

Supplementary Online Material

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Appendix S1

1. Eligibility Criteria of Primary cohort

Inclusion Criteria

1. Patients must have histologically-confirmed adenocarcinoma of prostate without neuroendocrine differentiation or small cell histology.
2. Patients have progressive disease despite “castration levels” of serum testosterone (<50 ng/dL) (≤ 1.73 nmol/L), and ongoing LHRH analogue treatment or prior surgical castration.
3. Progression as defined by at least two of the following: a rise in PSA, worsening symptoms, or radiological progression, namely, progression in soft tissue lesions measured by computed tomography imaging according to the modified Response Evaluation Criteria in Solid Tumors (RECIST) or progression on bone scanning according to criteria adapted from the Prostate Cancer Working Group (PCWG2) criteria.
4. Patients have not received radiotherapy, chemotherapy, or immunotherapy at least 30 days prior to the treatment.
5. Male, aged ≥ 18 years.
6. Life expectancy of greater than three months.
7. Eastern Cooperative Oncology Group (ECOG) Performance Status ≤ 2 .
8. Able to swallow the study drug whole as a tablet.
9. Willing to use a method of birth control with adequate barrier protection.
10. Patients must have normal organ and marrow function as defined below:
 - a. leukocytes $> 3,000/\text{mL}$
 - b. absolute neutrophil count $> 1,500/\text{mL}$
 - c. platelets $> 100,000/\text{mL}$
 - d. total bilirubin within normal institutional limits
 - e. AST(SGOT)/ALT(SGPT) $< 2.5 \times$ institutional upper limit of normal
 - f. creatinine within normal institutional limits
11. No evidence (within five years) of prior malignancies (except successfully treated basal cell or squamous cell carcinoma of the skin).
12. Participant is willing and able to give informed consent for participation in the study.

Exclusion Criteria

1. Patients who have had previous therapy with abiraterone and/or enzalutamide.
2. Concurrent use of other anticancer agents or treatments, with the following exceptions:
 - a. LHRH agonists or antagonists
 - b. denosumab or bisphosphonate (e.g., zoledronic acid).
3. Uncontrolled intercurrent illness including, but not limited to, ongoing or active infection, symptomatic congestive heart failure, unstable angina pectoris, cardiac arrhythmia, or psychiatric illness/social situations that would limit compliance with study requirements.
4. History of seizures or any disease that could predispose to seizure, including history of lost of consciousness or transient stroke in the last 12 months before inclusion (day 1).
5. Have a history of gastrointestinal disorders that may interfere with the absorption of the study agents.
6. Have a pre-existing condition that warrants long-term corticosteroid use in excess of study dose.
7. Have known allergies, hypersensitivity or intolerance to abiraterone acetate, prednisone, enzalutamide, or their excipients.
8. Other primary tumor (other than CRPC) including hematological malignancy present within the last five years (except non-melanoma skin cancer or low-grade superficial bladder cancer).

2. Eligibility Criteria of PREMIERE cohort

Inclusion criteria.

1. Age ≥ 18 years old.
2. Histologically or cytologically confirmed of prostate adenocarcinoma without neuroendocrine differentiation or small cell characteristics.
3. Ongoing androgen deprivation with GnRH analog or bilateral orchiectomy.
4. Testosterone serum levels ≤ 1.73 nmol/L or 50 ng/dL at the screening visit.
5. Patients receiving bisphosphonate therapy must have been on stable doses for at least four weeks before study entry.
6. Progressive disease at study entry, defined by one or more of the three following criteria while the patient was on androgen deprivation therapy:
 - a. PSA progression defined by a minimum of two rising PSA values with an interval of \geq one week between each determination. Patients that have received anti-androgen must be in progression upon anti-androgen withdrawal at least four weeks for flutamide and six weeks since the last dose of bicalutamide or nilutamide. PSA value should be ≥ 2 $\mu\text{g/L}$ (2 ng/mL).
 - b. Progression in soft tissue according to RECIST 1.1
 - c. Bone progression defined by the PCWG2 criteria, at least two new more lesions in the bone scan.
7. Metastatic disease documented by bone lesions in bone scan or by measurable soft tissue lesions by CT or MRI. Patients whose disease was limited to lymph nodes were required to have a lesion with a minor diameter of 2.5 cm.
8. No prior cytotoxic chemotherapy for prostate cancer.
9. Patients without previous abiraterone acetate treatment.
10. Asymptomatic or minimally symptomatic disease from prostate cancer (i.e., the score on Brief Pain Inventory question Short form question #3 must be < 4).
11. Eastern Cooperative Oncology Group (ECOG) performance status of 0-1.
12. Estimated life expectancy of \geq six months.
13. Patient able to swallow the study drug and to follow-up the study requirements.
14. Informed consent for the biomarker study: *TMPRSS2-ETS* rearrangement and the obtained samples.

Exclusion criteria.

1. Comorbidity, infection or severe concurrent disease, in the judgment of the investigator, that makes the patient not suitable for inclusion in the study.
2. Known or suspicion of brain or leptomeningeal disease.
3. History of another malignancy within the previous 5 years other than cured non-melanoma skin cancer.
4. Hematological count at screening selection:
 - a. Absolute neutrophil count $< 1,500/\mu\text{L}$
 - b. Platelet count $< 100,000/\mu\text{L}$
 - c. Haemoglobin < 5.6 mmol/L (9 g/dL)
5. Liver function at the screening visit: total bilirubin, aminotransferase (ALT) or aspartate aminotransferase (AST) > 2.5 times upper normal limit.
6. Renal function at the screening visit: creatinine > 177 $\mu\text{mol/L}$.
7. Albumin value < 30 g/L (3 g/dL) at the screening visit.
8. History of seizures or any disease that could predispose to seizure, including history of loss of consciousness or transient stroke in the last 12 months before inclusion (day 1).
9. Clinically significant cardiovascular disease, including:
 - a. Myocardial infarction within six months
 - b. Uncontrolled angina within three months

- c. Congestive heart failure New York Heart Association (NYHA) class III or IV or history of congestive heart failure class III or IV in the past, unless a screening echocardiogram or multi-gated acquisition scan performed within three months results in a left ventricular ejection fraction $\geq 45\%$.
- d. History of clinically significant ventricular arrhythmias (e.g., ventricular tachycardia)
- e. Heart block (Mobitz II or III without a permanent pace-maker in place).
- f. Hypotension at the screening visit, as indicated by systolic blood pressure < 86 mmHg)
- g. Bradycardia as indicate by a heart rate of < 50 beats per minute on the screening ECG.
- h. Uncontrolled hypertension as indicated by systolic blood pressure > 170 or diastolic blood pressure > 105 rpm at the screening visit.
- 10. Gastrointestinal disorder affecting absorption (e.g., gastrectomy, active peptic ulcer disease within three months).
- 11. Major surgery within last four months of inclusion.
- 12. Use of opioids for pain within four weeks before screening visit.
- 13. Use of radiotherapy for the treatment of the primary tumor within three weeks before treatment.
- 14. Use of radiotherapy for the treatment of metastasis within two months before study entry.
- 15. Use of radium-223 or other radionuclides for the treatment of bone disseminated disease.
- 16. Treatment with flutamide within four weeks of enrollment.
- 17. Treatment with bicalutamide or nilutamide within six weeks before enrollment in the study.
- 18. Treatment with 5- α reductase (finasteride, dutasteride), estrogens or ciproterone acetate within four weeks of enrollment.
- 19. Treatment with biological therapy for prostate cancer (other than bone targeted agents and GnRH analogues) or other drugs with antitumoral activity in the four weeks before study entry.
- 20. History of prostate cancer progression on ketoconazole.
- 21. Previous use, or participation in a clinical trial, of an investigational drug that blocks androgen synthesis (e.g., abiraterone, TAK-100, TAC 683, TAK-448) or target the androgen receptor (e.g., ARN507, BMS641988).
- 22. Participation in a clinical trial including enzalutamide.
- 23. Use of an investigational drug in the four weeks of enrollment.
- 24. Use of herbal products that may have hormonal anti-cancer activity or that modify PSA levels, systemic steroids at a dose higher than the equivalent of 10 mg of prednisone within four weeks of enrollment.
- 25. Hereditary fructose intolerance.
Any condition or reason that in the opinion of the investigator interferes with the ability of the patient to participate in the trial, which places the patient at undue risk, or complicates the interpretation of safety data.

Appendix S2. Detection of *AR* aberrations by digital droplet PCR in plasma samples

Circulating DNA was extracted from one to two ml of plasma with the QIAamp Circulating Nucleic Acid Kit (Qiagen). Total extracted plasma DNA was quantified with the Quant-iT high sensitivity PicoGreen double-stranded DNA Assay Kit (Invitrogen). DdPCR was performed on a QX200 ddPCR system (Bio-Rad). Copy number (CN) assays were performed for *AR* (Hs04121925_cn, FAM) and centromeric chromosome X gene *ZXDB* (Hs02220689_cn, FAM, Life Technologies) with *NSUN3* (dHsaCP2506682, HEX, Bio-Rad), *EIF2C1* (dHsaCP1000002, HEX, Bio-Rad), and *AP3B1* (dHsaCP1000001, HEX, Bio-Rad) as reference genes. We developed multiplex assays by varying the concentration of the fluorescent probes to differentiate droplets positive for respective genes on the basis of fluorescence intensity [1-3].

Rare mutation detection assays were performed for the *AR* mutations 2105T>A (p.L702H), 2632A>G (p.T878A), and 2629T>C (p.F876L) using a custom-made single nucleotide polymorphism (SNP) genotyping assay (Life Technologies), the SNP genotyping assay rs137852581 (Life Technologies), and the SNP genotyping assay rs137852578 (Life Technologies), respectively.

PCR reactions were prepared with 1-2 ng DNA, 10ul 2xSupermix and a total volume of primer probe assays of 2ul in a total volume of 20ul. PCR reactions were partitioned into ~20,000 droplets per sample with an Automated Droplet generator (Bio-Rad). Emulsified PCR reactions were run on a Mastercycler Nexus GSX1 (Eppendorf). For mutation assays, ddPCR conditions were optimized with a temperature gradient to identify the optimal annealing/extension temperature using wild-type DNA spiked with a synthetic oligonucleotide containing the mutation of interest. We selected the optimal temperature for incubation on the Mastercycler Nexus GSX1. Samples were incubated at 99°C for 10 min followed by 40 cycles of 95°C for 15 sec, 60°C for 60 sec, followed by 10 min incubation at 98°C for the *AR* copy number multiplex assay. For *AR* mutation detection, samples were incubated at 99°C for 10 min followed by 40 cycles of 95°C for 15 sec, 56-61°C for 60 sec, followed by 10 min incubation at 98°C.

Samples were read on a Bio-Rad QX200 droplet reader using QuantaSoft v1.3.2.0 software for *AR* CN analysis and mutation detection. At least two negative control wells with no DNA were included in every run. An oligo carrying the mutation of interest was used as a positive control for mutation assays. In addition, two wells with DNA from a germ line sample, characterized by the complete absence of mutation and normal *AR* CN status, were also included. Positive and negative clusters were gated using the FAM and VIC/HEX thresholds based on the amplitude of positive and negative controls that were ran concomitantly with each assay. Poisson distribution was used to estimate the average number of copies per reaction microliters. CN ratios of *AR* and reference genes and mutant vs wild-type were calculated for each sample to determine *AR* CN and the mutation allele fraction respectively as described previously [4].

Appendix S3. Statistical analysis

Using NGS we previously used *AR* amplicon variance in healthy volunteer plasma to set a cut-off of 1.91 for calling a patient CN gain. We do not observe variance with ddPCR and could therefore theoretically choose any cut-off >1. We performed a systematic search over all observed values of *AR* CN to identify the *AR* CN, which optimally splits the patients into two groups who have different prognosis of overall survival as we had hypothesized that *AR* gained patients have higher hazard rates than *AR* normal patients. *AR* mutant patients were excluded for this research. We used log-likelihood as correlative measure in a multivariable Cox proportional hazard model which included *AR* CN and serum lactate dehydrogenase as the second variable and was stratified by chemotherapy status of the patients. It has been shown that multivariable approach increases the accuracy of the cutpoint [5]. We used bootstrapping with replacement technique and iterated the search for the optimal cutpoint 30,000 times to estimate the measures of dispersion of the cutpoint. The search for cutpoint and the bootstrapping were performed using an in-house developed R script (supplementary Figure S2, available at *Annals of Oncology* online).

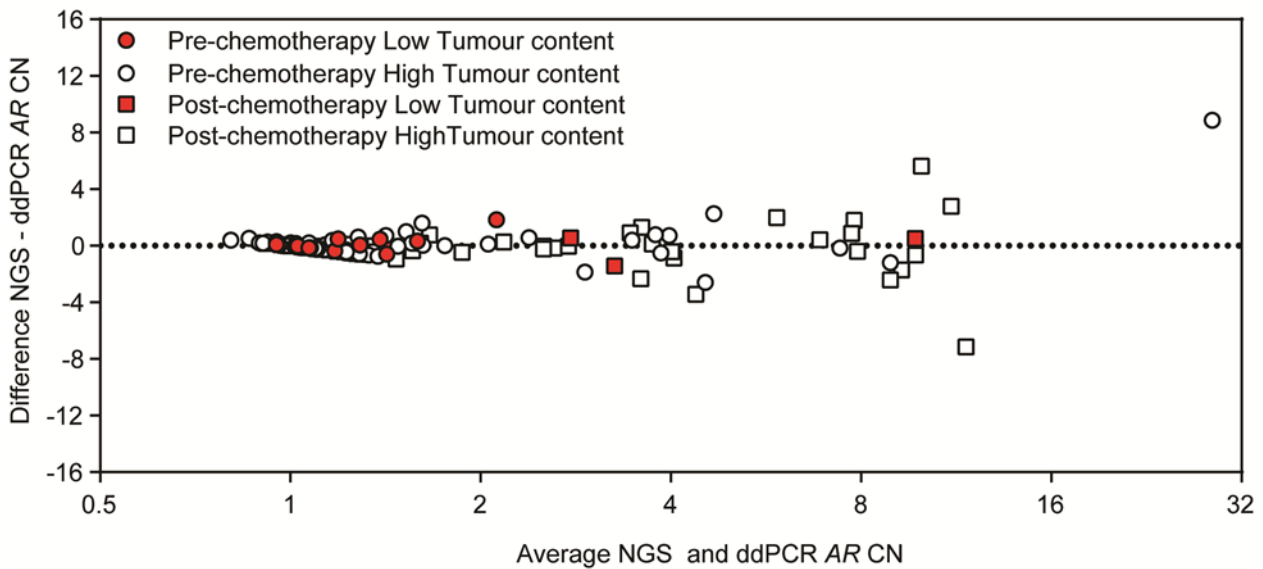
The association of *AR* status with progression-free survival (PFS) and overall survival (OS) was evaluated using univariable Cox regression. Survivor function of Time-to-event outcomes were also estimated using the Kaplan-Meier method. Differences between survivor functions of patients with *AR* CN gain vs *AR* CN normal (and *AR* mutant vs *AR* no mutant in docetaxel-treated patient group) were evaluated using the log-rank test. The association of *AR* status with time-to event outcomes was evaluated and hazard ratios (HRs) estimated from univariable and multivariable Cox proportional hazards regression methods (Figure 1A-C and Figure 2A-C).

Best PSA responses were depicted using standard waterfall plots; odds ratios (ORs) and the corresponding 95% confidence interval (CI) of PSA response were determined using a 2x2 contingency table and the Woolf logit method. Statistical significance was determined using Fisher's exact test (Figure 1E-F and Figure 2D).

The pre-treatment predictors evaluated for the multivariable Cox proportional hazards models included *AR* CN (gain vs normal), *AR* mutant (yes vs no), lactate dehydrogenase levels [>upper normal limit (UNL) vs ≤UNL], presence of liver metastases (yes vs no), presence of bone metastases (≤5 vs >5), neutrophil-to-lymphocyte ratio (>3 vs <3), alkaline phosphatase levels (>UNL vs ≤UNL), hemoglobin levels (≥UNL vs <UNL), albumin levels (>UNL vs ≤UNL), previous chemotherapy (yes vs no), dsDNA concentration (continuous variable), PSA levels (continuous variable), and patient age (continuous variable) (supplementary Table S4 and Table S5B, available at *Annals of Oncology* online). The final multivariable analyses were assessed using a proportional hazard model after stepwise backwards elimination by Akaike information criterion (Table 2).

Supplementary Figures

A



B

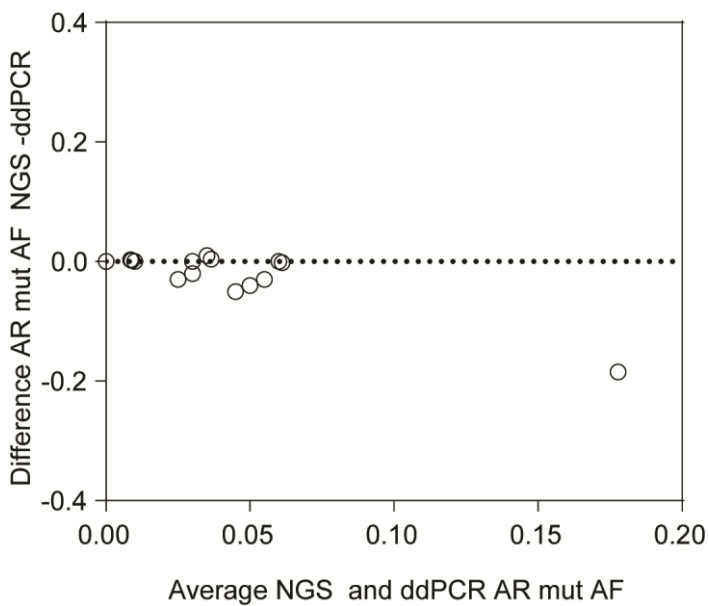
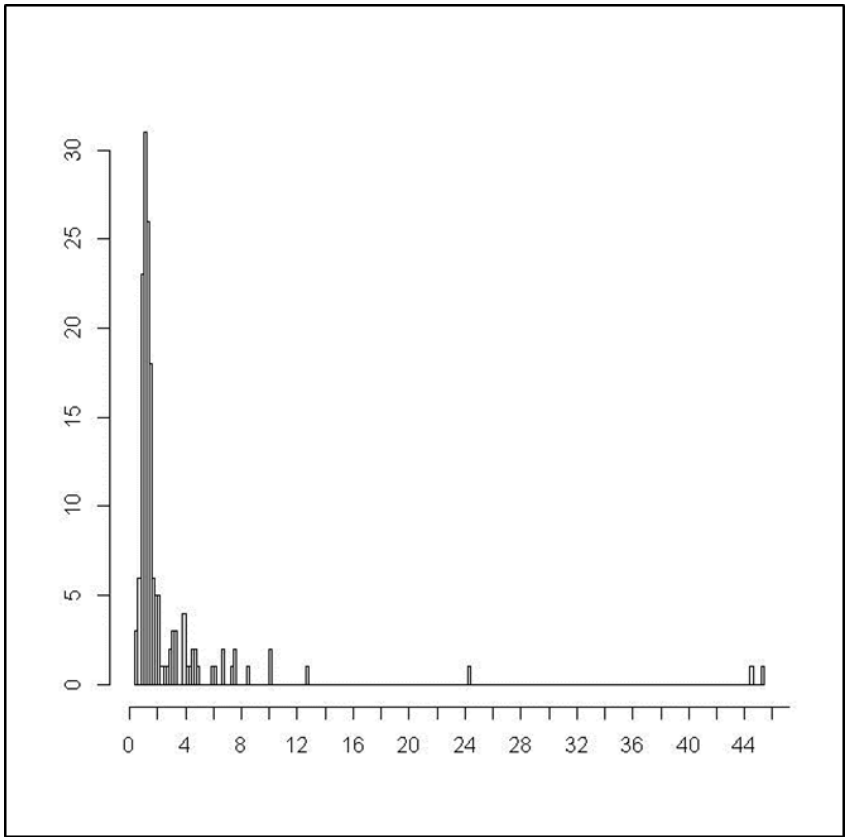


Figure S1. Evaluation of ddPCR copy number (CN) and mutation assay performance. Bland-Altman plot showing agreement of ddPCR and NGS AR copy number assessment, low tumor content samples had a tumor content fraction below 0.075 (A). Bland-Altman plot showing agreement of ddPCR and NGS AR mutation frequencies (B).

A



B

Histogram of The Highest Maximum Log-Likelihood

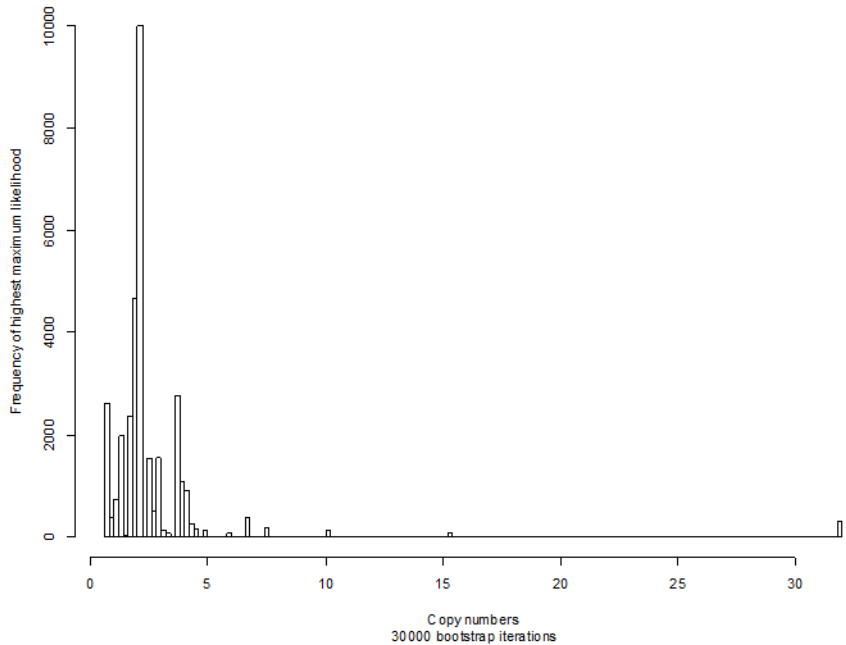


Figure S2. Selection of cut-off for AR CN gain by ddPCR. Range of AR CN across primary cohort (A), Cut-off analysis with maximum log-likelihood as the correlative statistic of the multivariable Cox proportional hazard model and boot-strapping with 30,000 iterations to provide the cut-off point dispersion (B).

Supplementary Tables

Table S1. Samples analysed by both NGS and ddPCR

		Pretreatment (<i>n</i>)	Progression (<i>n</i>)
NGS data included in previously published cohort [6]	Chemotherapy-naive	8	1
	Post-docetaxel	58	19
NGS data not in previously published cohort	Chemotherapy-naive	53	22
	Post-docetaxel	0	0
	Total	119	42

Abbreviations. ddPCR, droplet digital PCR; *n*, number; NGS, next generation sequencing.

Table S2. Agreement of AR CN gain call by ddPCR vs NGS

Chemotherapy-naive Cut off 2.01	AR Normal NGS	AR Gain NGS	NGS TC <0.075
AR Normal ddPCR	57	3	12
AR Gain ddPCR	1	10	0
Post-docetaxel Cut off 2.01	AR Normal NGS	AR Gain NGS	NGS TC<0.075
AR Normal ddPCR	37	1	12
AR Gain ddPCR	1	23	3

Abbreviations. AR, androgen receptor; ddPCR, digital droplet PCR; NGS, next generation sequencing; TC, tumor content.

Table S3. Univariate analysis in the primary cohort

	Overall Survival			Progression-free Survival		
	HR	CI 95%	<i>p</i>	HR	CI 95%	<i>p</i>
AR gain (yes vs no)	4.07	2.68-6.20	< 0.001	2.33	1.61-3.36	< 0.001
AR mutant (yes vs no)	4.81	2.02-11.44	< 0.001	2.86	1.24-6.59	0.014
Previous chemotherapy (yes vs no)	2.38	1.51-3.75	< 0.001	1.92	1.36-2.71	< 0.001
Pretreatment dsDNA concentration (continuous variable)	1.00	1.00-1.00	< 0.001	1.00	1.00-1.00	< 0.001
Pretreatment LDH (>UNL vs ≤UNL)	2.21	1.50-3.24	< 0.001	1.91	1.35-2.68	< 0.001
Liver metastases (yes vs no)	2.61	1.35-5.02	0.004	1.61	0.84-3.08	0.147
Bone metastases (>5 vs ≤5)	1.68	1.15-2.46	0.007	1.49	1.07-2.07	0.017
NLR (>3 vs <3)	1.67	1.13-2.46	0.010	1.34	0.96-1.87	0.080
ALP (>UNL vs ≤UNL)	2.00	1.36-2.93	0.010	2.09	1.48-2.94	< 0.001
Hb (<UNL vs ≥UNL)	1.80	1.20-2.69	0.004	1.50	1.03-2.18	0.031
Albumin (<UNL vs ≥UNL)	1.41	0.92-2.15	0.110	1.32	0.93-1.87	0.120
PSA (continuous variable)	1.00	1.00-1.00	0.009	1.00	1.00-1.00	0.002
Age (continuous variable)	0.98	0.95-1.00	0.054	0.98	0.96-1.00	0.104

Abbreviations. ALP, alkaline phosphatase; AR, androgen receptor; CI, confidence interval; dsDNA, double-stranded DNA; Hb, hemoglobin; HR, hazard ratio; LDH, lactate dehydrogenase; NLR, neutrophil to lymphocyte ratio; PSA, prostate specific antigen; UNL, upper normal limit.

Table S4. Multivariable Cox Proportional Hazard Analysis of predictors of overall survival and progression-free survival for primary cohort

	Overall Survival			Progression-free Survival		
	HR	CI 95%	<i>p</i>	HR	CI 95%	<i>p</i>
AR gain (yes vs no)	3.81	2.37-6.12	< 0.001	2.05	1.31-3.19	0.002
AR mutant (yes vs no)	3.12	1.32-7.40	0.010	2.23	0.98-5.08	0.056
Previous chemotherapy (yes vs no)	1.27	0.72-2.23	0.407	1.39	0.89-2.17	0.147
Pretreatment dsDNA concentration (continuous variable)	1.00	1.00-1.00	0.010	1.00	1.00-1.00	< 0.001
Pretreatment LDH (>UNL vs ≤UNL)	1.31	0.81-2.11	0.273	1.21	0.79-1.87	0.379
Liver metastases (yes vs no)	1.49	0.69-3.21	0.312	0.76	0.34-1.68	0.493
Bone metastases (>5 vs ≤5)	1.35	0.87-2.11	0.184	1.22	0.83-1.79	0.304
NLR (>3 vs <3)	1.37	0.89-2.11	0.156	1.06	0.73-1.54	0.759
ALP (>UNL vs ≤UNL)	1.32	0.85-2.05	0.222	1.43	0.95-2.14	0.086
Hb (<UNL vs ≥UNL)	0.91	0.55-1.50	0.705	0.79	0.49-1.26	0.314
Albumin (<UNL vs ≥UNL)	1.01	0.61-1.65	0.980	1.07	0.71-1.62	0.730
PSA (continuous variable)	1.00	1.00-1.00	0.458	1.00	1.00-1.00	0.766
Age (continuous variable)	0.99	0.96-1.02	0.386	0.99	0.96-1.01	0.309

Abbreviations. ALP alkaline phosphatase; AR, androgen receptor; CI, confidence interval; dsDNA, double-stranded DNA; Hb, hemoglobin; HR, hazard ratio; LDH, lactate dehydrogenase; NLR, neutrophil to lymphocyte ratio; PSA, prostate specific antigen; UNL, upper normal limit.

Table S5. Univariate analysis in PREMIERE. Biochemical PFS (A) and radiographic PFS (B)

A

	sPFS		
	HR	CI 95%	<i>p</i>
AR gain (yes vs no)	4.33	1.94-9.68	< 0.001
Pretreatment dsDNA concentration (continuous variable)	1.00	1.00-1.00	0.779
CTCs (AdnaTest®) (detected vs not detected)	3.40	1.76-6.56	< 0.001

B

	rPFS		
	HR	CI 95%	<i>p</i>
AR gain (yes vs no)	8.06	3.26-19.93	< 0.001
Pretreatment dsDNA concentration (continuous variable)	1.00	1.00-1.00	0.012
CTCs (AdnaTest®) (detected vs not detected)	7.09	2.61-19.25	< 0.001

Abbreviations. AR, androgen receptor; CI, confidence interval; CTC, circulating tumor cell; dsDNA, double-stranded DNA; HR, hazard ratio; sPFS, biochemical progression-free survival; rPFS, radiographic progression-free survival.

Supplementary References

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