Supplementary Methods

Patient blood samples

Blood samples were collected in 2.7 ml PAXgene™ blood RNA tubes (Qiagen) and stored at –80°C within 6 hours. The time point of sample collection was within 1 week before treatment initiation.

RNA purity

The NanoDrop 1000 spectrophotometer (Thermo Scientific) was used for quantification and purity assessment of RNA samples. All analyzed samples passed a quality control which included a ratio of absorbance at 260/280nm ≥ 2.0 in order to exclude DNA contamination and a ratio of absorbance at 260/230nm ≥ 1.9 to exclude the presence of contaminants.

AR-V7 and AR-FL assays

The following primers and probes were designed and purchased from Integrated DNA Technologies (IDT) for use in all droplet digital PCR (ddPCR) reactions:

Transcript	Type	Sequence
AR-FL	fwd	5’- ACT CCG TGC AGC CTA TTG -3’
	rev	5’- GGG CAC TTG CAC AGA GA -3’
	probe	5’- /5HEX/ CAC ATG GTG /ZEN/ AGC GTG GAC TTT /3IABkFQ/ -3’
AR-V7	fwd	5’- GGG ATG ACT CTG GGA GAA AA -3’
	rev	5’- CCC TCT AGA GCC CTC ATT TTG -3’
	probe	5’- /56-FAM/ CAG ACC CTG /ZEN/ AAG AAA GGC TGA C /3IABkFQ/ -3’

fwd, forward primer
rev, reverse primer
/5HEX/, 5' HEX fluorophore
/ZEN/, ZEN internal quencher
/3IABkFQ/, 3' Iowa Black FQ dark quencher
/56-FAM/, 5' 6-FAM fluorophore

PCR was used to validate the assays. Specificity of the primer design was assessed by a Nucleotide BLAST (blastn) search against the human Reference RNA sequences (refseq_rna) database at https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome. Synthetic DNA comprising the amplicon sequences (gBlocks) were purchased from IDT and used as positive controls in all ddPCR analyses. Purified nuclease-free water was used as negative, no-template control (NTC). Genomic DNA (gDNA) from peripheral blood leukocytes of healthy female and male subjects was used as negative control.

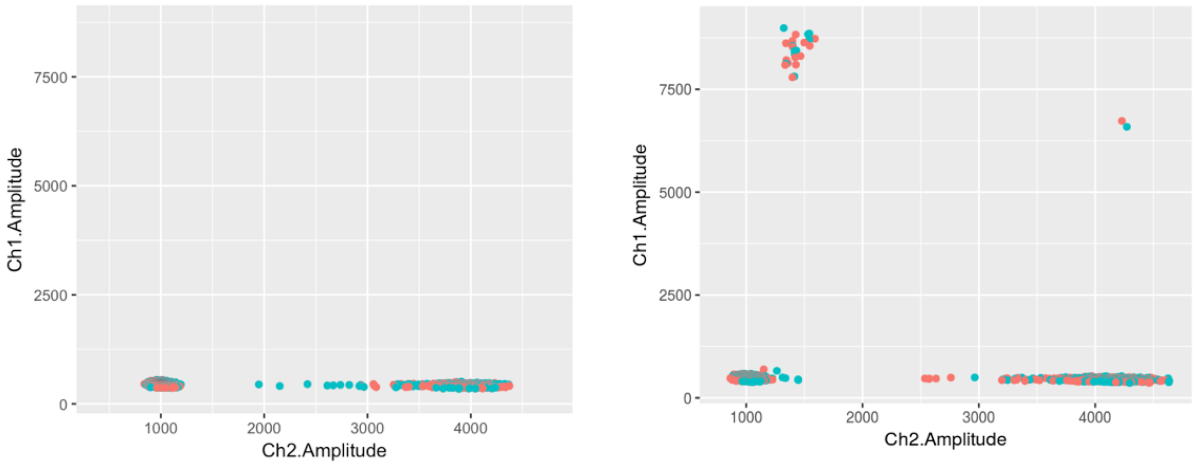
Droplet digital PCR reactions

AR-V7 and AR-FL transcript quantifications were carried out on a QX200 Droplet Digital PCR (ddPCR) system with automated droplet generation (Bio-Rad Laboratories). Reactions were carried out in 96-well microtiter plates (twin.tec PCR, Eppendorf). Each well contained 10.5 µl of ddPCR Supermix for Probes (no dUTP; Bio-Rad), 2 x 1.05 µl of target-specific primers (900 nmol/l), 2 x 1.05 µl of target-specific probe (250 nmol/l), and 6.3 µl of sample cDNA, for a total volume of 21 µl. Plates were sealed, spun down and loaded into a QX200 automated droplet generator (Bio-Rad). Immediately after droplet generation, 96-well plates containing droplet-partitioned samples were sealed and PCR was carried out on a C1000 Touch Thermal Cycler (Bio-Rad) using the following cycling protocol: enzyme activation at 95 °C for 5 minutes followed by 40 cycles of 94 °C for 30 seconds (for denaturation) and 60 °C for 60 seconds (for annealing/extension), followed by a final 10 minute incubation at 98 °C for enzyme deactivation. Ramp rate was 2 °C per second. Plates were then kept at 4 °C. Plates were read on a Bio-Rad QX200 droplet reader. All samples were measured in duplicates (2 wells) with a cDNA equivalent of 315 ng total RNA per well. Positive controls (gBlocks), NTC and negative controls (gDNA) were included in every run in duplicates as well.

The number of partitions (droplets) per reaction was 15354 on average (range 5308 to 21155, standard deviation (SD) 2275). With a droplet volume of 0.85 nl reported by Bio-Rad, the effective reaction size (total volume of partitions measured) was 13.1 µl on average (range 4.5 to 18 µl, SD 1.9 µl).

Droplet digital PCR data analysis

Raw droplet fluorescence intensity values for each droplet were exported from QuantaSoft droplet reader software v1.7.4 (Bio-Rad). To ensure automatic, unbiased, reproducible, and operator-independent thresholding (separating negative from positive droplets), custom scripts were developed and used to import droplet intensities and classify each droplet as positive or negative in R (version 3.2.3; http://www.r-project.org). Target mRNA concentrations *c* were then calculated for each well from the number of positive droplets *Np* and negative droplets *Nn* and the average droplet volume *V* = 0.85 nanoliter based on Poisson distribution statistics using the formula c = (ln(Np + Nn) – ln(Nn))/V. All operators involved in the measurements were blinded to the assignment of samples to healthy control subjects or patients and their outcome.



Examples of AR-V7 (Ch1, y-axis) and AR-FL (Ch2, x-axis) quantification in RNA from whole blood of a healthy control (left) and a patient (right). Each dot represents one droplet, and the two droplet colors mark data from two technical replicates.

AR-V7 positive cell line experiments

The limit of detection of our ddPCR assay was determined by spiking different cell numbers of the AR-V7 positive PCa cell line VCaP into 1 million leukocytes from pooled buffy coats of healthy subjects followed by quantification of AR-V7 and AR-FL mRNA. Cells were counted on a FACS machine (BD FACSAria III).

Statistical analysis

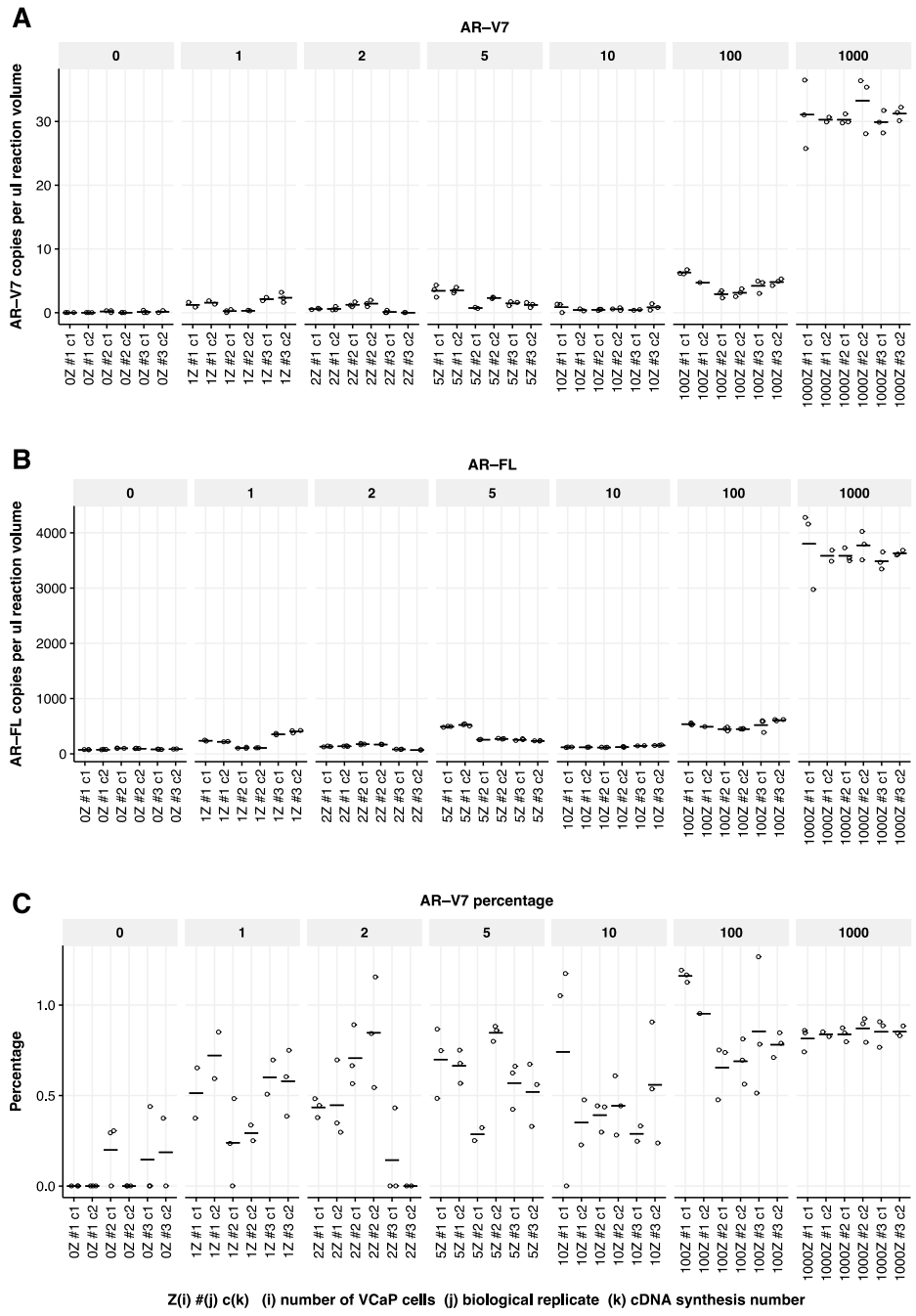
Data were analyzed using IBM SPSS Statistics version 24.0 and R version 3.2.3. Univariable association analysis of AR-V7 status with PSA response was performed with the use of Fisher's exact test. Multivariable Firth logistic regression [1] as implemented in the R package brglm [2] was used to model the influence of AR-V7 status adjusted for clinical variables on PSA response. Time-to-event outcomes (PSA-PFS, clinical PFS, and OS) were assessed using Kaplan–Meier curves with log-rank statistics. Multivariable Cox regression analyses using the R function coxph were carried out to calculate hazard ratio (HR) and 95% confidence intervals (CI) for the association of AR-V7 and clinical variables with time-to-event outcomes. As clinical variables, we included Eastern Cooperative Oncology Group (ECOG) performance score, prior treatment with abiraterone or enzalutamide, presence of visceral metastases, and serum PSA levels (all at baseline) as potential predictors in all regression analyses. All tests were two-sided and *P* values < 0.05 were considered statistically significant.

Supplementary References

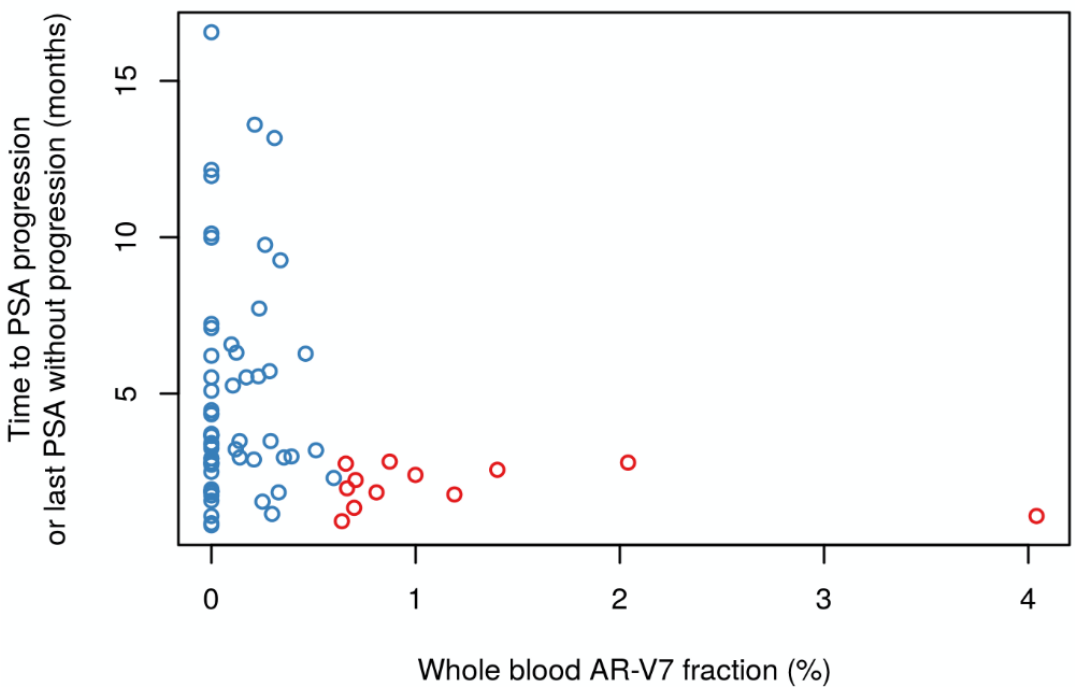
1. Firth D. Bias reduction of maximum likelihood estimates. Biometrika 1993;80:27–38.

2. Kosmidis I. *brglm: Bias reduction in binomial-response Generalized Linear Models*. Available at: http://www.ucl.ac.uk/~ucakiko/software.html

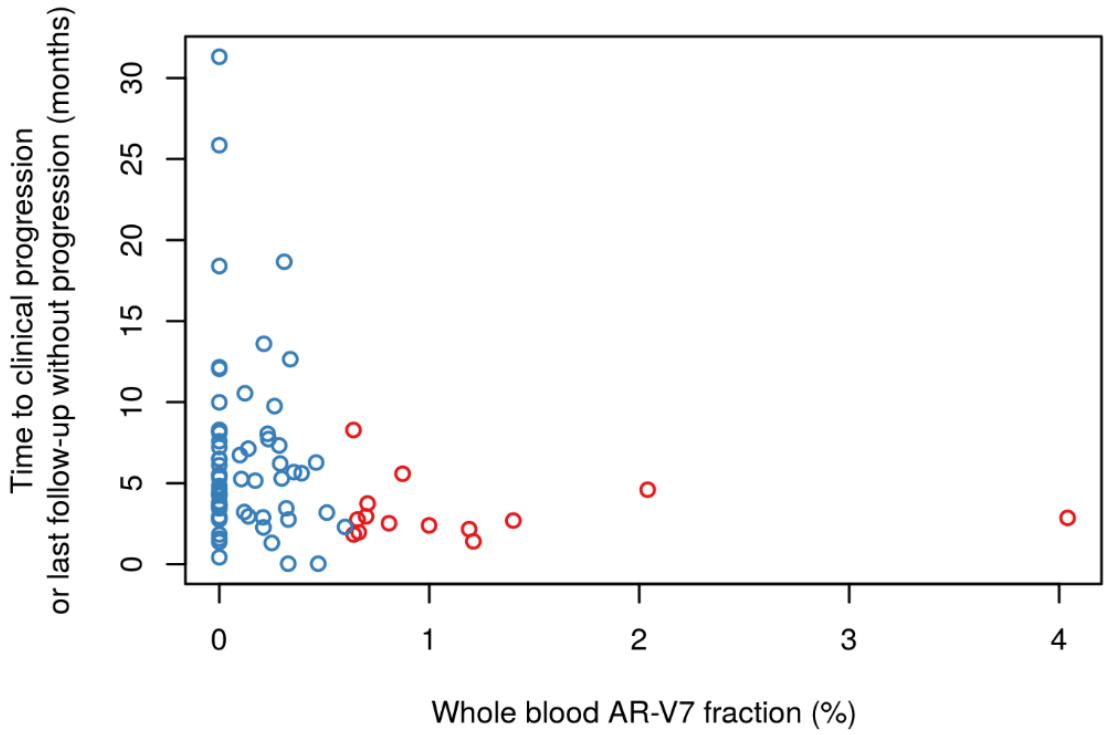
Supplementary Figures



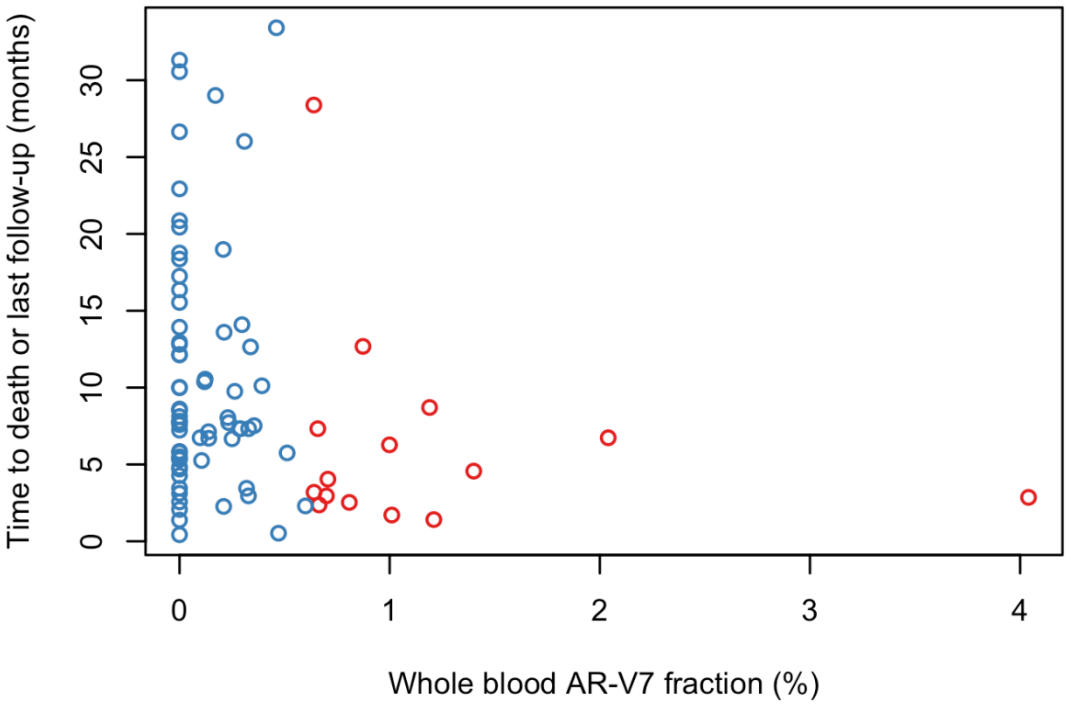
Supplementary Figure 1. AR-V7 and AR-FL quantification of VCaP cells in leukocytes. From samples with different numbers of VCaP cells (0, 1, 2, 5, 10, 100, and 1000) spiked into one million leukocytes, RNA was extracted, cDNA was synthesized from RNA and quantified for AR-V7 (A) and AR-FL (B) using our ddPCR assay. From this, the corresponding AR-V7 percentage was calculated as AR-V7 / (AR-V7 + AR-FL) (C). Each experiment was repeated three times (#1 to #3), with two separate cDNA synthesis steps (c1, c2) each, and ddPCR measurements were carried out in triplicates.



Supplementary Figure 2. Time to PSA progression or last PSA without progression vs AR-V7 levels. Patients with low AR-V7 levels are plotted in blue, patients with high AR-V7 levels are plotted in red.



Supplementary Figure 3. Time to clinical progression or last follow-up without progression vs AR-V7 levels. Patients with low AR-V7 levels are plotted in blue, patients with high AR-V7 levels are plotted in red.



Supplementary Figure 4. Time to death or last follow-up vs AR-V7 levels. Patients with low AR-V7 levels are plotted in blue, patients with high AR-V7 levels are plotted in red.

Supplementary Tables

Supplementary Table 1. Multivariable logistic regression analysis for PSA response with AR-V7 as continuous variable. AR-V7 as continuous variable, 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	Continuous	0.09 (0.01–0.85)	0.04
Pretreatment with abiraterone or enzalutamide	Yes vs No	0.26 (0.06–1.18)	0.08
ECOG	0, 1, or 2	0.55 (0.20–1.51)	0.24
Visceral metastases	Yes vs No	0.89 (0.25–3.21)	0.86
PSA	Continuous (units of 100 ng/ml)	1.02 (0.96–1.09)	0.51

Supplementary Table 2. Multivariable Cox regression analysis for PSA progression-free survival with AR-V7 as continuous variable. AR-V7 as continuous variable, prior treatment with abiraterone or enzalutamide, ECOG performance status, presence of visceral metastases, and serum PSA levels were assessed in one multivariable model.

Variable	Values	HR (95% CI)	p
AR-V7	Continuous	1.74 (0.81–3.70)	0.15
Pretreatment with abiraterone or enzalutamide	Yes vs No	1.22 (0.57–2.59)	0.61
ECOG	0, 1, or 2	2.24 (1.30–3.85)	0.004
Visceral metastases	Yes vs No	1.90 (0.99–3.67)	0.06
PSA	Continuous (units of 100 ng/ml)	0.99 (0.95–1.03)	0.58

Supplementary Table 3. Multivariable Cox regression analysis for clinical progression-free survival with AR-V7 as continuous variable. AR-V7 as continuous variable, prior treatment with abiraterone or enzalutamide, ECOG performance status, presence of visceral metastases, and serum PSA levels were assessed in one multivariable model.

Variable	Values	HR (95% CI)	p
AR-V7	Continuous	1.44 (0.99–2.12)	0.06
Pretreatment with abiraterone or enzalutamide	Yes vs No	1.23 (0.63–2.41)	0.54
ECOG	0, 1, or 2	1.83 (1.17–2.86)	0.01
Visceral metastases	Yes vs No	2.51 (1.40–4.47)	0.002
PSA	Continuous (units of 100 ng/ml)	1.00 (0.97–1.02)	0.83

Supplementary Table 4. Multivariable Cox regression analysis for overall survival with AR-V7 as continuous variable. AR-V7 as continuous variable, prior treatment with abiraterone or enzalutamide, ECOG performance status, presence of visceral metastases, and serum PSA levels were assessed in one multivariable model.

Variable	Values	HR (95% CI)	p
AR-V7	Continuous	1.66 (1.10–2.50)	0.016
Pretreatment with abiraterone or enzalutamide	Yes vs No	1.37 (0.62–3.05)	0.44
ECOG	0, 1, or 2	2.50 (1.50–4.15)	< 0.001
Visceral metastases	Yes vs No	1.27 (0.68–2.38)	0.45
PSA	Continuous (units of 100 ng/ml)	1.02 (0.99–1.04)	0.24