Androgen receptor gene status in plasma DNA associates with worse outcome on enzalutamide or abiraterone for castration-resistant prostate cancer: a multi-institution correlative biomarker study

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Abstract:	BACKGROUND There is an urgent need to identify biomarkers to guide personalized therapy in castration-resistance prostate cancer (CRPC). We aimed to clinically qualify androgen receptor (AR) status measurement in plasma DNA using multiplex droplet digital PCR (ddPCR) in pre- and post-chemotherapy CRPC. METHODS We optimised ddPCR assays for AR copy number and mutations and retrospectively analysed plasma DNA from patients recruited to one of three biomarker protocols with prospectively-collected clinical data. We evaluated associations between plasma AR and overall survival (OS) and progression-free survival (PFS) in 73 chemotherapy-naïve and 98 post-docetaxel CRPC patients treated with enzalutamide or abiraterone (Primary cohort) and 94 chemotherapy-naïve patients treated with enzalutamide (Secondary cohort; PREMIERE trial). RESULTS In the primary cohort, AR gain was observed in 10 (14%) chemotherapy-naïve and 33 (34%) post-docetaxel patients and associated with worse OS (Hazard Ratio (HR), 3.98; 95%CI, 1.74-9.10; p<0.001 and HR, 3.81; 95%CI, 2.28-6.37; p<0.001 respectively), PFS (HR, 2.18; 95%CI, 1.08-4.39; p=0.03, and HR, 1.95; 95%CI, 1.23-3.11; p=0.01 respectively) and rate of PSA decline ≥50% (Odds ratio (OR), 4.7; 95%CI, 1.17-19.17; p=0.035 and OR, 5.0; 95%CI, 1.70-14.91; p=0.003 respectively). AR mutations (2105T>A (p.L702H) and 2632A>G (p.T878A)) were observed in eight (11%) post-docetaxel but no chemotherapy-naïve abiraterone-treated patients and were also associated with worse OS (HR 3.26; 95%CI, 1.47-not reached; p=0.004). There was no interaction between AR and docetaxel status (p=0.83 for OS, p=0.99 for PFS). In the PREMIERE trial,

	11 patients (12%) with AR gain had worse sPFS (HR, 4.33; 95%CI, 1.94-9.68; p<0.001), rPFS (HR, 8.06; 95%CI, 3.26-19.93; p<0.001) and OS (HR, 11.08; 95%CI, 2.16-56.95; p=0.004). Plasma AR was an independent predictor of outcome on multivariate analyses in both cohorts. CONCLUSION Plasma AR status assessment using ddPCR identifies CRPC with worse outcome to enzalutamide or abiraterone. Prospective evaluation of treatment decisions based on plasma AR is now require

TITLE: Androgen receptor gene status in plasma DNA associates with worse outcome on enzalutamide or abiraterone for castration-resistant prostate cancer: a multi-institution correlative biomarker study

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ABSTRACT

BACKGROUND

There is an urgent need to identify biomarkers to guide personalized therapy in castration-resistance prostate cancer (CRPC). We aimed to clinically qualify androgen receptor (AR) status measurement in plasma DNA using multiplex droplet digital PCR (ddPCR) in pre- and post-chemotherapy CRPC.

METHODS

We optimised ddPCR assays for *AR* copy number and mutations and retrospectively analysed plasma DNA from patients recruited to one of three biomarker protocols with prospectively-collected clinical data. We evaluated associations between plasma *AR* and overall survival (OS) and progression-free survival (PFS) in 73 chemotherapy-naïve and 98 post-docetaxel CRPC patients treated with enzalutamide or abiraterone (Primary cohort) and 94 chemotherapy-naïve patients treated with enzalutamide (Secondary cohort; PREMIERE trial).

RESULTS

In the primary cohort, *AR* gain was observed in 10 (14%) chemotherapy-naïve and 33 (34%) post-docetaxel patients and associated with worse OS (Hazard Ratio (HR), 3.98; 95%CI, 1.74-9.10; $p<0.001$ and HR, 3.81; 95%CI, 2.28-6.37; $p<0.001$ respectively), PFS (HR, 2.18; 95%CI, 1.08-4.39; $p=0.03$, and HR, 1.95; 95%CI, 1.23-3.11; $p=0.01$ respectively) and rate of PSA decline ≥50% (Odds ratio (OR), 4.7; 95%CI, 1.17-19.17; $p=0.035$ and OR, 5.0; 95% CI, 1.70-14.91; $p=0.003$ respectively). *AR* mutations (2105T>A (p.L702H) and 2632A>G (p.T878A)) were observed in eight (11%) post-docetaxel but no chemotherapy-naïve abiraterone-treated patients and were also associated with worse OS (HR 3.26; 95%CI, 1.47-not reached; $p=0.004$). There was no interaction between *AR* and docetaxel status ($p=0.83$ for OS, $p=0.99$ for PFS). In the PREMIERE trial, 11 patients (12%) with *AR* gain had worse sPFS (HR, 4.33; 95%CI, 1.94-9.68; $p<0.001$), rPFS (HR, 8.06; 95% CI, 3.26-19.93; $p<0.001$) and OS (HR, 11.08; 95%CI, 2.16-56.95; $p=0.004$). Plasma *AR* was an independent predictor of outcome on multivariate analyses in both cohorts.

CONCLUSION

Plasma *AR* status assessment using ddPCR identifies CRPC with worse outcome to enzalutamide or abiraterone. Prospective evaluation of treatment decisions based on plasma *AR* is now required.

Clinical Trial number:NCT02288936 (PREMIERE trial)

Key words: castration-resistant prostate cancer, androgen receptor, plasma DNA, enzalutamide, abiraterone, biomarker

Key Message

We clinically qualified *AR* status in plasma DNA using an optimized multiplex droplet digital PCR assay. We studied a primary cohort of 171 pre- and post-docetaxel patients treated with abiraterone or enzalutamide and a second cohort of 94 chemotherapy-naïve patients treated with enzalutamide, confirming that detection of plasma *AR* aberrations predicted adverse outcome across the CRPC spectrum.

INTRODUCTION

Inhibition of androgen receptor (AR) signaling with abiraterone or enzalutamide is now standard treatment at emergence of castration-resistant prostate cancer (CRPC). However, the duration of response is variable and overall survival (OS) in unselected patients is modest despite some patients having responses that last several years [1, 2]. There is therefore an urgent need to develop biomarker strategies to *a priori* identify CRPC patients who will derive minimal benefit from AR targeting and offer them an alternative treatment paradigm. Testing for plasma Epidermal Growth Factor Receptor (EGFR) mutations has FDA clearance for selection of mutant lung cancer patients for EGFR tyrosine kinase inhibitors and studies of plasma DNA in multiple indications have suggested clinical utility for monitoring of mutations or copy number (CN) gain [3-6].

Next-generation sequencing (NGS) and PCR-based studies have identified associations between *AR* CN gain detected in plasma and worse outcome with abiraterone or enzalutamide, in predominantly post-docetaxel CRPC cohorts [7-12]. *AR* gene aberrations are rare prior to hormone therapy but occur in metastases harvested at rapid warm autopsy from up to 60% of patients [13]. Using NGS on sequential plasma samples, we have identified two *AR* point mutations (2105T>A (p.L702H) and 2632A>G (p.T878A)) as associating with resistance to abiraterone, shown previously to be activated by prednisone or progesterone respectively [7, 8, 14, 15]. For enzalutamide, the 2629T>C (p.F877L) point mutation has been reported as a resistance mechanism [16, 17] although a recent study suggested it is

very uncommon [12]. Following a well-described roadmap for implementation of a biomarker test into routine clinical practice [18], we aimed to optimize a droplet digital PCR (ddPCR) assay that is fit for purpose and can be widely implemented on plasma DNA in clinical laboratories. We sought to define *AR* CN and in a separate reaction, *AR* mutation status: 2105T>A and 2632A>G in patients considered for abiraterone and 2629T>C for patients treated with enzalutamide. We then aimed to obtain stage one biomarker clinical qualification for associations with clinical outcome on enzalutamide or abiraterone in chemotherapy-naïve and post-docetaxel CRPC patients treated in one of three biomarker protocols.

MATERIAL AND METHODS

Study design

This was a multi-institution analysis of plasma samples collected prospectively in studies with the primary aim of biomarker evaluation. The objectives were defined after sample collection but prior to plasma analysis. Our first objective was to determine the correlation between ddPCR testing for plasma *AR* and an orthogonal approach, next-generation sequencing (NGS), in samples collected prior to starting treatment and after disease progression. Our second objective was to evaluate associations between pre-treatment plasma *AR* and clinical outcome in a primary cohort, representative of both pre- and post-docetaxel patients, and test for interactions with prior chemotherapy exposure. As no trial to date has randomised patients between first-line enzalutamide or abiraterone and taxanes, we combined data from four cohorts of men recruited to two biomarker protocols and defined by treatment with enzalutamide or abiraterone and prior chemotherapy status. Our third objective was to test our ddPCR assay in a second cohort of chemotherapy-naïve men treated with enzalutamide in the PREMIERE trial.

Participants

The primary cohort included patients participating in one of two protocols separately approved by the Institutional Review Board of the Royal Marsden (RM), London, UK (REC 04/Q0801/6), and Istituto Scientifico Romagnolo per lo Studio e la Cura dei Tumori (IRST), Meldola, Italy (REC 2192/2013). Docetaxel in this cohort was only used in the CRPC setting. The second cohort was the PREMIERE trial (EudraCT: 2014-003192-28, NCT02288936) that was sponsored and conducted by the Spanish Genito-Urinary oncology Group (SOGUG). The trial was approved by the independent review board at each participating site. This trial was designed to analyse the predictive value of the gene fusion *TMPRSS2-ETS* in response to enzalutamide in patients with prostate cancer. Exploratory end-points included circulating cell-free DNA and circulating tumor cell (CTC) analysis. Data emerging after the trial was designed and initiated [7, 19, 20] led the PREMIERE Trial Management Group to prioritize two alternative biomarkers for evaluation, namely AR-V7 detected in CTCs as described previously [19] and plasma *AR*. *TMPRSS2-ETS* analyses are on-going and will be reported elsewhere. Preliminary AR-V7 data was presented in abstract form at the ESMO 2016 Annual Meeting [21] and will be published elsewhere. These analyses were based on the first censor cut-off, date May 2016. A second data analysis is planned at a predefined time-point when enough events have occurred to address the primary endpoint.

In both cohorts, patients were required to have histologically-confirmed prostate adenocarcinoma without neuroendocrine differentiation, progressive disease despite “castration levels” of serum testosterone (<50 ng/dL), on-going LHRH analogue treatment or prior surgical castration and no prior treatment with enzalutamide or abiraterone. Additional selection criteria by cohort are specified in the Supplementary Appendix S1, available at *Annals of Oncology* online. The choice of therapy in the primary cohort was at the discretion of the treating physician, either enzalutamide 160mg once a day or abiraterone 1g once a day and prednisone 5mg twice daily. In the PREMIERE trial, all patients received enzalutamide 160mg once a day. Treatment in both cohorts was administered continuously until

evidence of progression disease or unacceptable toxicity. The studies were conducted in accordance with the Declaration of Helsinki and the Good Clinical Practice guidelines of the International Conference of Harmonization. Written informed consent was obtained from all patients.

Procedures

Peripheral blood samples were collected within 30 days of treatment initiation and plasma aliquots stored at -80°C. ddPCR assays were performed as described in detail in Supplementary Appendix S2, available at *Annals of Oncology* online. For each individual sample *AR* CN was estimated using each of the reference genes *NSUN3*, *EIF2C1*, and *AP3B1* and using *ZXDB* at Xp11.21 as a control gene to determine X chromosome CN. *AR* mutation detection assays were performed for the *AR* mutations 2105T>A (p.L702H), 2632A>G (p.T878A), and 2629T>C (p.F877L) with a limit of detection of 1-2% using an input of 2 to 4 ng of DNA. For NGS on plasma and patient-matched germline DNA, we used a customized AmpliSeq targeted gene panel including *AR*, sequenced on an Ion Torrent Personal Genome Machine or Proton as described previously [7, 8]. Computational analysis estimating the plasma DNA tumor content, *AR* CN quantitation and point mutation detection (with a sensitivity of 98-99% depending on position and coverage) was performed as previously [8].

Serum prostate specific antigen (PSA) was assessed within one week of starting treatment and monthly thereafter. Radiographic disease was evaluated with the use of computed tomography and bone scan at the time of screening and every 12 weeks on treatment. In the primary cohort, serum lactate dehydrogenase (LDH) and alkaline phosphatase (ALP) were also measured within one week of starting treatment. In PREMIERE, CTCs were evaluated pre-treatment using the AdnaTest for Prostate Cancer (Qiagen GmbH, Germany) as described previously [21].

Outcomes

For the primary cohort, the primary endpoint was OS. The secondary endpoints were progression-free survival (PFS) (biochemical and/or radiographic and/or clinical) and PSA response. For PREMIERE, the primary endpoint was PSA-PFS (sPFS). Secondary endpoints included radiographic-PFS (rPFS), OS and PSA response. OS was calculated from initiation of therapy to death from any cause. Patients still alive at time of last follow-up were censored. PFS was calculated from the first day of enzalutamide or abiraterone therapy to the date of progression disease or death. Radiographic progression was defined using Response Evaluation Criteria in Solid Tumors version 1.1. PSA decline was evaluated according to Prostate Cancer Working Group (PCWG2) guidelines [22].

Statistical analyses

An R script [23] was developed to identify the optimal *AR* CN cut-point that associated with OS in the primary cohort, using maximum log-likelihood as correlative statistics in a multivariable Cox regression model by an approach described previously (Supplementary Appendix S3, available at *Annals of Oncology* online) [24]. The process was bootstrapped with 30,000 iterations to provide the measures of dispersion. Remaining analyses were conducted using Stata/MP 13.1 for Windows. Time-to-event outcomes were evaluated using Kaplan-Meier survivor estimates, log-rank test and univariate and multivariable Cox-proportional hazards models. The association of clinically relevant baseline factors (previously showed to be associated with prognosis [25, 26] with OS and PFS was examined using a univariate Cox regression model. A multivariate Cox regression model was then performed with a stepwise procedure to identify the prognostic factors for OS and PFS with a significance level of <0.05 for entry into the model. All tests were two-sided and an α-error of 5% was considered as significant. Odds ratios of PSA response were determined using a 2x2 contingency table and significant

differences using Fisher's exact test. (Supplementary Appendix S3, available at *Annals of Oncology* online).

RESULTS

Clinical Characteristics of the Primary Cohort

In the primary cohort, we had 171 men who started treatment with enzalutamide or abiraterone between Jan 31, 2011 and June 9, 2016, 73 prior to docetaxel and 98 after. All had received bicalutamide. Patient and treatment characteristics at the time of sample collection are detailed in Table 1.

Analytic Testing of Multiplex Droplet Digital PCR for Determination of Plasma *AR* Status

We used an optimized multiplex *AR* CN ddPCR assay on 2-4ng DNA from all pre-treatment samples and an additional 42 samples collected after disease progression. On a further 2-4ng DNA, we tested for *AR* mutations. From patients in the primary cohort with ddPCR data, we had NGS data available from our previous publication [8] for 86 samples and we performed NGS on an additional 75 (samples described in Supplementary Table S1, available at *Annals of Oncology* online). We observed a strong agreement between NGS and ddPCR for CN quantitation (n=161, Bland-Altman test: mean difference, -0.02, 95% CI Limits of agreement, -2.45 to 2.41) (Supplementary Figure S1A and Table S2, available at *Annals of Oncology* online). Estimation of *AR* mutation allelic frequency by ddPCR also displayed strong agreement with NGS (n=60, Bland-Altman test: mean difference -0.001, 95% CI limits of agreement, -0.015 to 0.016) with no cases of mutations detected by one approach but not the other (Supplementary Figure S1B, available at *Annals of Oncology* online).

Plasma *AR* status in the Primary Cohort

In our primary cohort, eight post-docetaxel (but no chemotherapy-naïve) abiraterone patients were *AR* point mutation positive prior to treatment (Table 1). We planned to analyse these separately for associations with outcome. All four patients with a 2105T>A (p.L702H) mutation had received at least six months of treatment with prednisone. We did not detect a 2629T>C (p.F877L) *AR* point mutation prior to treatment or in an additional 26 samples collected after progression on enzalutamide. Using maximum likelihood ratio as correlative statistics combined with boot-strapping, we identified an *AR* CN cut-point of 2.01 (interquartile range (IQR), 1.82-2.77 copies) for splitting patients into two distinct prognostic groups (Supplementary Figure S2, available at *Annals of Oncology* online). Use of this cut-off was also supported by 95.5% concordance between NGS and ddPCR for classifying *AR* CN status (Supplementary Table S2, available at *Annals of Oncology* online). Overall, 10 (14%) chemotherapy-naïve and 33 (34%) docetaxel-treated patients had *AR* gain (Table 1).

Plasma *AR* Associates with Worse Outcome in the Primary Cohort

There was a significant association for *AR* gain and OS in both chemotherapy-naïve (median, 12.40 months versus not reached; HR, 3.98; 95% CI, 1.74-9.10; $p < 0.001$) (Figure 1A), and post-docetaxel patients (median, 9.51 versus 21.80 months; HR, 3.81; 95% CI, 2.28-6.37; $p < 0.001$) (Figure 1B). For *AR* mutants in abiraterone-treated, post-docetaxel patients, a significant association with worse survival was also seen (median 4.06 months; HR, 3.26; 95% CI, 1.47-not reached; $p = 0.004$) (Figure 1B). We also observed a significant association between PFS and *AR* gain for chemotherapy-naïve patients treated with enzalutamide or abiraterone (median, 7.30 versus 9.20 months; HR, 2.18; 95% CI, 1.08-4.39; $p = 0.03$) (Figure 1C) and for post-docetaxel patients (median, 5.00 versus 7.36 months; HR, 1.95; 95% CI, 1.23-3.11; $p = 0.01$) (Figure 1D). A trend was seen for *AR* mutants to have worse PFS (median 4.10 months; HR, 2.10; 95% CI, 0.98-4.51; $p = 0.057$) (Figure 1D). Interactions between *AR* CN and treatment (abiraterone versus enzalutamide) ($p = 0.41$ for OS and $p = 0.11$ for PFS) or chemotherapy status ($p = 0.83$ for OS, $p = 0.99$ for PFS) examined in the Cox models were not

significant. We also evaluated the association of *AR* status with the rate of PSA decline in the chemotherapy-naïve and post-docetaxel groups. Chemotherapy-naïve patients with *AR* gain were 4.7 times less likely to have a ≥50% decline in PSA (95% CI, 1.17-19.17; $p = 0.035$) (Figure 1E). Plasma *AR* gain chemotherapy-treated patients were 5.0 times less likely to have a ≥50% decline in PSA (95% CI, 1.70-14.91; $p = 0.003$) (Figure 1F). For the eight *AR* mutant patients, a trend for a lower rate of ≥50% PSA decline was seen (odds ratio (OR), 6.3; 95% CI, 0.72-54.59; $p = 0.12$) (Figure 1F).

Plasma *AR* Independently Associates with Worse Outcome on Multivariate Analysis in the Primary Cohort.

Plasma *AR* status and 11 baseline characteristics previously shown to be clinically relevant [25, 26] were evaluated by both univariate and multivariate analyses on the whole primary cohort. Plasma *AR* gain or mutant were most significantly associated with OS or PFS (Supplementary Table S3 and Table S4 available at *Annals of Oncology* online). We then performed multivariate analysis with stepwise backwards elimination and the sole variables that remained significant were plasma *AR* status (HR, 4.10; 95% CI, 2.66-6.35; $p < 0.001$, and HR, 4.02; 95% CI, 1.87-8.66; $p < 0.001$, for *AR* CN and *AR* mutant, respectively, Table 2A) and total plasma DNA concentration for OS and plasma *AR* status (HR, 2.06; 95% CI, 1.36-3.12; $p = 0.001$, and HR, 2.20; 95% CI, 1.03-4.69; $p = 0.041$, for *AR* CN and *AR* mutant, respectively), total plasma DNA concentration and ALP levels for PFS (Table 2B).

Plasma *AR* status in the PREMIERE Cohort

The PREMIERE trial enrolled 98 patients in 16 sites between February 2015 through November 2015. Plasma was collected at study entry before starting enzalutamide from 94 patients who had a median follow-up of 10.6 months. Patient characteristics by plasma *AR* status are described in Table 3A.

Plasma *AR* Associates with Worse Outcome in the PREMIERE Cohort

Similar to our primary cohort pre-chemotherapy population, we observed *AR* gain in 11 (12%) patients. CTCs were detected in 35 patients (37%). *AR* gain was detected in seven (20%) CTC-positive and four (7%) CTC-negative patients (Table 3A). Plasma *AR* gain was significantly associated with shorter sPFS (median, 3.60 versus 15.5 months; HR, 4.33; 95% CI, 1.94-9.68; $p < 0.001$) (Figure 2A), rPFS (median, 3.90 months versus not reached; HR, 8.06; 95% CI, 3.26-19.93; $p < 0.001$) (Figure 2B) and OS (medians not reached; HR, 11.08; 95% CI, 2.16-56.95; P = 0.004) (Figure 2C) (Supplementary Table S5, available at *Annals of Oncology* online). Patients with *AR* gain were less likely to have a ≥50% decline in PSA (OR, 4.93; 95% CI, 1.30-18.75; $p = 0.025$) (Figure 2D).

Plasma *AR* Independently Associates with Worse Outcome on Multivariate Analysis in the PREMIERE Cohort

On multivariate analysis, the association of *AR* gain with the primary endpoint of sPFS was independent of plasma DNA concentration and the detection of CTCs (HR, 4.32; 95% CI 1.90-9.85; $p < 0.001$) (Table 3B). *AR* gain was also independently associated on multivariate analysis with rPFS (HR, 5.63; 95% CI, 2.15-14.74; $p < 0.001$) (Table 3B).

DISCUSSION

Several treatments are available for metastatic CRPC but to date, no approved biomarker to personalize therapy. Our analyses of plasma from 265 patients collected in three prospective biomarker protocols show that detection of *AR* CN gain prior to starting enzalutamide or abiraterone is associated with decreased OS and PFS regardless of prior chemotherapy status. We excluded samples from patient that had prior treatment with enzalutamide or abiraterone, given response rates and duration of benefit are very different when used sequentially [27]. Our previous study [8] suggests a similar

association between plasma *AR* and resistance in patients previously treated with enzalutamide or abiraterone and this requires further investigation in future studies.

We did not detect *AR* mutations (p.T878A or p.L702H) in chemotherapy-naïve patients. Our assay detects point mutations present in at least 2% of plasma DNA. Greater sensitivity is obtained with higher input DNA [28] although the clinical relevance of rarer mutations is uncertain. By using a multiplex ddPCR with four carefully selected reference genes, we have designed a robust assay that does not over-call gain due to loss in regions involving the reference gene. Our model for estimating the likelihood of the *AR* CN cut-off that best predicts associations with outcome was built with 171 patients. We plan to perform a meta-analysis of multiple trials when the data on *AR* CN acquired from different institutions and trials exceeds 1000 patients. We report the absence of an interaction between *AR* and chemotherapy status in non-randomized cohorts.

Detection of AR splice variants in CTCs is also associated with shorter PFS and OS with enzalutamide or abiraterone [19, 29]. *AR* CN is higher in the population with detectable CTCs although *AR* gain can also be observed in CTC-negative patients, accounting for one third of *AR* gained in the PREMIERE cohort. The overlap between AR-V7 positive and plasma *AR* gained patients and a comparison of the two tests in prospective trials is warranted to develop the best biomarker strategy for identifying resistant patients. Testing plasma *AR* status by ddPCR is affordable and can be widely implemented in clinical laboratories but does not control for plasma DNA tumor content [7, 8] that may introduce a bias. Nonetheless, multivariate analyses confirm that plasma *AR* by ddPCR provides information on the outcome of men starting enzalutamide or abiraterone that is independent of other factors previously reported to be prognostic [25, 26, 30]. In keeping with higher response rates to AR targeting in chemotherapy-naïve patients, the prevalence of plasma *AR* aberrations is 10-15% in this setting compared to 30-40% post-docetaxel. As our study is single arm, the associations we report are

prognostic although the association with PSA decline rate suggests plasma *AR* CN could identify patients resistant to enzalutamide or abiraterone. The aims of our study were defined after sample collection and therefore larger studies with a pre-specified primary objective of defining the association with outcome by plasma *AR* status could provide further supportive evidence for the role of *AR* CN as a biomarker in CRPC. For level one evidence to change clinical practice, our findings require confirmation in prospective trials where plasma *AR* CN defines treatment selection.

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Disclosure

The ICR developed abiraterone and therefore has a commercial interest in this agent. D.D. and G.A. are on the ICR list of rewards to inventors for abiraterone. G.A. has received honoraria, consulting fees, or travel support from Astellas, Medivation, Janssen, Millennium Pharmaceuticals, Ipsen, Ventana, ESSA Pharmaceuticals, and Sanofi-Aventis and grant support from Janssen, AstraZeneca, and Arno.

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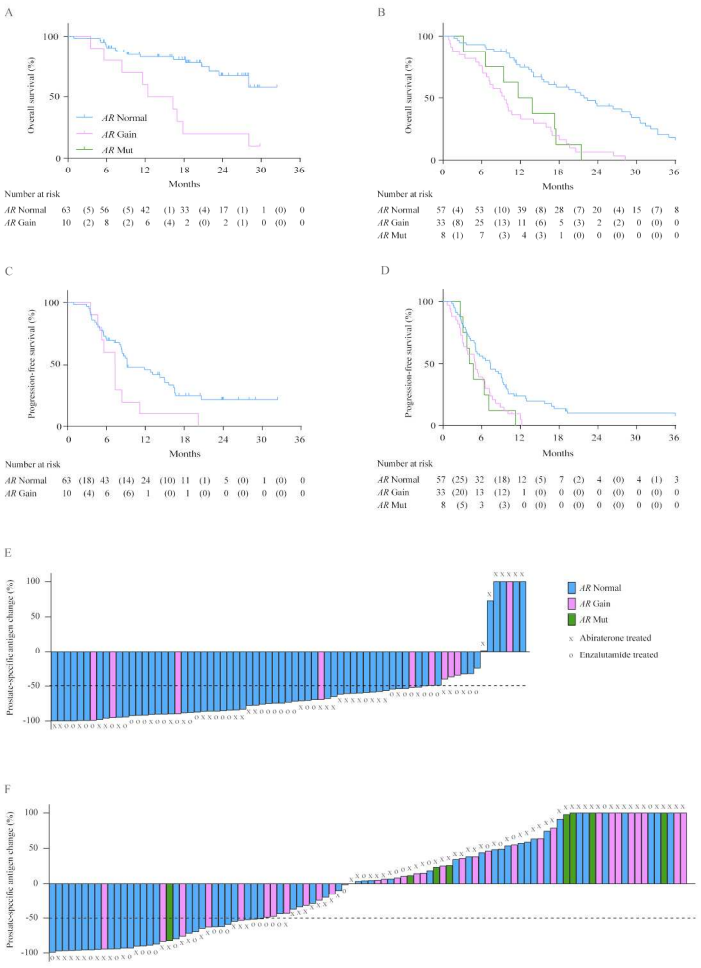
Legend to figures

Figure 1. Association of plasma *AR* status and outcome in the Primary cohort. Overall and progression-free survival for *AR* copy number normal, gain and mutated (Mut, p.L702H or p.T878A) chemotherapy-naïve (A, C) and post-docetaxel (B, D) castration-resistant prostate cancer patients treated with enzalutamide or abiraterone. PSA declines by *AR* status, waterfall plots of PSA declines for *AR* copy number normal, gain and mutated (Mut, p.L702H or p.T878A) chemotherapy-naïve (E) and post-docetaxel (F) castration-resistant prostate cancer patients. Bars were clipped at maximum 100%.

Figure 2. Association of plasma *AR* status and outcome in PREMIERE cohort. Biochemical progression-free survival (A), radiographic progression-free survival (B) and overall survival (C) for *AR* copy number normal versus *AR* gain patients. Waterfall plot (D) showing the magnitude of PSA decline by *AR* status. Bars were clipped at maximum 100%.

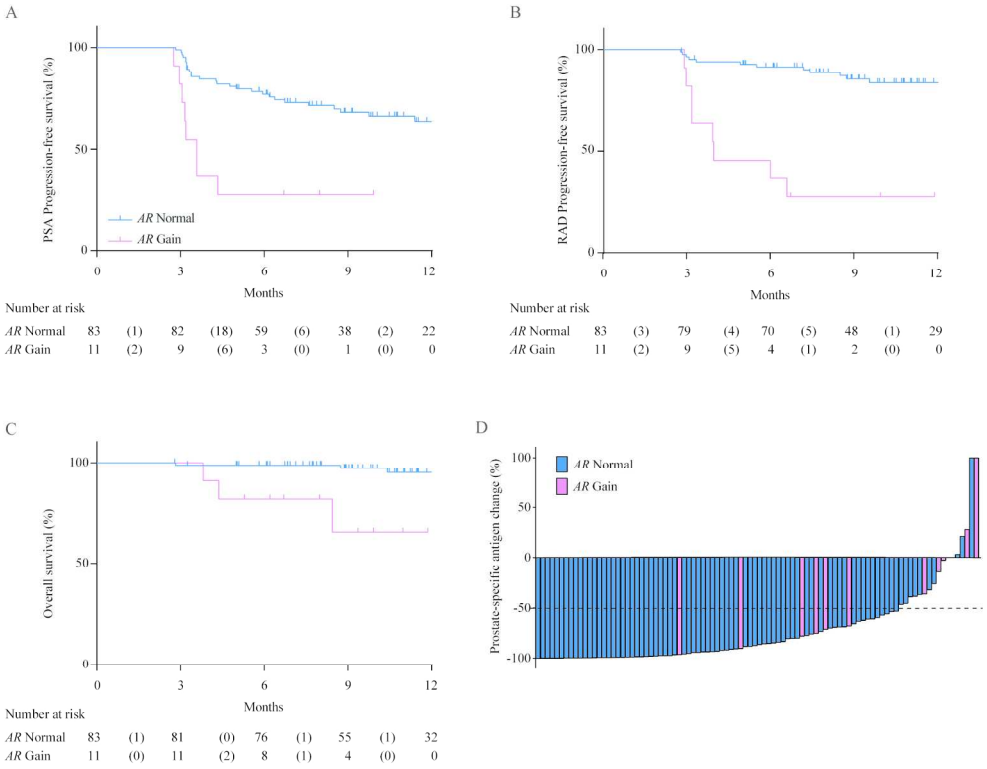
FIGURE 1



Association of plasma AR status and outcome in the Primary cohort.

210x297mm (300 x 300 DPI)

FIGURE 2



Association of plasma AR status and outcome in PREMIERE cohort.

172x136mm (300 x 300 DPI)

Table 1. Baseline characteristics of the primary cohort by *AR* status

	Enzalutamide chemotherapy-naive (*n*=35)		Abiraterone chemotherapy-naive* (*n*=38)		Enzalutamide post-docetaxel (*n*=27)		Abiraterone post-docetaxel (*n*=71)		
n **(%)**	***AR*** **normal 29 (83)**	***AR*** **gain 6 (17)**	***AR*** **normal 34 (89)**	***AR*** **gain 4 (11)**	***AR*** **normal 20 (74)**	***AR*** **gain 7 (26)**	***AR*** **normal 37 (52)**	***AR*** **gain 26 (37)**	***AR*** **mutant 8 (11)**
Age, years Median (range)	73 (63-91)	71.5 (63-81)	75 (56-87)	75 (66-86)	78 (59-87)	81 (65-85)	75 (41-82)	73 (41-91)	77 (63-86)
Pretreatment PSA, mg/liter Median (range)	28 (2-1555)	110 (32-298)	15 (1-191)	313 (126-797)	23 (2-1899)	252 (11-893)	56 (1-3211)	142 (2-3150)	144 (1-803)
Pretreatment LDH, U/liter Median (range)	164 (80-915)	169 (137-253)	154 (77-253)	219 (134-312)	154 (78-234)	201 (167-245)	172 (106-417)	222 (135-968)	250 (157-650)
Pretreatment ALP, U/liter Median (range)	76 (44-531)	65 (55-188)	92 (51-426)	175 (102-255)	90 (55-531)	241 (87-890)	93.5 (61-934)	96 (36-1040)	119 (39-891)
Previous cabazitaxel treatment, *n* (%)	-	-	-	-	2 (10)	1 (14)	0 (0)	3 (11)	1 (12.5)
Sites of metastases, *n* (%), visceral metastases, n (%) **≤ 5 bone metastases** **>5 bone metastases** **Lymph node, no bone metastases**	6 (21),0 (0) 4 (14),0 (0) 4 (14), 0 (0)	1 (17), 0 (0) 2 (33), 0 (0) 0 (0), 0 (0)	13 (38), 0 (0) 14 (41), 2 (6) 5 (15), 1 (3)	1(25), 0 (0) 3 (75), 0 (0) 0 (0), 0 (0)	5 (40), 0 (0) 12 (60), 2 (10) 1 (5), 0 (0)	3 (43), 0 (0) 4 (57), 1 (14) 0 (0), 0 (0)	12 (32), 3 (8) 17 (46), 2 (5) 6 (16), 1 (3)	8 (31), 2 (8) 17 (65), 4 (15) 1 (4), 1 (4)	3 (37.5), 1 (12.5) 5 (62.5), 1 (12.5) 0 (0), 0 (0)
Pretreatment dsDNA concentration, ng Median (range)	17 (6-577)	15 (11-27)	19 (6-103)	39 (29-134)	27 (7-190)	40 (9-121)	24 (4-783)	65 (7-2566)	32 (11-550)
Time of follow-up, months Median (range)	27.8 (5.2-33.0)		18.5 (0.9-28.5)		26.1 (0.8-39.9)		44.5 (1.1-68.0)		

* No *AR* (p.L702H or p.T878A) mutation detected.

Abbreviations: ALP alkaline phosphatase; *AR*, Androgen receptor; dsDNA, double strand DNA; LDH, lactate dehydrogenase; *n*, number; NLR, neutrophil to lymphocyte ratio; PSA, prostate specific antigen.

Table 2. Multivariable Cox Proportional Hazard Analysis of Predictors of Overall Survival (A) and Progression-free Survival (B) for Primary Cohort after stepwise backwards elimination

A

	Overall Survival		
	HR	**CI 95%**	***p***
***AR* gain** (yes versus no)	4.26	2.76-6.55	< 0.001
***AR* mutant** (yes versus no)	3.80	1.77-8.15	0.001
Pretreatment dsDNA concentration (continuous variable)	1.00	1.00-1.00	< 0.001

B

	Progression-free Survival		
	HR	**CI 95%**	***p***
***AR* gain** (yes versus no)	2.22	1.48-3.34	< 0.001
***AR* mutant** (yes versus no)	2.59	1.24-5.44	0.012
ALP (>UNL versus ≤UNL)	1.64	1.13-2.36	0.009
Pretreatment dsDNA concentration (continuous variable)	1.00	1.00-1.00	< 0.001

Abbreviations. ALP alkaline phosphatase; *AR*, androgen receptor; HR, hazard ratio; CI, confidence interval; dsDNA, double-stranded DNA; UNL, upper normal limit.

Table 3. PREMIERE cohort. Baseline characteristics of patients according to *AR* status (A). Multivariable Cox proportional hazard analysis of predictors of PSA progression-free survival (B).

A

***n* (%)**	**PREMIERE (*n*=94)**	
	***AR* normal 83 (88)**	***AR* gain 11 (12)**
Age, years Median (range)	77 (57-95)	80 (60-88)
PSA, mg/liter Median (range)	24 (3-4319)	59 (2-254)
Prior bicalutamide at CRPC, n (%)	69 (83)	9 (82)
Sites of metastases, *n* (%), visceral metastases, n (%)		
≤ 5 bone metastases	57 (69), 10 (12)	8 (73), 1 (9)
>5 bone metastases	12 (15), 1 (1)	1 (9), 0 (0)
Lymph node, no bone metastases	12 (15), 2 (2)	1 (9), 0 (0)
dsDNA concentration, ng/mL Median (range)	19.4 (0.5-134.7)	23.1 (4.4-1584.9)
CTC detection, n (%)		
Yes	28 (34)	7 (64)
No	55 (66)	4 (36)
Time of follow-up, months Median (range)	10.8 (2.8-16.7)	

B

	sPFS			**rPFS**		
	HR	**CI 95%**	***p***	**HR**	**CI 95%**	***p***
***AR* gain** (yes versus no)	4.32	1.90-9.85	< 0.001	5.63	2.15-14.74	< 0.001
Pretreatment dsDNA concentration (continuous variable)	1.00	1.00-1.00	0.240	1.00	1.00-1.00	0.853
CTC detection (AdnaTest®) (yes versus no)	3.18	1.63-6.20	0.001	5.74	2.08-15.90	0.001

Abbreviations. AR, androgen receptor; CI, confidence interval; CRPC, castration resistant prostate cancer; CTC, circulating tumor cell; dsDNA, double-stranded DNA; HR, hazard ratio; PSA, prostate specific antigen; rPFS, Radiographic Progression-free Survival; sPFS, Progression-Free Survival.

Supplementary Online Material

Appendix S1.

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/mL
    b. absolute neutrophil count >1,500/mL
    c. platelets >100,000/mL
    d. total bilirubin within normal institutional limits
    e. AST(SGOT)/ALT(SGPT) <2.5 X 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 bisfosfonate 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 ≥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 µg/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 ≥ 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/µL
    b. Platelet count <100,000/µL
    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 µmol/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 lost 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 ≥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 n*ormal 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).

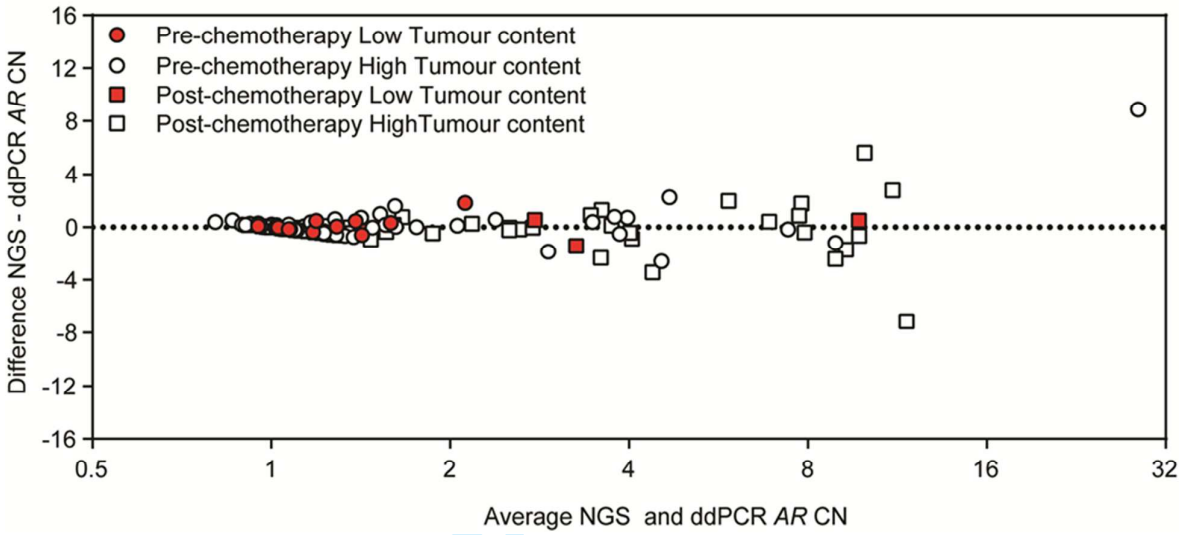
The association of *AR* status with progression-free survival (PFS) and overall survival (OS) was evaluated using univariable Cox regression. Survivor function of Time-to-event outcomes were also estimated using the Kaplan-Meier method. Differences between survivor functions of patients with *AR* CN 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

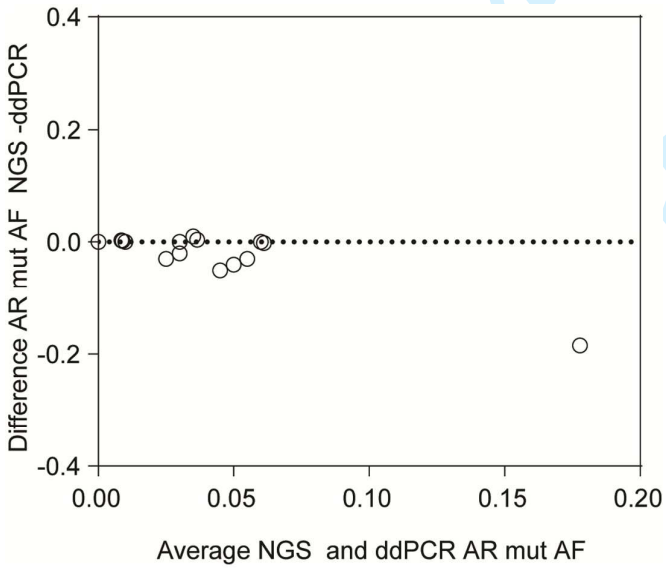
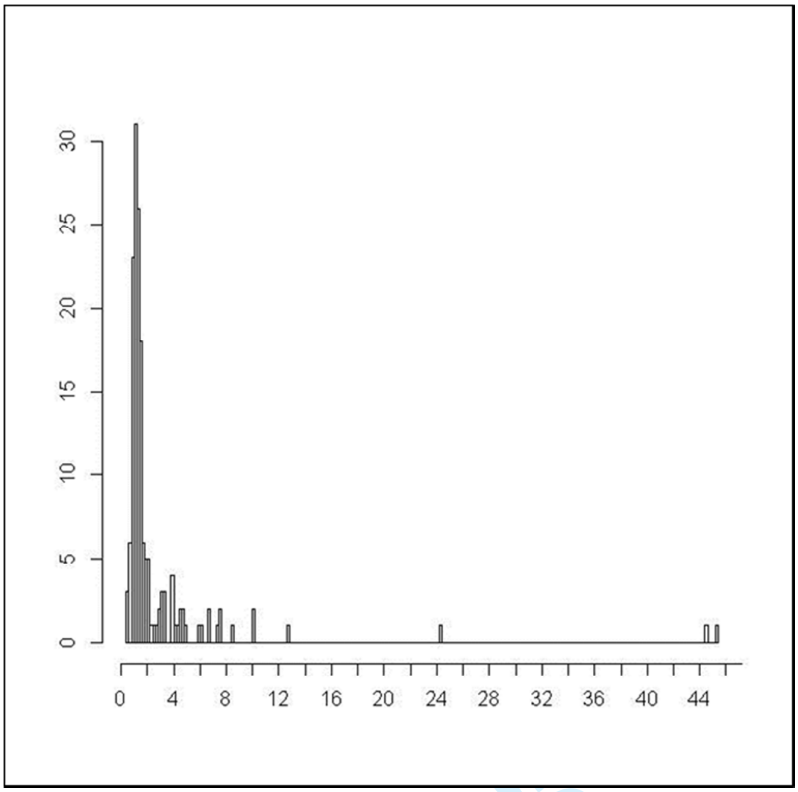


Figure S1. Evaluation of ddPCR copy number (CN) and mutation assay performance. Bland-Altman plot showing agreement of ddPCR and NGS *AR* copy number assessment, low tumor content samples had a tumor content fraction below 0.075 (A). Bland-Altman plot showing agreement of ddPCR and NGS *AR* mutation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

A



B

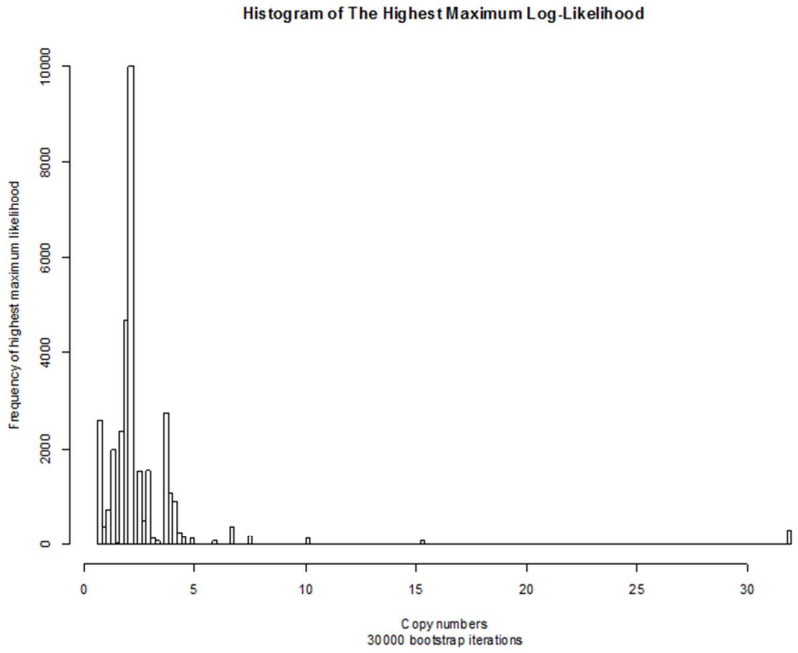


Figure S2. Selection of cut-off for *AR* CN gain by ddPCR. Range of *AR* CN across primary cohort (A), Cut-off analysis with maximum log-likelihood as the correlative statistic of the multivariable Cox proportional hazard model and boot-strapping with 30,000 iterations to provide the cut-off point dispersion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

Supplementary Tables

Table S1. Samples analysed by both NGS and ddPCR

		Pretreatment (*n*)	Progression (*n*)
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%**	***p***	**HR**	**CI 95%**	***p***
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%**	***p***	**HR**	**CI 95%**	***p***
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%**	***p***
***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%**	***p***
***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.

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TITLE: Androgen receptor gene status in plasma DNA associates with worse outcome on enzalutamide or abiraterone for castration-resistant prostate cancer: a multi-institution correlative biomarker study

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ABSTRACT

BACKGROUND

There is an urgent need to identify biomarkers to guide personalized therapy in castration-resistance prostate cancer (CRPC). We aimed to clinically qualify androgen receptor (AR) status measurement in plasma DNA using multiplex droplet digital PCR (ddPCR) in pre- and post-chemotherapy CRPC.

METHODS

We optimised ddPCR assays for *AR* copy number and mutations and retrospectively analysed plasma DNA from patients recruited to one of three biomarker protocols with prospectively-collected clinical data. We evaluated associations between plasma *AR* and overall survival (OS) ~~survival (OS)~~ and ~~survival (OS) and~~ progression-free survival (PFS) in 73 chemotherapy-naïve and ~~and~~ 98 ~~98~~ post-docetaxel CRPC patients treated with enzalutamide or abiraterone (Primary cohort) and 94 chemotherapy-naïve patients treated with enzalutamide (Secondary cohort; PREMIERE trial).~~treated with enzalutamide or abiraterone.~~

RESULTS

In the primary cohort, *AR* gain was observed in 10 (14%) chemotherapy-naïve and 33 (34%) post-docetaxel patients and associated with worse OS (Hazard Ratio (HR), 3.98; 95%CI, 1.74-9.10; $p<0.001$ and HR, 3.81; 95%CI, 2.28-6.37; $p<0.001$ respectively), PFS (HR, 2.18; 95%CI, 1.08-4.39; $p=0.03$, and HR, 1.95; 95%CI, 1.23-3.11; $p=0.01$ respectively) and rate of PSA decline ≥50% (Odds ratio (OR), 4.7; 95%CI, 1.17-19.17; $p=0.035$ and OR, 5.0; 95% CI, 1.70-14.91; $p=0.003$ respectively). *AR* mutations (2105T>A (p.L702H) and 2632A>G (p.T878A)) were observed in eight (11%) post-docetaxel but no chemotherapy-naïve abiraterone-treated patients and were also associated with worse OS (HR 3.26; 95%CI, 1.47-not reached; $p=0.004$). There was no interaction between *AR* and docetaxel status ($p=0.83$ for OS, $p=0.99$ for PFS). In the PREMIERE trial, 11 patients (12%) with *AR* gain had worse sPFS (HR, 4.33; 95%CI, 1.94-9.68; $p<0.001$), rPFS (HR, 8.06; 95% CI, 3.26-19.93; $p<0.001$) and OS (HR, 11.08; 95%CI, 2.16-56.95; $p=0.004$). Plasma *AR* was an independent predictor of outcome on multivariate analyses in both cohorts.

CONCLUSION

Plasma *AR* status assessment using ddPCR identifies CRPC with worse outcome to enzalutamide or abiraterone. Prospective evaluation of treatment decisions based on plasma *AR* is now required.

Clinical Trial number:NCT02288936 (PREMIERE trial)

Key words: castration-resistant prostate cancer, androgen receptor, plasma DNA, enzalutamide, abiraterone, biomarker

INTRODUCTION

Inhibition of androgen receptor (AR) signaling with abiraterone or enzalutamide is now standard treatment at emergence of castration-resistant prostate cancer (CRPC). However, the duration of response is variable and overall survival (OS) in unselected patients is modest despite some patients having responses that last several years [1, 2]. There is therefore an urgent need to develop biomarker strategies to *a priori* identify CRPC patients who will derive minimal benefit from AR targeting and offer them an alternative treatment paradigm. Testing for plasma Epidermal Growth Factor Receptor (EGFR) mutations has FDA clearance for selection of mutant lung cancer patients for EGFR tyrosine kinase inhibitors and studies of plasma DNA in multiple indications have suggested clinical utility for monitoring of mutations or copy number (CN) gain [3-6].

Next-generation sequencing (NGS) and PCR-based studies have identified associations between *AR* CN gain detected in plasma and worse outcome with abiraterone or enzalutamide, in predominantly post-docetaxel CRPC cohorts [7-12]. *AR* gene aberrations are rare prior to hormone therapy but occur in metastases harvested at rapid warm autopsy from up to 60% of patients [13]. Using NGS on sequential plasma samples, we have identified two *AR* point mutations (2105T>A (p.L702H) and 2632A>G (p.T878A)) as associating with resistance to abiraterone, shown previously to be activated by prednisone or progesterone respectively [7, 8, 14, 15]. For enzalutamide, the 2629T>C (p.F877L) point mutation has been reported as a resistance mechanism [16, 17] although a recent study suggested it is very uncommon [12]. Following a well-described roadmap for implementation of a biomarker test into routine clinical practice [18], we aimed to optimize a droplet digital PCR (ddPCR) assay that is fit for purpose and can be widely implemented on plasma DNA in clinical laboratories. We sought to define *AR* CN and in a separate reaction, *AR* mutation status: 2105T>A and 2632A>G in patients considered for abiraterone and 2629T>C for patients treated with enzalutamide. We then aimed to obtain stage one biomarker clinical qualification for associations with clinical outcome on enzalutamide or

abiraterone in chemotherapy-naïve and post-docetaxel CRPC patients treated in one of three biomarker protocols.

MATERIAL AND METHODS

Study design

This was a multi-institution analysis of plasma samples collected prospectively in studies with the primary aim of biomarker evaluation. The objectives were defined after sample collection but prior to plasma analysis. Our first objective was to determine the correlation between ddPCR testing for plasma *AR* and an orthogonal approach, next-generation sequencing (NGS), in samples collected prior to starting treatment and after disease progression. Our second objective was to evaluate associations between pre-treatment plasma *AR* and clinical outcome in a primary cohort, representative of both pre- and post-docetaxel patients, and test for interactions with prior chemotherapy exposure. As no trial to date has randomised patients between first-line enzalutamide or abiraterone and taxanes, we combined data from four cohorts of men recruited to two biomarker protocols and defined by treatment with enzalutamide or abiraterone and prior chemotherapy status. Our third objective was to test our ddPCR assay in a second cohort of chemotherapy-naïve men treated with enzalutamide in the PREMIERE trial.

Participants

The primary cohort included patients participating in one of two protocols separately approved by the Institutional Review Board of the Royal Marsden (RM), London, UK (REC 04/Q0801/6), and Istituto Scientifico Romagnolo per lo Studio e la Cura dei Tumori (IRST), Meldola, Italy (REC 2192/2013). Docetaxel in this cohort was only used in the CRPC setting. The second cohort was the PREMIERE trial (EudraCT: 2014-003192-28, NCT02288936) that was sponsored and conducted by the Spanish Genito-Urinary oncology Group (SOGUG). The trial was approved by the independent review board at

each participating site. This trial was designed to analyse the predictive value of the gene fusion *TMPRSS2-ETS* in response to enzalutamide in patients with prostate cancer. Exploratory end-points included circulating cell-free DNA and circulating tumor cell (CTC) analysis. Data emerging after the trial was designed and initiated [7, 19, 20] led the PREMIERE Trial Management Group to prioritize two alternative biomarkers for evaluation, namely AR-V7 detected in CTCs as described previously [19] and plasma *AR*. *TMPRSS2-ETS* analyses are on-going and will be reported elsewhere. Preliminary AR-V7 data was presented in abstract form at the ESMO 2016 Annual Meeting [21] and will be published elsewhere. These analyses were based on the first censor cut-off, date May 2016. A second data analysis is planned at a predefined time-point when enough events have occurred to address the primary endpoint.

In both cohorts, patients were required to have histologically-confirmed prostate adenocarcinoma without neuroendocrine differentiation, progressive disease despite "castration levels" of serum testosterone (<50 ng/dL), on-going LHRH analogue treatment or prior surgical castration and no prior treatment with enzalutamide or abiraterone. Additional selection criteria by cohort are specified in the Supplementary Appendix S1, available at *Annals of Oncology* online. The choice of therapy in the primary cohort was at the discretion of the treating physician, either enzalutamide 160mg once a day or abiraterone 1g once a day and prednisone 5mg twice daily. In the PREMIERE trial, all patients received enzalutamide 160mg once a day. Treatment in both cohorts was administered continuously until evidence of progression disease or unacceptable toxicity. The studies were conducted in accordance with the Declaration of Helsinki and the Good Clinical Practice guidelines of the International Conference of Harmonization. Written informed consent was obtained from all patients.

Procedures

Peripheral blood samples were collected within 30 days of treatment initiation and plasma aliquots stored at -80°C. ddPCR assays were performed as described in detail in Supplementary Appendix S2, available at *Annals of Oncology* online. For each individual sample *AR* CN was estimated using each of the reference genes *NSUN3*, *EIF2C1*, and *AP3B1* and using *ZXDB* at Xp11.21 as a control gene to determine X chromosome CN. *AR* mutation detection assays were performed for the *AR* mutations 2105T>A (p.L702H), 2632A>G (p.T878A), and 2629T>C (p.F877L) with a limit of detection of 1-2% using an input of 2 to 4 ng of DNA. For NGS on plasma and patient-matched germline DNA, we used a customized AmpliSeq targeted gene panel including *AR*, sequenced on an Ion Torrent Personal Genome Machine or Proton as described previously [7, 8]. Computational analysis estimating the plasma DNA tumor content, *AR* CN quantitation and point mutation detection (with a sensitivity of 98-99% depending on position and coverage) was performed as previously [8].

Serum prostate specific antigen (PSA) was assessed within one week of starting treatment and monthly thereafter. Radiographic disease was evaluated with the use of computed tomography and bone scan at the time of screening and every 12 weeks on treatment. In the primary cohort, serum lactate dehydrogenase (LDH) and alkaline phosphatase (ALP) were also measured within one week of starting treatment. In PREMIERE, CTCs were evaluated pre-treatment using the AdnaTest for Prostate Cancer (Qiagen GmbH, Germany) as described previously [21].

Outcomes

For the primary cohort, the primary endpoint was OS. The secondary endpoints were progression-free survival (PFS) (biochemical and/or radiographic and/or clinical) and PSA response. For PREMIERE, the primary endpoint was PSA-PFS (sPFS). Secondary endpoints included radiographic-PFS (rPFS), OS and PSA response. OS was calculated from initiation of therapy to death from any cause. Patients

still alive at time of last follow-up were censored. PFS was calculated from the first day of enzalutamide or abiraterone therapy to the date of progression disease or death. Radiographic progression was defined using Response Evaluation Criteria in Solid Tumors version 1.1. PSA decline was evaluated according to Prostate Cancer Working Group (PCWG2) guidelines [22].

Statistical analyses

~~An R script [23] was developed to identify the optimal *AR* CN cut-point that associated with OS in the primary cohort, using maximum log-likelihood as correlative statistics in a multivariable Cox regression model by an approach described previously (Supplementary Appendix S3, available at *Annals of Oncology* online) [24]. The process was boot-strapped with 30,000 iterations to provide the measures of dispersion. Remaining analyses were conducted using Stata/MP 13.1 for Windows. Qualitative variables were compared using the Fisher's exact test. Time-to-event outcomes were evaluated using Kaplan-Meier survivor estimates, log-rank test and univariate and multivariable Cox-proportional hazards models. Selected clinically relevant baseline factors previously associated with prognosis were assessed for significant association with OS and PFS using an univariate Cox regression model. A multivariate Cox regression model was performed with a stepwise procedure to identify the prognostic factors for OS and PFS with a significance level of 0.05 for entry into the model. All tests were two-sided and an α-error of 5% was considered as significant (Supplementary Appendix S3, available at *Annals of Oncology* online).~~

An R script [23] was developed to identify the optimal *AR* CN cut-point that associated with OS in the primary cohort, using maximum log-likelihood as correlative statistics in a multivariable Cox regression model by an approach described previously (Supplementary Appendix S3, available at *Annals of Oncology* online) [24]. The process was bootstrapped with 30,000 iterations to provide the measures of dispersion. Remaining analyses were conducted using Stata/MP 13.1 for Windows. Time-to-event outcomes were evaluated using Kaplan-Meier survivor estimates, log-rank test and univariate and

multivariable Cox-proportional hazards models. The association of ~~a selected set of~~ clinically relevant baseline factors (previously showed to be associated with prognosis [25, 26] with OS and PFS was examined using a univariate Cox regression model. A multivariate Cox regression model was then performed with a stepwise procedure to identify the prognostic factors for OS and PFS with a significance level of <0.05 for entry into the model. All tests were two-sided and an α-error of 5% was considered as significant. Odds ratios of PSA response were determined using a 2x2 contingency table and significant differences using Fisher's exact test. (Supplementary Appendix S3, available at *Annals of Oncology* online).

RESULTS

Clinical Characteristics of the Primary Cohort

In the primary cohort, we had 171 men who started treatment with enzalutamide or abiraterone between Jan 31, 2011 and June 9, 2016, 73 prior to docetaxel and 98 after. All had received bicalutamide. Patient and treatment characteristics at the time of sample collection are detailed in Table 1.

Analytic Testing of Multiplex Droplet Digital PCR for Determination of Plasma *AR* Status

We used an optimized multiplex *AR* CN ddPCR assay on 2-4ng DNA from all pre-treatment samples and an additional 42 samples collected after disease progression. On a further 2-4ng DNA, we tested for *AR* mutations. From patients in the primary cohort with ddPCR data, we had NGS data available from our previous publication [8] for 86 samples and we performed NGS on an additional 75 (samples described in Supplementary Table S1, available at *Annals of Oncology* online). We observed a strong agreement between NGS and ddPCR for CN quantitation (n=161, Bland-Altman test: mean difference, -0.02, 95% CI Limits of agreement, -2.45 to 2.41) (Supplementary Figure S1A and Table S2, available at *Annals of Oncology* online). Estimation of *AR* mutation allelic frequency by ddPCR also displayed

strong agreement with NGS (*n*=60, Bland-Altman test: mean difference -0.001, 95% CI limits of agreement, -0.015 to 0.016) with no cases of mutations detected by one approach but not the other (Supplementary Figure S1B, available at *Annals of Oncology* online).

Plasma *AR* status in the Primary Cohort

In our primary cohort, eight post-docetaxel (but no chemotherapy-naïve) abiraterone patients were *AR* point mutation positive prior to treatment (Table 1). We planned to analyse these separately for associations with outcome. All four patients with a 2105T>A (p.L702H) mutation had received at least six months of treatment with prednisone. We did not detect a 2629T>C (p.F877L) *AR* point mutation prior to treatment or in an additional 26 samples collected after progression on enzalutamide. Using maximum likelihood ratio as correlative statistics combined with boot-strapping, we identified an *AR* CN cut-point of 2.01 (interquartile range (IQR), 1.82-2.77 copies) for splitting patients into two distinct prognostic groups (Supplementary Figure S2, available at *Annals of Oncology* online). Use of this cut-off was also supported by 95.5% concordance between NGS and ddPCR for classifying *AR* CN status (Supplementary Table S2, available at *Annals of Oncology* online). Overall, 10 (14%) chemotherapy-naïve and 33 (34%) docetaxel-treated patients had *AR* gain (Table 1).

Plasma *AR* Associates with Worse Outcome in the Primary Cohort

There was a significant association for *AR* gain and OS in both chemotherapy-naïve (median, 12.40 months versus not reached; HR, 3.98; 95% CI, 1.74-9.10; $p < 0.001$) (Figure 1A), and post-docetaxel patients (median, 9.51 versus 21.80 months; HR, 3.81; 95% CI, 2.28-6.37; $p < 0.001$) (Figure 1B). For *AR* mutants in abiraterone-treated, post-docetaxel patients, a significant association with worse survival was also seen (median 4.06 months; HR, 3.26; 95% CI, 1.47-not reached; $p = 0.004$) (Figure 1B). We also observed a significant association between PFS and *AR* gain for chemotherapy-naïve patients treated with enzalutamide or abiraterone (median, 7.30 versus 9.20 months; HR, 2.18; 95% CI, 1.08-

4.39; p = 0.03) (Figure 1C) and for post-docetaxel patients (median, 5.00 versus 7.36 months; HR, 1.95; 95% CI, 1.23-3.11; p = 0.01) (Figure 1D). A trend was seen for *AR* mutants to have worse PFS (median 4.10 months; HR, 2.10; 95% CI, 0.98-4.51; p = 0.057) (Figure 1D). Interactions between *AR* CN and treatment (abiraterone versus enzalutamide) (p = 0.41 for OS and p = 0.11 for PFS) or chemotherapy status (p = 0.83 for OS, p = 0.99 for PFS) examined in the Cox models were not significant. We also evaluated the association of *AR* status with the rate of PSA decline in the chemotherapy-naïve and post-docetaxel groups. Chemotherapy-naïve patients with *AR* gain were 4.7 times less likely to have a ≥50% decline in PSA (95% CI, 1.17-19.17; p = 0.035) (Figure 1E). Plasma *AR* gain chemotherapy-treated patients were 5.0 times less likely to have a ≥50% decline in PSA (95% CI, 1.70-14.91; p = 0.003) (Figure 1F). For the eight *AR* mutant patients, a trend for a lower rate of ≥50% PSA decline was seen (odds ratio (OR), 6.3; 95% CI, 0.72-54.59; p = 0.12) (Figure 1F).

Plasma *AR* Independently Associates with Worse Outcome on Multivariate Analysis in the Primary Cohort.

~~In our pre-specified~~Plasma *AR* status and 11 baseline characteristics previously shown to be clinically relevant [25,26] ~~and~~were evaluated by both univariate and multivariate analyses on the whole primary cohort. P~~p~~lasma *AR* gain or mutant were most significantly associated with OS or PFS ~~univariate and complete multivariate analyses~~ multivariate analysis (Supplementary Table S3 and Table S4 available at *Annals of Oncology* online). ~~and S4, available at *Annals of Oncology* online)~~ We then performed~~and~~ multivariate analysis with~~after~~ stepwise backwards elimination and the sole variables that remained significant were~~including~~ plasma *AR* status (HR, 4.10~~26~~; 95% CI, 2.6~~77~~6-6.3~~55~~; p < 0.001, and HR, 4.02~~3.80~~; 95% CI, 1.8~~77~~-8.66; ~~p~~p < 0.001 ~~= 0.011~~, for *AR* CN and *AR* mutant, respectively, Table 2A) and total plasma DNA concentration for OS and plasma *AR* status (HR, 2.06; 95% CI, 1.36-3.12~~48-3.34~~; p = 0.001, and HR, 2.20~~59~~; 95% CI, 1.03~~24~~-4.69~~5.44~~; p = 0.041~~12~~, for *AR* CN and *AR* mutant, respectively), total plasma DNA concentration and ALP levels for PFS (Table 2B). ~~serum LDH and~~

chemotherapy status (univariate analyses included in Supplementary Table S3, available at *Annals of Oncology* online), *AR* status was independently associated with the primary endpoint of OS (HR, 4.263.77; 95% CI, 2.7642-6.555.88; *P p* < 0.001, and HR, 3.802.76; 95% CI, 1.7726-8.156.07; *P p* = 0.011, for *AR* CN and *AR* mutant, respectively) (Table 2A) and PFS (HR, 2.221.96; 95% CI, 1.4832-3.342.93; *P p* <= 0.001, and HR, 2.59; 95% CI, 1.24-5.44; *p* = 0.012, for *AR* CN and *AR* mutant, respectively) (Table 2B).

Plasma *AR* status in the PREMIERE Cohort

The PREMIERE trial enrolled 98 patients in 16 sites between February 2015 through November 2015. Plasma was collected at study entry before starting enzalutamide from 94 patients who had a median follow-up of 10.6 months. Patient characteristics by plasma *AR* status are described in Table 3A.

Plasma *AR* Associates with Worse Outcome in the PREMIERE Cohort

Similar to our primary cohort pre-chemotherapy population, we observed *AR* gain in 11 (12%) patients. CTCs were detected in 35 patients (37%). *AR* gain was detected in seven (20%) CTC-positive and four (7%) CTC-negative patients (Table 3A). Plasma *AR* gain was significantly associated with shorter sPFS (median, 3.60 versus 15.5 months; HR, 4.33; 95% CI, 1.94-9.68; $p < 0.001$) (Figure 2A), rPFS (median, 3.90 months versus not reached; HR, 8.06; 95% CI, 3.26-19.93; $p < 0.001$) (Figure 2B) and OS (medians not reached; HR, 11.08; 95% CI, 2.16-56.95; P = 0.004) (Figure 2C) (Supplementary Table S5, available at *Annals of Oncology* online). Patients with *AR* gain were less likely to have a ≥50% decline in PSA (OR, 4.93; 95% CI, 1.30-18.75; $p = 0.025$) (Figure 2D).

Plasma *AR* Independently Associates with Worse Outcome on Multivariate Analysis in the PREMIERE Cohort

On multivariate analysis, the association of *AR* gain with the primary endpoint of sPFS was independent of plasma DNA concentration and the detection of CTCs (HR, 4.32; 95% CI 1.90-9.85; $p < 0.001$) (Table 3B). *AR* gain was also independently associated on multivariate analysis with rPFS (HR, 5.63; 95% CI, 2.15-14.74; $p < 0.001$) (Table 3B).

DISCUSSION

Several treatments are available for metastatic CRPC but to date, no approved biomarker to personalize therapy. Our analyses of plasma from 265 patients collected in three prospective biomarker protocols show that detection of *AR* CN gain prior to starting enzalutamide or abiraterone is associated with decreased OS and PFS regardless of prior chemotherapy status. We excluded samples from patient that had prior treatment with enzalutamide or abiraterone, given response rates and duration of benefit are very different when used sequentially [27]. Our previous study [8] suggests a similar association between plasma *AR* and resistance in patients previously treated with enzalutamide or abiraterone and this requires further investigation in future studies.

We ~~find that~~did not detect *AR* mutations (p.T878A or p.L702H) ~~are uncommon~~ in chemotherapy-naïve patients ~~and p.L702H is only detected in patients previously treated with prednisone~~. Our assay detects point mutations present in at least 2% of plasma DNA. Greater sensitivity is obtained with higher input DNA [2~~8~~6] although the clinical relevance of rarer mutations is uncertain. B~~Critically b~~y using a multiplex ddPCR with four carefully selected reference genes, we have designed a robust assay that does not over-call gain due to loss in regions involving the reference gene. Our model for estimating the likelihood of the *AR* CN cut-off that best predicts associations with outcome was built with 171 patients. We plan to perform a meta-analysis of multiple trials when the data on *AR* CN acquired from different institutions and trials exceeds 1000 patients. We report the absence of an interaction between *AR* and

chemotherapy status in non-randomized cohorts. ~~Randomization between docetaxel and *AR* targeting agents could be challenging without pre-defined molecular selection so we here used cohorts of post-docetaxel patients treated prior to marketing approval of abiraterone or enzalutamide for chemotherapy-naïve CRPC.~~

Detection of AR splice variants in CTCs is also associated with shorter PFS and OS with enzalutamide or abiraterone [19, 29]. *AR* CN is higher in the population with detectable CTCs although *AR* gain can also be observed in CTC-negative patients, accounting for one third of *AR* gained in the PREMIERE cohort. The overlap between AR-V7 positive and plasma *AR* gained patients and a comparison of the two tests in prospective trials is warranted to develop the best biomarker strategy for identifying resistant patients. Testing plasma *AR* status by ddPCR is affordable and can be widely implemented in clinical laboratories but does not control for plasma DNA tumor content [7, 8] that may introduce a bias. Nonetheless, multivariate analyses confirm that plasma *AR* by ddPCR provides information on the outcome of men starting enzalutamide or abiraterone that is independent of other factors previously reported to be prognostic ~~including serum LDH and CTC detection~~ [25, 26, 30]. In keeping with higher response rates to AR targeting in chemotherapy-naïve patients, the prevalence of plasma *AR* aberrations is 10-15% in this setting compared to 30-40% post-docetaxel. As our study is single arm, the associations we report are prognostic although the association with PSA decline rate suggests ~~Overall, our analyses provide strong supportive evidence for the role of~~ plasma *AR* CN could ~~for~~ identify~~ing~~ patients resistant to enzalutamide or abiraterone. The aims of our study were defined after sample collection and therefore larger studies with a pre-specified primary objective of defining the association with outcome by plasma *AR* status could provide further supportive evidence for the role of *AR* CN as a biomarker in CRPC. ~~Our results in patients at development of castration resistance suggest a role for plasma *AR* to select patients for taxane chemotherapy or alternative novel agents in preference to standard AR targeting at a key decision point in the treatment pathway., despite the~~

~~retrospective design of the study and the small number of patients showing *AR* aberrations, especially in chemotherapy-naïve patient group.~~ ~~F~~For level one evidence to change clinical practice, our findings ~~now~~ require confirmation in prospective ~~larger~~ trials where plasma *AR* CN defines treatment selection. ~~In addition, larger studies with pre-specified primary objectives could significantly evidence the role of *AR* CN as biomarker of resistance to anti-*AR* therapies.~~

Legend to figures

Figure 1. Association of plasma *AR* status and outcome in the primary cohort. Overall and progression-free survival for *AR* copy number normal, gain and mutated (Mut, p.L702H or p.T878A) chemotherapy-naïve (A, C) and post-docetaxel (B, D) castration-resistant prostate cancer patients treated with enzalutamide or abiraterone. PSA declines by *AR* status, waterfall plots of PSA declines for *AR* copy number normal, gain and mutated (Mut, p.L702H or p.T878A) chemotherapy-naïve (E) and post-docetaxel (F) castration-resistant prostate cancer patients. Bars were clipped at maximum 100%.

Figure 2. Association of plasma *AR* status and outcome in PREMIERE cohort. Biochemical progression-free survival (A), radiographic progression-free survival (B) and overall survival (C) for *AR* copy number normal versus *AR* gain patients. Waterfall plot (D) showing the magnitude of PSA decline by *AR* status. Bars were clipped at maximum 100%.

Key Message

There is an urgent need to identify biomarkers to guide personalized therapy in castration-resistance prostate cancer (CRPC). This is particularly important in chemotherapy-naïve CRPC, where no biomarker is available and biopsies can be challenging. Following a well predefined road-map for biomarker development, we aimed to clinically qualify androgen receptor (*AR*) status measurement in plasma DNA using an optimized multiplex droplet digital PCR (ddPCR) assay that includes four carefully selected reference genes and prevents to overcall gain due to loss in regions covered by the reference genes. Overall, 265 CRPC patients were studied in two cohorts: the primary cohort included 73 chemotherapy-naïve and 98 post-docetaxel patients treated with abiraterone or enzalutamide and independently recruited to two biomarker protocols at the Royal Marsden (UK) and IRST (Italy) between January 2011 and June 2016; the second cohort was composed of 94 asymptomatic or oligo-symptomatic chemotherapy-naïve patients recruited between February and November 2015 to the PREMIERE trial (NCT02288936), a Spanish Oncology Genitourinary Group (SOGUG) sponsored trial involving 16 Spanish hospitals. In the primary cohort, *AR* gain was observed in 10 (14%) chemotherapy-naïve and 33 (34%) post-docetaxel patients and was associated with a worse OS (Hazard Ratio (HR), 3.98; 95% CI, 1.74-9.10; p<0.001 and HR, 3.81; 95% CI, 2.28-6.37; p<0.001 respectively), PFS (HR, 2.18; 95% CI, 1.08-4.39; p=0.03, and HR, 1.95; 95% CI 1.23-3.11; p=0.01 respectively) and rate of PSA decline ≥50% (Odds ratio (OR), 4.7; 95% CI, 1.17-19.17; p=0.035 and OR, 5.0; 95% CI, 1.70-14.91; p=0.003 respectively). *AR* mutations (2105T>A (p.L702H) and 2632A>G (p.T878A)) were observed in eight (11%) post-docetaxel but no chemotherapy-naïve abiraterone-treated patients and were also associated with worse OS (HR 3.26; 95% CI, 1.47-not reached; p=0.004). There was no interaction between *AR* and docetaxel status (p=0.83 for OS, p=0.99 for PFS). In the PREMIERE trial, 11 patients (12%) had *AR* gain that had worse sPFS (HR, 4.33; 95% CI 1.94-9.68; <0.001), rPFS (HR, 8.06; 95% CI, 3.26-19.93; p<0.001) and OS (HR, 11.08; 95% CI, 2.16-56.95; p=0.004). Plasma *AR* was an independent predictor of outcome on multivariate analyses in both

~~cohorts. In conclusion, detection in plasma of *AR* aberrations, using a robust multiplex ddPCR method, predicts an adverse outcome in chemotherapy-naïve and post-docetaxel CRPC~~There is an urgent need to identify biomarkers to guide personalized therapy in CRPC. We clinically qualified androgen receptor (*AR*) status in plasma DNA using an optimized multiplex droplet digital PCR assay. We studied a primary cohort of 171 pre- and post-docetaxel patients treated with abiraterone or enzalutamide and a second cohort of 94 chemotherapy-naïve patients treated with enzalutamide, showing that detection of plasma *AR* aberrations predicted an adverse outcome in pre- and post-docetaxel CRPC.

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We would like to acknowledge all the staff at SOGUG for their support to run the PREMIERE trial and APICES for data management. We are grateful to Astellas for supporting the PREMIERE trial. We thank the participating men and their families who suffered from metastatic prostate cancer and nonetheless gave the gift of participation so that others might benefit.

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Disclosure

The ICR developed abiraterone and therefore has a commercial interest in this agent. D.D. and G.A. are on the ICR list of rewards to inventors for abiraterone. G.A. has received honoraria, consulting fees, or travel support from Astellas, Medivation, Janssen, Millennium Pharmaceuticals, Ipsen, Ventana, ESSA Pharmaceuticals, and Sanofi-Aventis and grant support from Janssen, AstraZeneca, and Arno.

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