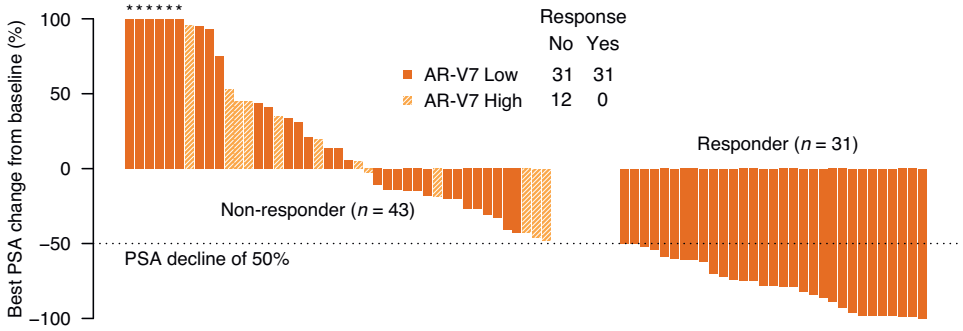

Fig. 2 – Waterfall plot of best prostate-specific antigen (PSA) changes and androgen receptor splice variant 7 (AR-V7) status. The dotted line depicts the threshold for defining a PSA response (≥50% reduction in PSA serum level from baseline). Asterisks indicate an increase of >100% in best PSA change. 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.

Table 2 – Multivariable logistic regression analyses [a]

Variable	Odds ratio (95% CI)	*p* value
AR-V7 (high vs low)	0.03 (0.00–0.70)	0.03
Abiraterone/enzalutamide pretreatment (yes vs no)	0.25 (0.06–1.09)	0.06
ECOG performance score (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

[a] AR-V7 status, prior treatment with abiraterone or enzalutamide, Eastern Cooperative Oncology Group (ECOG) performance status, presence of visceral metastases, and serum prostate-specific antigen (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).
CI = confidence interval.

3.4. 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 mo [95% confidence interval {CI} 1.8–3.0] vs 3.7 mo [95% CI 2.3–3.1]; $p < 0.001$; Fig. 3A), shorter clinical PFS (2.7 mo [95% CI 2.3–3.1] vs 5.5 mo [95% CI 4.4–6.6]; $p < 0.001$; Fig. 3B), and shorter OS (4.0 mo [95% CI 2.0–6.0] vs 13.9 mo [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 a continuous variable. We observed some

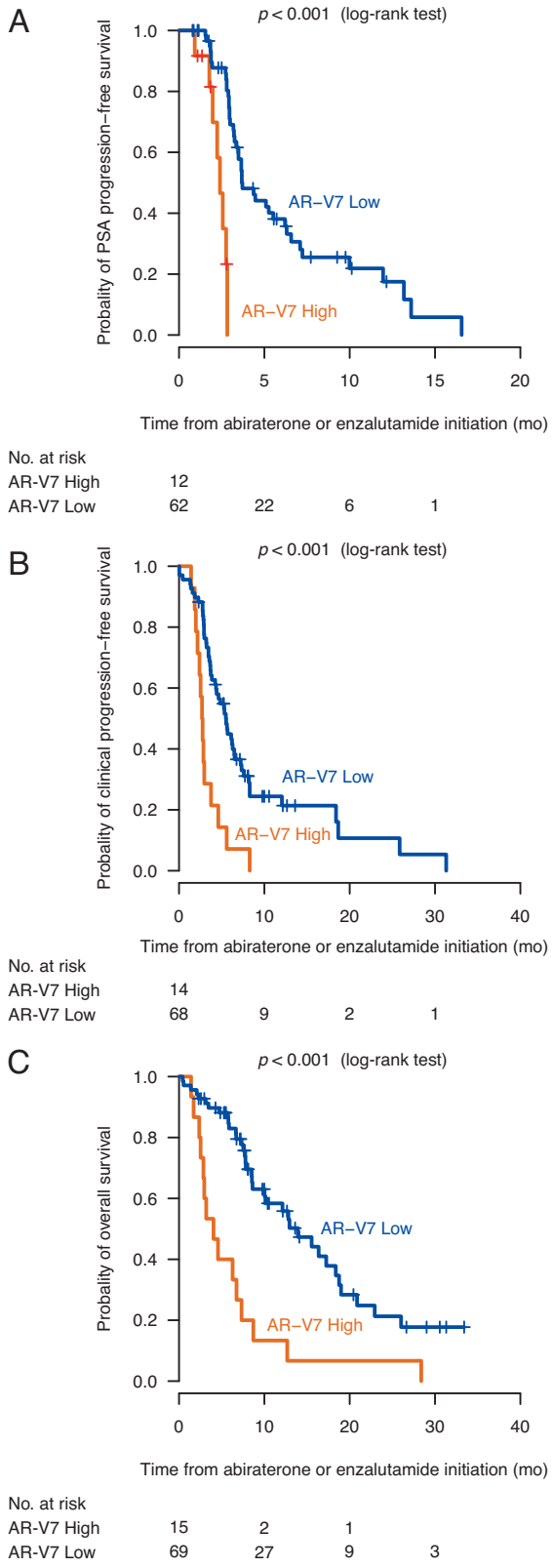

Fig. 3 – Kaplan-Meier analysis. (A) Prostate-specific antigen (PSA) progression-free survival, (B) clinical or radiographic progression-free survival, and (C) overall survival according to androgen receptor splice variant 7 (AR-V7) levels in whole blood.

evidence of poor PSA-PFS ($p = 0.15$) and clinical PFS ($p = 0.06$) with increasing AR-V7 expression, although this association did not meet conventional levels of statistical significance (Supplementary Tables 2 and 3, Supplementary Figs. 2 and 3). Moreover, increasing AR-V7 expression was confirmed as an independent prognostic factor for poor OS ($p = 0.02$; Supplementary Table 4, Supplementary Fig. 4).

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 from mCRPC patients from a prospective biorepository, we demonstrated 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 a PSA response, as well as shorter PSA-PFS, clinical PFS, and OS on multivariable analysis. To the best of 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 by Liu et al [13]. They compared AR-V7 detection rates using RNA isolated from either whole blood or CTCs enriched via leukocyte depletion for ten 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 higher sensitivity for the whole-blood RNA approach compared to CTC enrichment via leukocyte depletion. Moreover, in support of our data, Todenhöfer et al [14] reported an association of AR-V7 status in whole blood with PSA-PFS and OS in 37 mCRPC patients undergoing abiraterone treatment. While none of the four AR-V7–positive patients achieved a PSA response with a decline $\geq$50%, the statistical analysis did not reach significance, potentially due to the small sample size. Furthermore, Qu et al [15] used ddPCR to quantify mRNA levels of AR-V7 in whole blood from mCRPC patients treated with abiraterone ($n = 81$) or enzalutamide ($n = 51$) and found an association with time to treatment failure. A limitation of their study was that the 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 from healthy men [14]. This emphasizes the necessity of determining robust and clearly defined thresholds for AR-V7 levels in whole blood for translation into clinical routine testing and standardization of clinical decision-making. By contrast, solely qualitative AR-V7 detection in whole blood may lead to conflicting data as

Table 3 – Multivariable Cox regression analyses [a]

Variable	PSA-PFS		Clinical PFS		Overall survival	
	HR (95% CI)	*p* value	HR (95% CI)	*p* value	HR (95% CI)	*p* value
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
Abi/Enza pretreatment (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

[a] For the outcomes prostate-specific antigen progression-free survival (PSA-PFS), clinical PFS, and overall survival, one multivariable model for association of the covariates AR-V7, prior treatment with abiraterone (Abi) or enzalutamide (Enza), Eastern Cooperative Oncology Group (ECOG) performance status, presence of visceral metastases, and serum PSA levels with the outcome variable was created.
HR = hazard ratio; CI = confidence interval,

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 high variation, ranging between 11% and 68% [8,11–15]. This is attributable to various causes, most importantly the variety of methods used, including CTC-derived RNA- or protein-based assays as well as different whole-blood assays. However, the optimal method for determining 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 rates may be attributable to the heterogeneity of patient cohorts. In our study, AR-V7 positivity ranged from 0% to 30% corresponding to a range of zero to three 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], the 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 [11] described one patient who showed a PSA response to abiraterone despite CTC positivity for AR-V7 mRNA. Likewise, Bernemann et al [27] from the same group conducted a retrospective study in which PSA response to abiraterone or enzalutamide was assessed in 21 patients with CTCs positive for AR-V7 mRNA. In their cohort, four patients (19%) achieved a PSA decline ≥50%. One potential explanation is that AR-V7–positive patients achieving a 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 patients with high AR-V7 levels who had a close to 50% PSA decline (43%, 46%, and 48%), all of whom were treated with abiraterone. However, these patients did not experience prolonged benefit from their treatment. Two of the patients developed clinical progression within 3 mo, and the third patient experienced clinical progression after 4 mo and died after 6 mo.

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 approximately 4 d, and storage at −80 °C over long time periods. Furthermore, it has been shown that digital PCR is reproducible across laboratories [28] with greater precision, better day-to-day reproducibility, and similar sensitivity compared to quantitative real-time PCR.

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

5. Conclusions

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 no PSA response and shorter PSA-PFS, clinical PFS, and OS. These results support AR-V7 as a predictive biomarker for nonresponse 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 to determine the clinical utility of AR-V7 as a resistance marker and quantify the survival benefit of AR-V7–guided therapy selection.

Author contributions: Matthias M. Heck 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: Heck, Winter.
Acquisition of data: Thoene, Secci, Winter, Seitz, Nawroth, Tauber, Thalgott, Schmid, Heck.
Analysis and interpretation of data: Heck, Winter, Bietenbeck, Seitz.
Drafting of the manuscript: Heck, Seitz, Winter.
Critical revision of the manuscript for important intellectual content: Thoene, Bietenbeck, Tauber, Nawroth, Retz, Gschwend, Ruland.
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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.2017.07.024.

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