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ORIGINAL RESEARCH

Longitudinal assessment of plasma androgen receptor copy number predicts overall survival in subsequent treatment lines in castration-resistant prostate cancer: analysis from a prospective trial

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Background: Baseline plasma androgen-receptor copy number (*AR*-CN) is a promising biomarker for metastatic castration-resistant prostate cancer (mCRPC) outcome and treatment response; however, the role of its longitudinal testing is unproven. We aimed to evaluate the prognostic role of *AR*-CN assessed before subsequent treatment lines in mCRPC patients.

Methods: A subgroup analysis of a prospective multicenter biomarker trial (IRSTB030) was carried out. Plasma *AR*-CN status (classified as normal or gain, cut-off value = 2) was assessed with digital PCR before each treatment line.

Results: Forty mCRPC patients receiving sequentially docetaxel, cabazitaxel and an AR signaling inhibitor (abiraterone or enzalutamide) were analyzed. At multivariate analysis, at each assessment overall survival (OS) was independently correlated with *AR*-CN status [first line: hazard ratio (HR) 4.1 [95% confidence interval (CI) 1.6-10.5]; second line: HR 2.4 (95% CI 1.1-5.3); third line: HR 2.1 (95% CI 1.0-4.3)] and median prostate-specific antigen [first line: HR 4.4 (95% CI 1.8-10.9); second line: HR 3.4 (95% CI 1.6-7.2); third line: HR 2.5 (95% CI 1.2-5.6)]. In the three subsequent assessments, *AR*-CN status changed from normal to gain in 15 (38%) patients. These patients had longer OS (47 months) compared with patients presenting *AR*-CN gain from first assessment (36 months), but shorter than those maintaining normal *AR*-CN (69 months) ($P = 0.003$).

Conclusions: Plasma *AR*-CN correlates with survival not only at baseline (before first treatment), but also in the assessments before the following lines. Interestingly, *AR*-CN status may change from normal to gain across subsequent treatments in a significant number of cases, identifying a group of patients with intermediate outcomes. Longitudinal assessment of *AR*-CN status could represent a promising method to capture mCRPC intrinsic heterogeneity and to improve clinical management.

Key words: liquid biopsy, copy number alterations, abiraterone acetate, enzalutamide, docetaxel

BACKGROUND

The treatment scenario for metastatic castration-resistant prostate cancer (mCRPC) has been rapidly expanding and many therapeutic options are currently available.[1-8] With the exception of poly(ADP-ribose) polymerase (PARP)-inhibitors in patients harboring DNA damage repair alterations, however, treatment selection and sequencing are still mainly directed by clinical parameters at the physicians' choice, due to the lack of valid biomarkers to guide clinical management. Thus, the identification of valid biomarkers, possibly non-invasive and easily repeatable, is urgently needed.

Plasma androgen receptor (AR) status is a promising biomarker for prognosis and response in prostate cancer (PC) patients receiving an AR signaling inhibitor (ARSI) or taxanes and it may change during the natural history of the disease, probably under the pressure of treatments and at disease progression.[9-13] The clinical impact of these dynamic changes has not been thoroughly explored so far, however, and the utility of repeated sampling of plasma *AR* status before the start of different treatment lines has not been investigated.

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METHODS

The aim of this study was to evaluate the role of repeated assessment of plasma *AR* copy number (CN) status as a prognostic liquid biopsy biomarker in patients with mCRPC treated with three subsequent treatment lines. Moreover, we aimed at evaluating the frequency of *AR*-CN status change across different treatments and exploring a possible correlation of the dynamics of *AR* status with clinical outcomes.

Between December 2014 and December 2018, 104 mCRPC patients who were candidates for cabazitaxel treatment were enrolled in the prospective multicenter biomarkers trial IRSTB030 (NCT03381326). We carried out a subgroup analysis of this cohort, selecting patients treated sequentially with docetaxel, cabazitaxel and an ARSI (abiraterone acetate or enzalutamide) in our center; treatment sequencing was chosen according to clinical practice. Clinical and biological data have been collected and evaluated.

Inclusion criteria were: age >18 years, histologically confirmed diagnosis of PC, presence of castration resistance status [progression of disease despite castration levels of serum testosterone (<50 ng/dl)], advanced disease, Eastern Cooperative Oncology Group performance status (ECOG PS) 0-2, adequate cardiac, renal, hepatic and bone marrow function. All patients were treated with cabazitaxel, docetaxel and one ARSI. Treatment administration was carried out according to National Comprehensive Cancer Network (NCCN) and European Society of Medical Oncology (ESMO) guidelines.[14,15] Dose reductions were permitted and managed by the physicians as clinically indicated.

The trial was approved by the Institutional Review Board of Istituto Scientifico Romagnolo per lo Studio e la Cura dei Tumori (IRST) IRCCS, Meldola, Italy (protocol code: IRSTB030) and was conducted in accordance with the Declaration of Helsinki and Good Clinical Practice guidelines of the International Conference of Harmonization. All patients signed informed consent for the biomarker research.

Plasma samples were collected immediately before each treatment start (before cycle 1). Clinical data [radiological disease status, serum prostate specific antigen (PSA), serum lactate dehydrogenase (LDH) and *AR*-CN status] were assessed before each treatment start. PSA and LDH were assessed pretreatment and before every subsequent cycle. The value of LDH was dichotomized at the upper limit of normal (ULN >225 U/l).

Disease was evaluated radiographically with the use of total body computed tomography (CT) scans and technetium bone scintigraphy or with positron emission tomography—CT (PET/CT) with ^{11}C-choline or ^{68}Ga-prostate-specific membrane antigen (PSMA) (according to physicians' choice), before each treatment start and every 12 weeks on treatment. Progression of disease was defined according to Prostate Cancer Clinical Trials Working Group (PCWG3) criteria, considering radiographic evidence of new lesions by bone scintigraphy or CT or PET/CT scans.[16]

DNA isolation and quantification

Plasma DNA was extracted with the QIAamp Circulating Nucleic Acid Kit (Qiagen, Milan, Italy) according to the manufacturer's instruction, using 1 ml of plasma. Total extracted DNA was quantified by a spectrophotometer (Nanodrop ND-1000, Celbio, Milan, Italy) using 2 µl of DNA.

Digital PCR analysis

Analyses of *AR*-CN were carried out with the QuantStudio3D digital Polymerase Chain Reaction PCR (dPCR) System (Thermo Fisher Scientific, Waltham, MA) in a duplex assay using FAM and VIC fluorescent probes. *AR*-CN was evaluated with two assays (AR1: Hs04107225; AR2:Hs04511283) and two reference genes were selected as control genes: RNaseP, TaqMan Copy Number Reference Assay, and AGO1 (Hs02320401) modified with VIC-labeled probe. DNA samples from three healthy male donors were pooled and used as a calibrator. Data were analyzed using QuantStudio 3D AnalysisSuite Cloud Software (Thermo Fisher Scientific). The average number of copies per reaction microliters was determined using Poisson distribution. A ratio of target copies and reference copies was measured for each sample, and then a ratio between the sample and calibrator was calculated. *AR*-CN status was defined as the mean value of the result of the two assays. *AR*-CN gain status was defined using a cut-point value >2.01 for gain, as previously described.[9]

Statistical analysis

Overall survival (OS) was calculated from each treatment start to death from any cause or the date of last follow-up; progression-free survival (PFS) was defined as the time between the date of each treatment start and the date of radiological, clinical or biochemical progression or last tumor evaluation. Data cut-off for OS and PFS was 31 March 2021.

PFS and OS were estimated using the Kaplan—Meier method and compared using the logrank test. *P* values were two-sided and $P < 0.05$ was considered as statistically significant. Statistical analyses were carried out with SAS statistical software, version 9.4 (SAS Institute, Cary, NC).

In order to determine an appropriate sample size for our study, a power analysis [SAS statistical software (SAS Institute)] was carried out. We aimed to detect a hazard ratio (HR) >2.00 (biomarker positive versus negative), with a significance level (α) set at 0.05 and a power (1-β) of 0.80. Taking into account preliminary data and previous studies in mCRPC patients, we estimated a 30% prevalence of *AR* alterations.[17,18] Using these parameters, the power analysis identified that a total sample size of 40 patients and 40 events (death) would be required to achieve adequate statistical power.[19,20]

RESULTS

Forty patients were included in the subgroup analysis. Median age at enrollment was 70 years [interquartile range (IQR) 64-75 years]. Median follow-up was 89.0 months (95% CI 11.4-111.0 months). ECOG-PS was 0 for 28 (70%) patients. Gleason score at diagnosis was ≥8 in 26 (65%) cases. Median PFS was 6.8 months (95% CI 5.4-8.9 months) for ARSI (in any line), 6.5 months (95% CI 5.4-7.0 months) for docetaxel, and 5.4 months (95% CI 3.6-6.8 months) for cabazitaxel.

Table 1. Patients' characteristics assessed before each treatment line

Line of treatment	First	Second	Third
	N (%)	*N* (%)	*N* (%)
Type of treatment			
ARSI	17 (42.5)	11 (27.5)	12 (30.0)
Docetaxel	23 (57.5)	17 (42.5)	0
Cabazitaxel	0	12 (30.0)	28 (70.0)
LDH			
<225 U/l (<ULN)	32 (80.0)	28 (70.0)	18 (45.0)
≥225 U/l (≥ULN)	8 (20.0)	12 (30.0)	22 (55.0)
Presence of bone metastases			
No	5 (12.5)	3 (7.5)	2 (5.0)
Yes	35 (87.5)	37 (92.5)	38 (95.0)
Presence of visceral metastases			
No	34 (85.0)	34 (85.0)	31 (77.5)
Yes	6 (15.0)	6 (15.0)	9 (22.5)
AR-CN status			
Normal	30 (75.0)	25 (62.5)	20 (50.0)
Gain	10 (25.0)	15 (37.5)	20 (50.0)
PSA, ng/ml: median value (range, IQR)	21 (1.8-531, 10.5-115)	25 (0.5-580; 8.5-136)	39 (0.4-593; 18-115.5)

AR-CN, androgen receptor copy number; ARSI, androgen receptor signaling inhibitor; IQR, interquartile range; LDH, lactate dehydrogenase; PSA, prostate-specific antigen; ULN, upper limit of normal.

Patients' characteristics assessed before each treatment line are reported in Table 1.

First-line assessment

A total of 23 (58%) patients received docetaxel, whereas 17 (42%) received an ARSI. Six (15%) patients had visceral metastases. Median PSA was 21 ng/ml (IQR 10.5-115 ng/ml). The level of LDH was >ULN in eight (20%) cases. A total of 10 (25%) patients presented with a CN gain of *AR* before first line; 30 (75%) patients had a normal *AR*-CN. Overall median PFS for first assessment treatment was 6.8 months (95% CI 5.4-7.3 months), whereas median OS was 50.8 months (95% CI 35.9-53.6 months). Median PFS was 8.9 months (95% CI 3.9-13.8 months) in patients treated with an ARSI and 6.6 months (95% CI 5-7.3 months) in patients treated with docetaxel at this assessment.

Second-line assessment

Patients received taxanes (17 docetaxel, 12 cabazitaxel) in 29 (73%) cases. Eleven (27%) patients received an ARSI. Six (15%) patients had visceral metastases. Median PSA was 25 ng/ml (IQR 8.5-136 ng/ml). LDH was higher than ULN in 12 (30%) patients. A gain in *AR*-CN was identified in 15 (38%) patients.

Median PFS and OS were 6.6 months (95% CI 5.5-7.0 months) and 28.4 months (95% CI 21.3-37.1 months), respectively. At this assessment, median PFS was 7.5 months (95% CI 3.5-12.4 months), 6.3 months (95% CI 2.1-7.0 months) and 5.8 months (95% CI 2.1-7.9 months) for patients treated with ARSI, docetaxel and cabazitaxel, respectively.

Third-line assessment

A total of 28 (70%) patients were treated with cabazitaxel, whereas 12 (30%) received an ARSI. Visceral metastases affected nine (22%) patients.

Median PSA was 39 ng/ml (IQR 18-115.5 ng/ml); LDH was >ULN in 22 (55%) patients. Twenty (50%) patients had *AR*-CN gain. Median PFS and OS were 5.2 months (95% CI 2.9-6.2 months) and 16.6 months (95% CI 10.0-20.3 months), respectively. In particular, at this assessment, median PFS was 5.6 months (95% CI 3.9-16.0 months), 6.0 months (95% CI 3.8-7.3 months) and 4.6 months (95% CI 2.4-6.8 months) for patients treated with an ARSI, docetaxel and cabazitaxel, respectively.

AR-CN status changes during subsequent treatments

The status of *AR* has been evaluated longitudinally through a repeated assessment before the start of three subsequent treatment lines for all 40 patients included in this subgroup analysis. *AR*-CN status (normal versus gain) for every patient before each line of treatment is represented in Figure 1. In detail, at the first assessment, 10 patients out of 40 (25%) presented *AR* gain; at the second one, 15 (38%) had *AR* gain; at the third one, 20 patients (50%) had *AR* gain.

Nineteen patients (48%) changed their *AR*-CN status in at least one of the three evaluated timepoints; 15 patients (38%) with baseline normal AR-CN acquired a gain in a subsequent sample.

Six patients presented *AR*-CN gain from baseline sampling and remained unchanged in the other two timepoint samples. A total of 15 patients remained *AR*-CN normal throughout all three samples. Four patients acquired a CN gain at second-line sampling and remained 'gained' thereafter, whereas nine patients acquired *AR*-CN gain only at third-line sampling. Interestingly, six patients presenting with *AR*-CN gain at any timepoint converted to *AR*-CN normal in at least one following sample. Among these patients, four received taxanes in the line preceding the conversion from gain to normal.

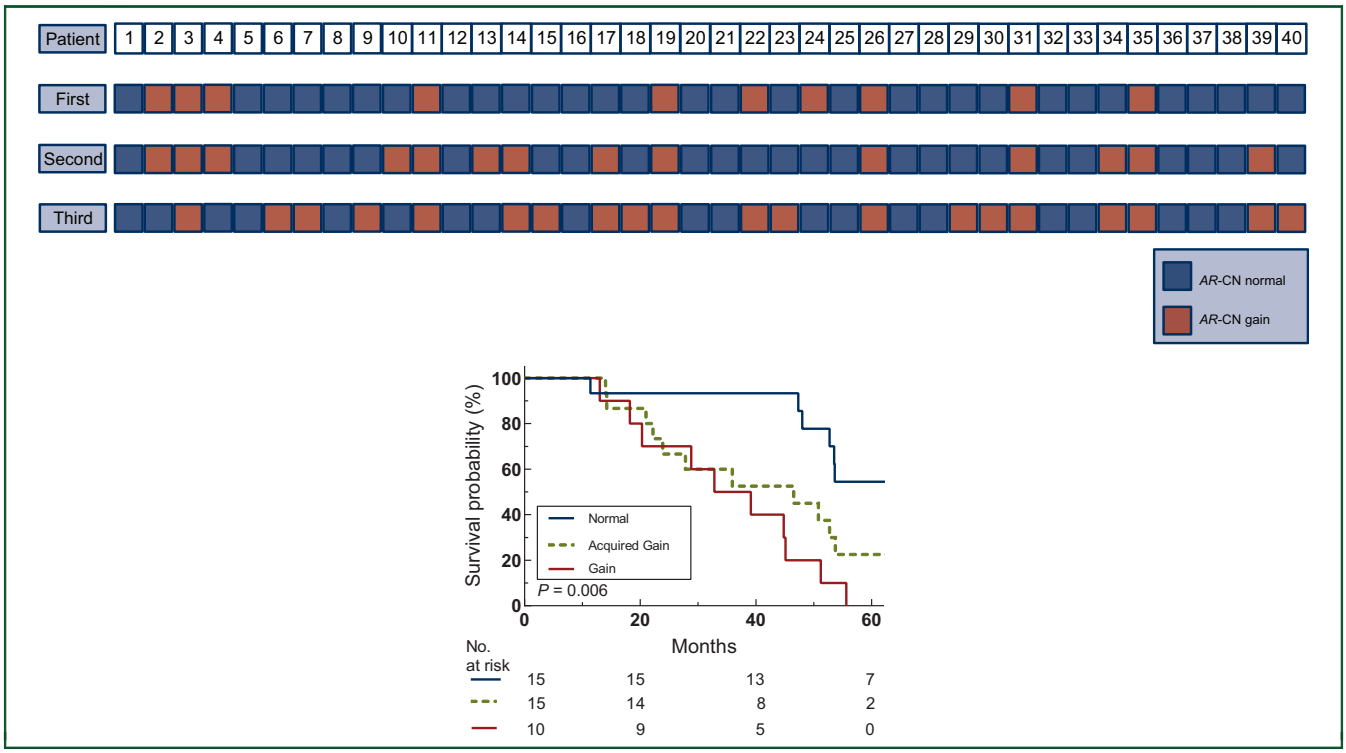

Figure 1. **Top: androgen receptor copy number (*AR*-CN) status for each patient assessed before first, second and third treatment line. Blue: *AR*-CN normal; red squares: *AR*-CN gain. Bottom: the effect of *AR*-CN status changes during subsequent treatment lines on overall survival. Red line: patients with *AR*-CN gain in all three assessments; Blue line: patients with *AR*-CN normal in all three assessments; Dotted line: patients acquiring *AR*-CN gain at second or third assessment.**

Prognostic factors for OS

Median PSA value and *AR*-CN status assessed before each treatment line start significantly correlated with OS in all three evaluations: in particular, patients with *AR*-CN gain and higher pre-treatment PSA had shorter OS when evaluated at each timepoint. Data are expressed as median, HR and 95% CI. Kaplan—Meier survival curves for *AR*-CN status assessed before first, second and third treatment are reported in Figure 2.

At first line, patients with a PSA value (measured before cycle 1) higher than median PSA (21 ng/ml) had a significantly shorter OS (HR 2.66, 95% CI 1.20-5.87; $P = 0.016$) (Supplementary Table S1, available at https://doi.org/10.1016/j.esmoop.2023.102036).

Patients with *AR*-CN gain before first-line treatment had a significantly shorter OS (HR 3.11, 95% CI 1.39-6.97; $P = 0.006$). In particular, median OS was 35.9 months in patients with a gain in *AR*-CN and 52.7 months in patients with normal *AR*-CN.

Presence of visceral metastases before first-line treatment significantly correlated with OS (HR 2.81, 95% CI 1.13-7.02; $P = 0.026$). Patients with visceral metastases had a median OS of 25.5 months, whereas patients with no visceral metastases had a median OS of 51.1 months. Age, LDH values, presence of bone metastases and type of treatment were not significantly related with OS.

At multivariate analysis (Table 2), PSA value (HR 4.40, IQR 1.77-10.93; $P = 0.001$) and *AR* status (HR 4.14, 95% CI 1.64-10.47; $P = 0.03$) at first-line assessment remained independently related to OS.

At second-line assessment, PSA values measured before cycle 1 higher than median PSA (25 ng/ml) significantly correlated with OS (HR 3.45, 95% CI 1.63-7.30; $P = 0.001$). Median OS was 39.3 months in patients with PSA <25 ng/ml, and 17.9 months for patients with PSA ≥ 25 ng/ml (Supplementary Table S1, available at https://doi.org/10.1016/j.esmoop.2023.102036).

The status of *AR*-CN evaluated before second line was significantly related to OS (HR 2.63, 95% CI 1.22-5.64; $P = 0.013$). Median OS was 35.9 and 20.1 months in patients with normal *AR*-CN and in those with a gain in *AR*-CN, respectively. Age, LDH value, presence of visceral metastases and type of treatment were not significantly related to OS at univariate analysis.

At multivariate analysis (Table 2), PSA value (HR 3.36, 95% CI 1.56-7.21; $P = 0.002$) and *AR* status (HR 2.37, 95% CI 1.05-5.33; $P = 0.037$) were independently related to OS at second-line assessment.

At third-line assessment, patients with a PSA value higher than median PSA (39 ng/ml) had a significantly poorer OS (12.3 versus 19.1 months for patients with PSA lower than median). This correlation was statistically significant (HR 2.24, 95% CI 1.07-4.69; $P = 0.033$) (Supplementary Table S1, available at https://doi.org/10.1016/j.esmoop.2023.102036).

AR status remained significantly correlated with OS also at third-line assessment: patients with *AR*-CN gain had a shorter OS (10 months) compared with those with normal *AR*-CN (19.1 months) (HR 2.19, 95% CI 1.07-4.49; $P = 0.033$).

The presence of visceral metastases at third-line assessment was a predictor of poorer OS (9.4 versus 20 months

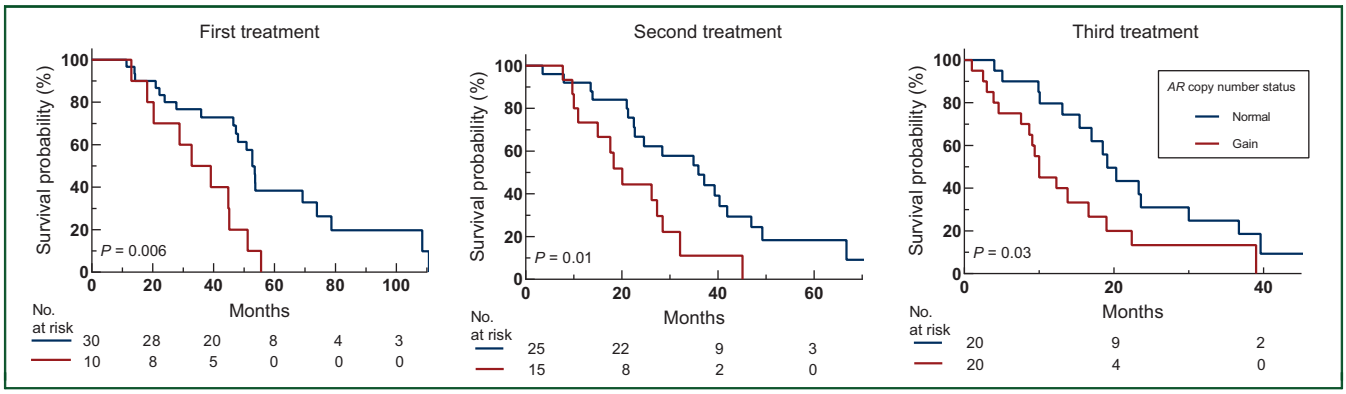

Figure 2. Correlation between androgen receptor copy number status assessed before first, second and third treatment line and overall survival.
AR, androgen receptor.

for patients with no visceral metastases) (HR 2.59, 95% CI 1.11-6.04; $P = 0.028$). Age, LDH value and treatment type were not significantly related to OS at univariate analysis. At multivariate analysis (Table 2), PSA value (HR 2.54, 95% CI 1.15-5.58; $P = 0.020$), *AR* status (HR 2.08, 95% CI 1.01-4.30; $P = 0.049$) and presence of visceral metastases (HR 2.83, 95% CI 1.17-6.84; $P = 0.021$) were independent prognostic factors for OS at third-line assessment.

Prognostic factors for PFS

At first-line evaluation, the presence of visceral metastases correlated with PFS (5.1 versus 7.2 months; HR 4.05 (95% CI 1.50-10.91); $P = 0.006$] (Supplementary Table S1, available at https://doi.org/10.1016/j.esmoop.2023.102036). The type of treatment also significantly correlated with PFS: patients treated with an ARSI had a longer PFS than those treated with docetaxel (7.6 versus 6.4 months, respectively; HR 2.02, 95% CI 1.03-3.98; $P = 0.042$).

At multivariate analysis (Table 2), only the presence of visceral metastases independently correlated with first-line PFS (HR 4.12, 95% CI 1.47-11.58; $P = 0.007$).

At second-line assessment, the type of second-line treatment correlated with PFS (Supplementary Table S1, available at https://doi.org/10.1016/j.esmoop.2023.102036): patients treated with ARSI experienced a longer PFS (8.9 months) than those treated with taxanes (docetaxel or cabazitaxel) (6.4 months) (HR 2.85, 95% CI 1.22-6.68; $P = 0.016$).

Also, PSA correlated with PFS (7 months for patients with higher than median value, 6 months for those with lower) (HR 1.9, 95% CI 0.98-3.70; $P = 0.05$).

At multivariate analysis (Table 2), PSA value was the only independent factor for second-line PFS (HR 2.42, 95% CI 1.17-5.00; $P = 0.017$).

At third line assessment (Supplementary Table S1, available at https://doi.org/10.1016/j.esmoop.2023.102036), PSA value higher than median PSA (39 ng/ml) was significantly related to third-line PFS. Patients with higher PSA values had shorter PFS (3.4 versus 5.6 months) (HR 2.22, 95% CI 1.12-4.41; $P = 0.022$).

The presence of visceral metastases also correlated with PFS (2.8 versus 5.6 months) (HR 2.36, 95% CI 1.07-5.50; $P = 0.033$). At multivariate analysis (Table 2), only PSA value was an independent predictor for third-line PFS (HR 2.13, 95% CI 1.06-4.29; $P = 0.034$).

Correlation of AR-CN status changes on OS

As discussed before, it was not infrequent to see a change in *AR*-CN status during treatment (Figure 1). A gain in *AR*-CN

Table 2. Multivariate analysis of progression-free survival and overall survival in the three treatment lines

	PFS HR (95% CI)	*P*	OS HR (95% CI)	*P*
First treatment				
PSA (≥21 versus <21)	1.05 (0.54-2.05)	0.881	**4.40 (1.77-10.93)**	**0.001**
Visceral mets (yes versus no)	**4.12 (1.47-11.58)**	**0.007**	2.09 (0.77-5.68)	0.150
AR-CN status (gain versus normal)	0.99 (0.45-2.16)	0.974	**4.14 (1.64-10.47)**	**0.003**
Second treatment				
PSA (≥25 versus <25)	**2.42 (1.17-5.00)**	**0.017**	**3.36 (1.56-7.21)**	**0.002**
Visceral mets (yes versus no)	0.62 (0.24-1.57)	0.314	1.25 (0.48-3.25)	0.642
AR-CN status (gain versus normal)	0.66 (0.33-1.33)	0.244	**2.37 (1.05-5.33)**	**0.037**
Third treatment				
PSA (≥39 versus <39)	**2.13 (1.06-4.29)**	**0.034**	**2.54 (1.15-5.58)**	**0.020**
Visceral mets (yes versus no)	2.19 (0.97-4.93)	0.058	**2.83 (1.17-6.84)**	**0.021**
AR-CN status (gain versus normal)	1.42 (0.71-2.84)	0.316	**2.08 (1.01-4.30)**	**0.049**

PSA values are expressed in ng/ml. Statistically significant results (*P* values <0.05) are indicated in bold.
AR, androgen receptor; CI, confidence interval; CN, copy number; HR, hazard ratio; mets, metastases; OS, overall survival; PFS, progression-free survival; PSA, prostate specific antigen.

Table 3. Androgen receptor copy number status changes effect on overall survival

	Median OS months (95% CI)	*P*	HR (95% CI)	*P*
AR-CN normal from baseline	69.2 (48.0-nr)		1.00	
Acquired *AR*-CN Gain	46.5 (21.0-53.7)		2.40 (1.00-5.81)	
AR-CN gain from baseline	35.9 (13.0-45.1)	0.003	4.87 (1.84-12.85)	0.006

AR-CN, androgen receptor copy number; CI, confidence interval; HR, hazard ratio; nr, not reached; OS, overall survival.

was detected in 25% of patients at the first assessment, in 38% at the second, and in 50% at the third one. Almost half of patients in our cohort changed their status through the three assessments. We analyzed the correlation of outcomes with the change of *AR*-CN status during treatment (Table 3).

Patients with normal *AR*-CN at all three timepoints had a longer OS (69.2 months) compared with those with *AR*-CN gain from the first assessment (35.9 months).

Interestingly, patients changing their status from normal to gain at the second or third assessment, had an intermediate OS (46.5 months) compared with the above-mentioned groups. (Figure 1). These differences in OS were statistically significant ($P = 0.006$).

DISCUSSION

This analysis of a subgroup of patients from a prospective multicenter biomarkers study (IRSTB030)[21] represents the first evidence that repeated assessment of *AR*-CN status before subsequent treatment lines can represent a valid prognostic biomarker for OS in mCRPC patients treated with ARSI, docetaxel and cabazitaxel.

Prostate cancer is a biologically heterogeneous and dynamic disease, with strong intra-patient tumor heterogeneity and clonal evolution occurring at various stages of disease.[22,23]

Acquired alterations of *AR*-CN have been demonstrated as one of the main mechanisms of the transition from hormone sensitivity to resistance.[24] It has been demonstrated how the presence of *AR* aberrations is both prognostic and predictive. In fact, the occurrence of *AR* aberrations is correlated with shorter survival and to lower response rates to ARSI, due to altered activation of the mutated *AR*.[15,25,26] The use of *AR* status as a biomarker, however, has not been validated prospectively and thus is not recommended in clinical practice.

Several studies have observed that plasma *AR* gain identified through next-generation sequencing or digital-droplet PCR is associated with resistance to ARSI and worst outcomes in terms of PFS and OS in chemotherapy-naive and post-docetaxel mCRPC.[8] Also, patients with *AR* gain seem to benefit more from taxane-based regimens compared with ARSI.[10] The evidence of the role of *AR*-CN status in predicting prognosis and response in mCRPC patients treated with cabazitaxel, however, is much lower. A retrospective analysis of 155 patients treated with cabazitaxel has reported shorter OS [10.5 versus 14.1 months, HR 1.44 (95% CI 0.98-2.13)] and PFS [4 versus 5 months, HR 1.47 (95% CI 1.05-2.07)] in *AR*-gained patients compared with *AR*-normal patients.[27] A recent meta-analysis including 16 papers and >1000 patients has confirmed the role of plasma *AR*-CN gain in mCRPC as a robust prognostic biomarker for the response to ARSI, with a fixed effect in different treatment lines.[28] The authors hypothesized that patients with plasma *AR*-CN gain might benefit more from cabazitaxel (or first-line docetaxel) than from ARSI, whereas patients with normal *AR*-CN could still benefit from ARSI even in later lines.

In our study, we observed that *AR*-CN status is a dynamic biomarker: it can change during different treatments, probably due to treatment pressure on prostate cancer cells leading to treatment resistance. In our population, a substantial number of patients (48%) changed *AR*-CN status throughout subsequent treatments and disease progression. Notably, patients with a normal *AR*-CN at baseline acquiring *AR*-CN gain at second or third evaluation, have an intermediate OS compared with those with *AR*-CN normal or gain throughout the three assessments.

We observed that change from gain to normal *AR*-CN in the three assessments was not infrequent; in fact, six patients converted their *AR*-CN status from gain to normal. Due to the low number of events, however, no conclusions can be drawn. Moreover, it cannot be excluded that this event could be due to a decrease in circulating tumor DNA at some timepoints, as a result of treatment response.

It is known how PC has an intrinsic heterogeneity and that its biological features may vary during the natural history of the disease.[22,23] This is one of the reasons why liquid biopsy represents a valid tool, depicting the various biological changes of the disease.

These findings suggest that evaluating *AR*-CN status only at baseline could not reflect properly the dynamic and heterogenic nature of CRPC and therefore a patient's status should be longitudinally assessed before each treatment, in order to have updated information on its biological status and to improve clinical management and treatment selection.

We demonstrate that *AR*-CN status variation correlates with OS if assessed before three subsequent treatment lines, and was prognostic both for ARSI and taxanes. Although the role of *AR* aberrations on ARSI response has been widely demonstrated, their impact on taxanes is much less investigated.

The worse outcomes in *AR*-gained patients treated with taxanes observed in our series could be related to the high prevalence of co-occurrence of androgen receptor splice variant 7 (AR-V7) and *AR* gains (reported to be up to four-fold higher), especially in more advanced disease stages.[29] It is known that the presence of AR-V7 confers resistance to ARSI, but recent studies have demonstrated that this alteration could also negatively affect taxane response.[30,31] In particular, in a previous prospective study from our group on cabazitaxel-treated mCRPC patients, we have observed that AR-V7 expression on circulating tumor cells was

significantly associated with *AR*-CN gain.[21] In the same cohort, we have demonstrated not only that patients harboring *AR*-CN gain before cabazitaxel start have shorter OS and PFS, but also that AR-V7-positive patients have worse outcomes when treated with reduced chemotherapy doses. Therefore, in our cohort of advanced and pretreated patients, the potential high prevalence and progressively accumulating co-occurrence of *AR* gain with AR-V7 could further explain why patients with detectable *AR* gain have poor outcomes regardless of treatment type. Nevertheless, the predictive role of the variations of *AR*-CN status needs to be investigated by further and larger studies, designated for this purpose.

The role of PSA as a prognostic factor has been widely demonstrated and is used routinely in daily clinical practice. Its role as a predictor for OS in mCRPC patients treated with ARSI or chemotherapy, however, is less clear.[32-34] In our study, PSA remained a good prognostic factor for OS in each treatment line. Similarly, a retrospective study on 45 patients with mCRPC has reported that, besides the presence of visceral metastasis, PSA >100 ng/ml before cabazitaxel treatment is an independent factor associated to OS;[35] a recent systematic review has confirmed that PSA absolute value and kinetics are significant predictors for OS and PFS for patients treated with ARSI and taxanes.[36]

We acknowledge some limitations of this study: although its unique design and the statistical power analysis requirements were met, a larger cohort of patients could strengthen and confirm our findings. Also, the heterogeneity of treatment regimens in each line might undermine the power of the results.

Although in our cohort *AR*-CN status has been shown to be independently related to OS even when tested with markers of high tumor burden (PSA, presence of visceral metastases), the values of *AR*-CN have not been controlled for circulating tumor DNA fraction; this issue could represent a bias since the detection of *AR*-gained clones could be related to higher fractions of circulating tumor DNA, which are themselves known to be prognostic.[37,38] Lastly, only *AR*-CN gains were considered, thus excluding other known *AR* aberrations, such as somatic point mutations or splice variants, which could give additional information.

Although the clinical utility of these findings should be validated in *ad hoc* clinical trials, they provide a proof-of-concept framework for investigating in real time the molecular and biological bases of treatment resistance. The sequential assessment of plasma *AR*-CN in patients on an individual basis throughout subsequent treatment lines could enable a real-time tracking of emerging clones resistant to treatments and ultimately an improvement of patients' stratification and tailored clinical management.

The status of plasma *AR*-CN could allow a more precise identification of patients with higher risk of early progression and for whom closer monitoring could be advised. Recent studies have shown the usefulness of integrating plasma *AR*-CN status with other circulating biomarkers and functional imaging to obtain a multimodal approach to help outcome prediction.[38,39]

The assessment of plasma *AR*-CN could also represent a valuable tool to refine the selection of patients with lower chances of response to standard treatments and who could benefit from treatment intensification through the use of combinatorial approaches or novel agents, preferably within clinical trials. Finally, the detection of *AR* gains could help clinicians to select patients for whom the use of taxanes at reduced doses should be avoided, according to what suggested in previous studies.[18,21]

CONCLUSIONS

The results of our study confirm that *AR*-CN status represents a valid prognostic liquid biopsy biomarker in mCRPC. Our results show that repeated plasma *AR*-CN assessment before subsequent treatment lines may represent a useful and feasible approach to optimize prognostication and treatment management in patients affected by mCRPC.

Plasma *AR*-CN correlates with OS not only before first line, but also in the assessments prior to later treatment lines, across different treatment types. Interestingly, *AR*-CN status may change from normal to gain across subsequent treatments in a significant number of cases, thus identifying a group of patients with intermediate outcomes. Longitudinal assessment of *AR*-CN status, thanks to its non-invasiveness and the possibility of being easily repeatable, could represent a promising and feasible method to capture mCRPC intrinsic heterogeneity and dynamic biological landscape through the journey of patients during different therapies, disease progression and treatment resistance occurrence.

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DISCLOSURE

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DATA SHARING

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The trial was approved by the Institutional Review Board of Istituto Scientifico Romagnolo per lo Studio dei Tumori (IRST) IRCCS, Meldola, Italy (protocol code: IRSTB030) and

was conducted in accordance with the Declaration of Helsinki and Good Clinical Practice guidelines of the International Conference of Harmonization. All patients signed informed consent for the biomarker research.

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