Prognostic significance of total plasma cell-free DNA level and androgen receptor amplification in castration-resistant prostate cancer

（去勢抵抗性前立腺癌における cell-free DNA 総量、
アンドロゲン受容体増幅の予後因子としての有効性）

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Abstract

Purpose: To investigate the prognostic significance of total cell-free DNA (cfDNA) level and androgen receptor amplification (*AR*-amp) in patients with castration-resistant prostate cancer (CRPC).

Methods: We retrospectively compared the total cfDNA level and *AR*-amp in 42 individuals without prostate cancer, 57 patients with localized prostate cancer without androgen-deprivation therapy (ADT), 97 patients with castration-sensitive prostate cancer (CSPC) with ADT, and 97 patients with CRPC. The association of these cfDNA biomarkers on disease status and overall survival was evaluated using Kaplan–Meier analysis and multivariable Cox regression analysis. Finally, a simple risk model was developed including total cfDNA and *AR*-amp to predict poor prognosis

Results: The median total cfDNA level and *AR*-amp in patients with CRPC was 387 pg/μL and 1.07 copies, respectively. The total cfDNA levels and *AR*-amp were significantly higher in the patients with CRPC than in individuals without prostate cancer, patients with localized prostate cancer without ADT, and patients with CSPC with ADT. Total cfDNA-high (>600 pg/μL) and *AR*-amp-high (>1.26 copies) were significantly associated with poor overall survival. Multivariable Cox regression analysis showed cfDNA-high and *AR*-amp-high were

significantly associated with poor overall survival in patients with CRPC. We developed a risk model using cfDNA-high (score 1) and *AR*-amp-high (score 1). The risk score 1-2 was significantly associated with worse overall survival than score 0.

Conclusion: Total cfDNA level and *AR*-amp are potential biomarkers for poor prognosis in patients with CRPC.

Introduction

Prostate cancer (PC) is the major cancer in men in Western countries and Japan [1-3]. As the progress to metastatic castration-resistant PC (CRPC) remains a major cause of deaths [4], there is an urgent need for practical tumor biomarkers to guide treatment [5-8]. Androgen receptor (AR) gene copy number variations (CNV) from plasma cell-free DNA (cfDNA) and circulating tumor DNA (ctDNA) are promising tools for identifying tumor-specific mutations in patients with CRPC [9-15]. However, plasma *AR* status alone is inadequate as a robust CRPC biomarker because not all CRPCs exhibit *AR* amplification (*AR*-amp) [16,17]. Also, ctDNA analysis has several limitations in clinical practice including definition, detection, genome coverages, and costs. Conversely, total cfDNA is a simple predictor of systemic inflammatory and cell death. Although total cfDNA level is not the specific biomarker for CRPC, recent studies suggested the potential association of total cfDNA with tumor aggressiveness [10,18,19]. Accordingly, we hypothesized that the combination of total cfDNA level and *AR*-amp have a potential to predict poor prognosis in patients with CRPC. In this study, we retrospectively compared total cfDNA level and *AR*-amp and investigated the prognostic significance of total cfDNA and *AR*-amp in patients with CRPC.

Materials and methods

This retrospective study was performed under the ethical standards of the Declaration of Helsinki and was approved by the Ethics Review Board of the Hirosaki University School of Medicine (authorization number: 2019–094).

Study population and patient selection

We evaluated 42 healthy individuals and 251 patients with PC who were treated at Hirosaki University Hospital and related hospitals between January 2001 and February 2020. Of the 251 patients with PC, 57 had localized PC without androgen-deprivation therapy (ADT), 97 had castration-sensitive PC under ADT (CSPC-ADT group), and 97 had CRPC (CRPC group). The CRPC group was further classified into two subgroups: nonmetastatic CRPC (M0CRPC, n = 19), and metastatic CRPC (M1CRPC, n = 78). The inclusion criteria for PC patients were: 1) histologically proven or clinically diagnosed PC, and 2) patients who were treated with surgery, radiotherapy, ADT ± bicalutamide, or life-extending therapy (ADT + second-generation AR axis-targeted therapy and/or taxane-based chemotherapy) as a standard of care. The exclusion criteria for PC patients were: 1) patients with no cfDNA samples and 2) those with insufficient baseline clinical information.

Variable evaluations

The following variables were analyzed at diagnosis: age, year of diagnosis, Gleason score, serum prostate-specific antigen (PSA) at diagnosis and cfDNA evaluation, serum testosterone level at cfDNA evaluation, and prognosis. The tumor stage and grade were assigned based on the 2009 TNM classification of the Union of International Cancer Control. Metastatic status was evaluated via chest and body computed tomography and bone scintigraphy before initiating ADT. Bone metastatic volume was evaluated by the extent of disease on bone scintigraphy. In patients with metastatic disease, the high-volume disease was defined using the CHAARTED criteria: The presence of visceral metastases, or ≥4 bone lesions with ≥1 beyond the vertebral body and pelvis. The overall survival was evaluated from the date of cfDNA evaluation to the date of any cause of death or final follow up.

Treatment protocol

Patients with localized PC were initially treated with surgery (with or without 6-9 months neoadjuvant therapy), radiotherapy with concomitant ADT (6-18 months), or ADT alone. Patients with metastatic PC were initially treated with ADT (with or without bicalutamide). CRPC was defined according to the recommendations of the Cancer Clinical Trials Working Group 2 [20]. Following the CRPC diagnosis, patients underwent sequential therapy based on the

decision of the attending physicians, including ADT + AR axis-targeted therapy and/or taxane-based chemotherapy. Indication of life-extending therapy for CRPC in Japan was reported in the previous study [21].

Plasma cfDNA extraction and cfDNA characteristics

Plasma samples were isolated using a BD vacutainer CPT cell preparation tube with sodium citrate. Isolated plasma was then subject to cfDNA extraction: cfDNA was extracted from the sample (1 mL) using AB MagMAX Cell-Free DNA Isolation Kit (Applied Biosystems, Foster City, CA, USA). The length and concentration of cfDNA were analyzed using an Agilent High Sensitivity DNA Kit and Agilent Bioanalyzer 2100 (Agilent Technologies Japan, Ltd Tokyo, Japan).

CNV of the *AR* gene in cfDNA

All reagents and equipment used for droplet digital PCR (ddPCR) were from Bio-Rad Laboratories (Hercules, CA, USA). The copy number variation value of the AR for each cfDNA sample was normalized to the reference gene (aquaporin-5, AQP5). The PCR probes for copy number variation of human androgen receptor (AR CNV, copies) (LBx® Probe AR CNV, A109), and human aquaporin-5 (AQP5) (LBx® Probe AQP5 CNV, A124, as a control) were purchased from RIKEN GENESIS CO LTD (Tokyo Japan).

Outcomes of total cfDNA levels and *AR*-amp

We compared the total cfDNA and *AR*-amp (*AR* CNV, copies) levels among the healthy individuals, localized PC, without ADT, CSPC-ADT, and CRPC. We investigated the association among the cfDNA and *AR*-amp and clinical variables in CRPC patients. Overall survival (OS) was compared between the total cfDNA-high and -low patients, between the *AR*-amp-high and -low patients.

Risk model development using cfDNA parameters

The effect of total cfDNA level and *AR*-amp on prognosis in patients with CRPC was evaluated using multivariable Cox regression analysis including age and CHAARTED high-volume disease. We then developed a risk model to predict poor prognosis using total cfDNA and *AR*-amp.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 7.00 (GraphPad Software, San Diego, CA, USA), BellCurve for Excel (Social Survey Research Information Co., Ltd., Tokyo, Japan), and R 3.3.2 (The R Foundation for Statistical Computing, Vienna, Austria). The statistical difference was compared using the Student t-test, Mann–Whitney U test, Fisher exact test, or Chi-squared test. To estimate the high value of total cfDNA and *AR*-amp in the CRPC patients, we evaluated the optimal cutoff values of total cfDNA and *AR*-

amp for any cause of death using a receiver operating characteristic (ROC) curve and the area under the curve (AUC). A linear relationship between the two variables was evaluated by scatterplot and linear regression analysis with correlation coefficient (R). Absolute R values of 0.00-0.24, 0.25-0.49, 0.50-0.74, and 0.75-1.00 were defined as none to a very weak, weak, moderate, and strong linear relationship. OS was estimated and compared using the Kaplan–Meier curve and the log-rank test, respectively. Multivariable Cox regression analyses were performed and the hazard ratio with 95% confidence interval was calculated. P-values < 0.05 were considered statistically significant.

Results

The median age of healthy individuals and patients with PC was 51 (interquartile range: IQR, 39-51) and 74 (69-80) years, respectively. The median months from CRPC diagnosis to cfDNA evaluation in the CRPC group was 12 (IQR, 4-12) months. There were 1 and 33 patients with any cause of death in the CSPC-ADT and CRPC groups (**Table 1**), respectively. We found 7 patients (7/251, 2.8%) who could not evaluate total cfDNA but can detect *AR*-amp (localized PC n = 3 and CSPC-ADT n = 4). We excluded those patients from the *AR*-amp analysis but included in the total cfDNA analysis. Finally, the number of patients in the total cfDNA analysis was 54 in the localized PC, 93 in the CSPC-

ADT, and 97 in the CRPC groups. The median total cfDNA (**Fig. 1A**) and *AR*-amp (**Fig. 1B**) levels were significantly higher in the CRPC group than in the other groups. We compared the total cfDNA level and *AR*-amp between the healthy individuals and patients with localized PC without ADT and confirmed that there was no significant difference between the two groups (**Fig. 1A and 1B**).

Of 97 patients with CRPC, the total cfDNA level was significantly associated with *AR*-amp level ($P < 0.001$), while it showed a weak relationship between them ($R = 0.48$) (**Fig. 1C**). The serum testosterone level was not significantly associated with *AR*-amp level ($P = 0.184$), and demonstrated none to a very weak relationship ($R = 0.02$) (**Fig. 1C**). The total cfDNA level was significantly associated with PSA level at the cfDNA evaluation ($P < 0.001$), while a weak relationship was observed between them ($R = 0.41$) (**Fig. 1D**). The *AR*-amp level was not significantly associated with PSA level at the cfDNA evaluation ($P = 0.294$), and demonstrated none to a very weak relationship ($R = 0.04$) (**Fig. 1D**).

The total cfDNA level was not significantly higher in the M1CRPC (median, 396 pg/μL) group compared to the M0CRPC (median, 275 pg/μL) group ($P = 0.065$) (**Fig. 1E**). The *AR*-amp level was not significantly different between the M0CRPC (median 1.03 copies) and M1CRPC (median 1.08 copies) groups (P

= 0.511) (**Fig. 1E**). The number of treatment lines after CRPC diagnosis was not significantly associated with the total cfDNA level and *AR*-amp (**Fig. 1F**). Also, the total cfDNA and *AR*-amp were not significantly different between the pre-docetaxel and post docetaxel status (**Fig. S1A**). The median time from CRPC diagnosis to cfDNA evaluation was 12 months. There was no significant difference in the total cfDNA (P = 0.201) and AR-amp (P = 0.896) levels between the <12 and ≥12 months (**Fig. S1B**). The optimal cutoff value of total cfDNA and *AR*-amp for any cause of death was defined as > 600 pg/mL with an AUC value of 0.713 (**Fig. S1C**), and >1.26 copies with an AUC value of 0.616 (**Fig. S1D**). OS was significantly shorter in total cfDNA-high patients (>600 pg/μL) than in total cfDNA-low patients (≤600 pg/μL) (P < 0.001) (**Fig. 1G**). Similarly, OS was significantly shorter in *AR*-amp-high patients (>1.26 copies) than in *AR*-amp-low patients (≤1.26 copies) (P < 0.001) (**Fig. 1H**).

Age-adjusted multivariable Cox regression analysis showed that total cfDNA-high and *AR*-amp-high were significantly associated with poor OS (**Fig. 2A**). We developed a risk model including total cfDNA-high (1 point) and *AR*-amp-high (1 point) (**Fig. 2B**). The risk score of 1-2 was significantly associated with poor prognosis than that of 0 in CRPC patients (P < 0.001) (**Fig. 2C**).

Discussion

We evaluated the utility of the total cfDNA and *AR*-amp as a biomarker in patients with CRPC. We found that total cfDNA and *AR*-amp were significantly higher in the CRPC group. Furthermore, the combination of total cfDNA-high and *AR*-amp-high were significantly associated with poor OS.

The disadvantage of total cfDNA is the presence of nonspecific biological noise, such as that resulting from tissue damage or inflammation. Circulating-tumor DNA analysis is necessary to detect tumor specific aggressiveness. However, ctDNA analysis has several limitations in clinical practice. Accordingly, we focused on the clinical implication of total cfDNA level in patients with CRPC. A recent study showed a synchronize trend of ctDNA and total cfDNA levels in patients with CRPC [10]. We demonstrate that total cfDNA can be used as a prognostic biomarker for CRPC with the optimal cutoff of total cfDNA (>600 pg/μL) and *AR*-amp (>1.26 copies). Our results showed a total cfDNA level of >600 pg/μL might be feasible to indicate any abnormal reaction within the body such as disease progression based on the maximum value of total cfDNA in the healthy individuals [22-24]. However, not enough evidence is available for the utility of total cfDNA and *AR*-amp in the patients with CRPC. Therefore, further

studies are necessary to confirm the utility of the total cfDNA level on prognosis in patients with CRPC.

Our results showed significantly poor OS in patients with the *AR*-amp-high than those with *AR*-amp-low. The cutoff of >1.26 copies for *AR*-amp was similar to that reported by previous studies (*AR*-amp >1.38 [25], > 1.54 [14] or > 2.01 copies [26]). Because *AR*-amp was the most frequent gene modification in patients with CRPC [27], it may be a hallmark of gene alternation in CRPC cells. However, *AR*-amp status alone is unable to capture non-*AR* related disease progression, such as neuroendocrine differentiation [16]. Therefore, we need to use quantitative and qualitative evaluation of ctDNA in patients with neuroendocrine differentiation to optimize treatment selection [7,28,29].

The number of treatment lines or pre- or post-docetaxel setting was not significantly associated with total cfDNA level and *AR*-amp. Initially, we hypothesized that cfDNA alterations would be high at the late line of treatment. However, we found no clear relationship between the time from CRPC diagnosis and cfDNA alterations (**Fig. S1B**). However, longitudinal studies are necessary to clarify the mechanisms for the acquired resistance to CRPC treatments.

Tumor-specific biomarkers such as DNA from exosomes and/or circulating-tumor cells are desirable to develop an accurate risk model. AR

splice variants 7 is one of the most promising biomarkers in mCRPC [30]. However, there still be challenges in the equipment, running costs, genome coverage, and the mixture of genomic and somatic mutations in clinical practice. Therefore, we focused on the simple combination of total cfDNA and *AR*-amp in this study. However, we agree with the limitation of our method using nonspecific biomarkers. Our next study needs to address these limitations.

The limitations of this study include the retrospective study design, small sample size, selection bias, and unmeasurable confounding factors. Moreover, we could not define the predictive value of cfDNA due to the cross-sectional sampling and mixed treatments in our cohorts. Despite these limitations, our results demonstrated the combination of simple biomarkers is useful for predicting the prognosis of patients with CRPC. Further studies are warranted to clarify whether it can be utilized as a simple cfDNA biomarker in CRPC.

Conclusion

Total cfDNA level and *AR*-amp are potential biomarkers for poor prognosis in patients with CRPC.

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Conflicts of Interest: None of the authors have any conflicts of interest to declare.

Ethics statement:

The present retrospective, multicenter study was performed in accordance with the ethical standards of the Declaration of Helsinki, and it was approved by the ethics review board of the Hirosaki University School of Medicine (authorization number: 2018–062) and all hospitals. Pursuant to the provisions of the ethics committee and the ethics guidelines in Japan, written informed consent is not required for public disclosure of study information in the case of a retrospective and/or observational study using materials, such as existing documents (opt-out approach).

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Figure legends:

Figure 1 Clinical implication of total cfDNA level and *AR*-amp status

We found 7 patients (7/251, 2.8%) who could not evaluate total cfDNA but can detect AR-amp (localized PC n = 3 and CSPC-ADT n = 4). We included those patients in the AR-amp analysis but excluded the total cfDNA analysis. Finally, the number of patients in the total cfDNA analysis was 54 in the localized PC, 93 in the CSPC-ADT, and 97 in the CRPC groups. Total cfDNA (**A**) and *AR*-amp (**B**) levels were compared among the healthy individuals, and patients with localized PC, CSPC-ADT, and CRPC. A linear relationship between the *AR*-amp and total cfDNA levels, and between the *AR*-amp and total testosterone levels was evaluated by scatterplot and correlation coefficient (**C**). A linear relationship between the PSA and total cfDNA levels, and between the PSA levels and *AR*-amp was evaluated by scatterplot and correlation coefficient (**D**). Total cfDNA and *AR*-amp were compared between the M0CRPC and M1CRPC groups (**E**). Total cfDNA level and *AR*-amp were compared between the patients with first-line treatment and second or more line treatments after CRPC (**F**). Overall survival was compared between the total cfDNA-high (>600 pg/μL) and total cfDNA-low (≤600 pg/μL) patients (**G**). Overall survival was compared between the *AR*-amp-high (>1.26 copies) and *AR*-amp-low (≤1.26 copies) patients (**H**).

Figure 2 Development of the total cfDNA and *AR*-amp risk model in patients with CRPC

Multivariate Cox regression analysis for overall survival including age, CHAARTED criteria (high or low), total cfDNA level (high or low) and *AR*-amp (high or low) (**A**). The risk model including total cfDNA-high (score 1 point), *AR*-amp-high (score 1 point) is shown on the 2-dimensional scatterplot (**B**). Overall survival was stratified among patients with cfDNA risk scores of 0, 1, and 2 (**C**).

Figure S1 Supplementary figures

The total cfDNA and *AR*-amp were compared between the pre-docetaxel and post docetaxel status (**A**). The median time from CRPC diagnosis to cfDNA evaluation was 12 months. The relationship between the time from CRPC diagnosis and cfDNA parameters was evaluated (**B**). There were no significant differences in the total cfDNA level and *AR*-amp between <12 and ≥12 months. The optimal cutoff value of total cfDNA (**C**) and *AR*-amp (**D**) for any cause of death were defined using a ROC curve and the AUC.

Fig.1

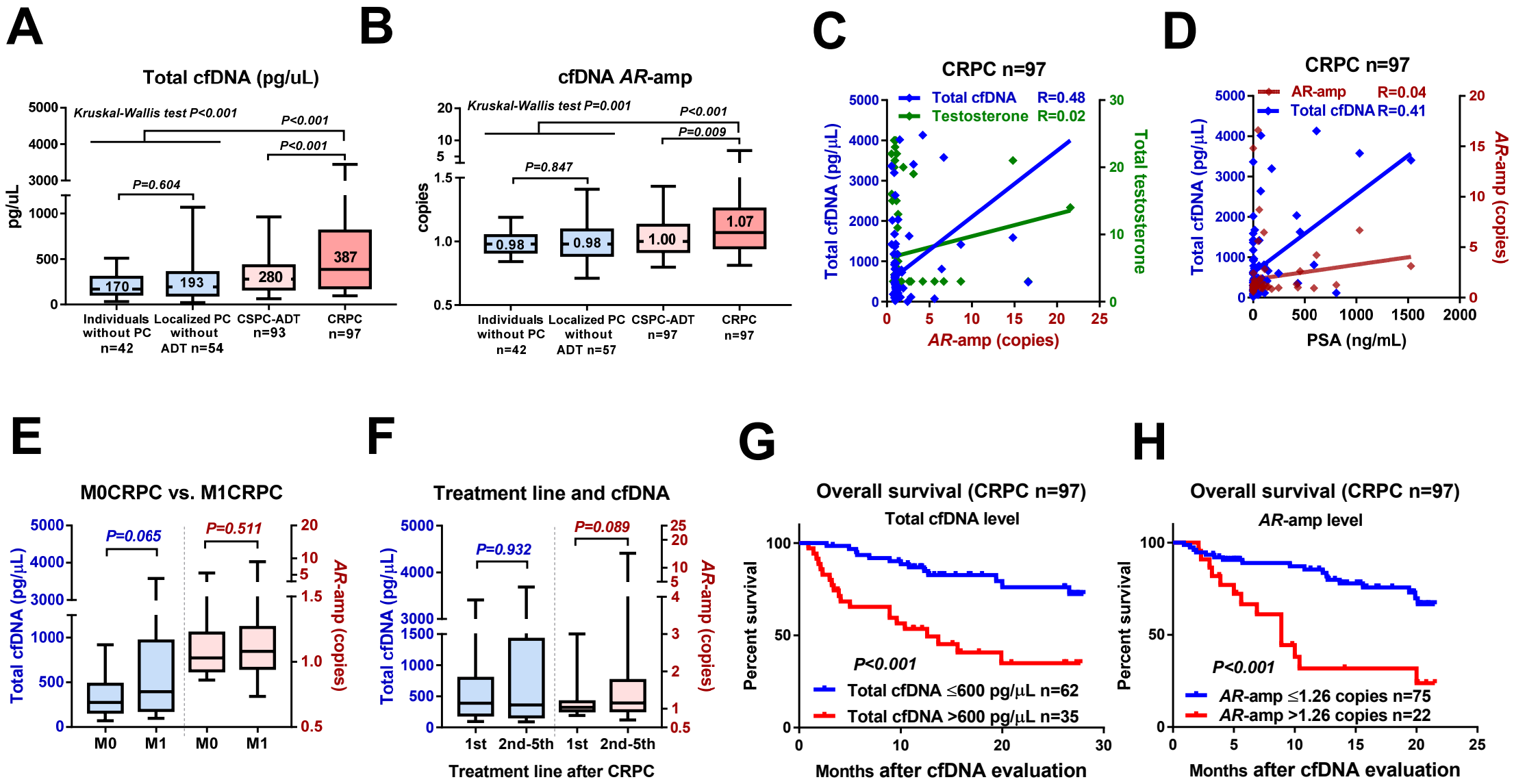
A
Total cfDNA (pg/uL)
Kruskal-Wallis test P<0.001
P<0.001
P<0.001
P=0.604
pg/uL
5000
4000
3000
1000
500
0
170
193
280
387
Individuals without PC n=42
Localized PC without ADT n=54
CSPC-ADT n=93
CRPC n=97
B
cfDNA AR-amp
Kruskal-Wallis test P=0.001
P<0.001
P=0.009
P=0.847
copies
20
10
5
1.5
1.0
0.5
0.98
0.98
1.00
1.07
Individuals without PC n=42
Localized PC without ADT n=57
CSPC-ADT n=97
CRPC n=97
C
CRPC n=97
Total cfDNA R=0.48
Testosterone R=0.02
Total cfDNA (pg/μL)
Total testosterone
AR-amp (copies)
D
CRPC n=97
AR-amp R=0.04
Total cfDNA R=0.41
Total cfDNA (pg/μL)
AR-amp (copies)
PSA (ng/mL)
E
M0CRPC vs. M1CRPC
P=0.065
P=0.511
Total cfDNA (pg/μL)
AR-amp (copies)
M0
M1
M0
M1
F
Treatment line and cfDNA
P=0.932
P=0.089
Total cfDNA (pg/μL)
AR-amp (copies)
1st
2nd-5th
1st
2nd-5th
Treatment line after CRPC
G
Overall survival (CRPC n=97)
Total cfDNA level
Percent survival
P<0.001
Total cfDNA ≤600 pg/μL n=62
Total cfDNA >600 pg/μL n=35
Months after cfDNA evaluation
H
Overall survival (CRPC n=97)
AR-amp level
Percent survival
P<0.001
AR-amp ≤1.26 copies n=75
AR-amp >1.26 copies n=22
Months after cfDNA evaluation

Fig.2

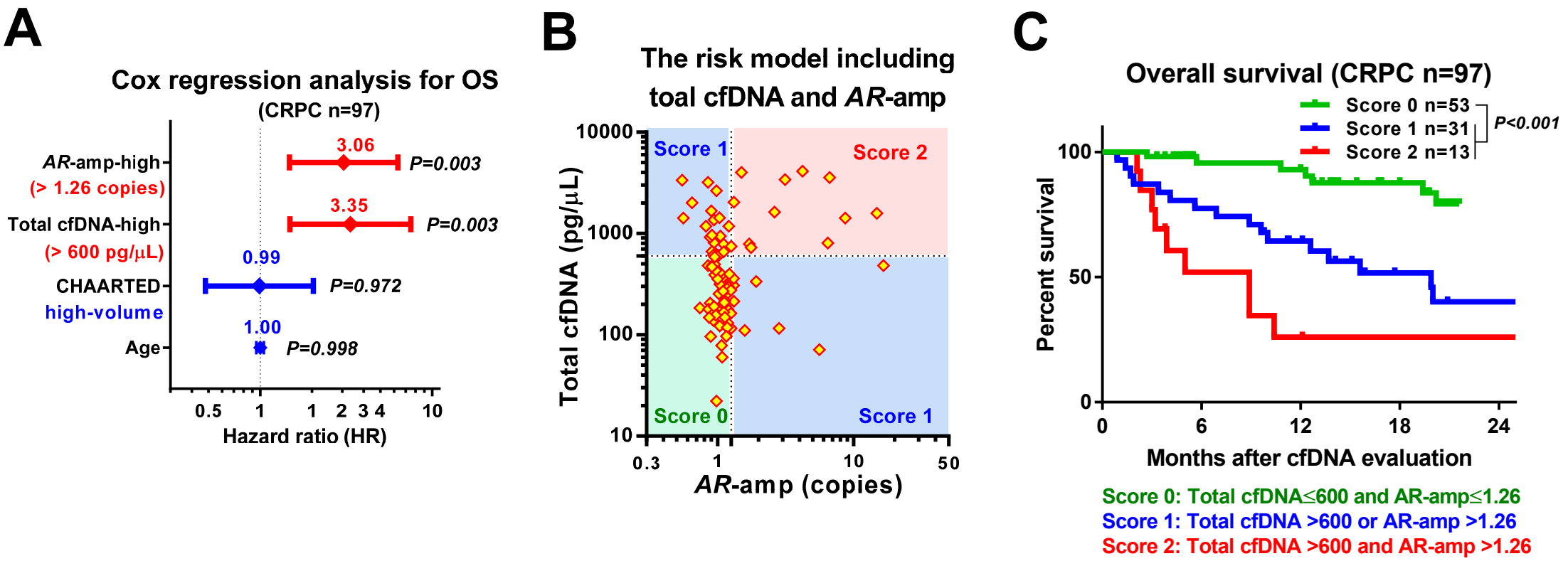
A
Cox regression analysis for OS
(CRPC n=97)
AR-amp-high
(> 1.26 copies)
3.06
P=0.003
Total cfDNA-high
(> 600 pg/μL)
3.35
P=0.003
CHAARTED
high-volume
0.99
P=0.972
Age
1.00
P=0.998
0.5
1
1
2
3
4
10
Hazard ratio (HR)
B
The risk model including
toal cfDNA and AR-amp
Score 1
Score 2
Score 0
Score 1
10000
1000
100
10
Total cfDNA (pg/μL)
0.3
1
10
50
AR-amp (copies)
C
Overall survival (CRPC n=97)
Score 0 n=53
Score 1 n=31
Score 2 n=13
P<0.001
100
50
0
Percent survival
0
6
12
18
24
Months after cfDNA evaluation
Score 0: Total cfDNA≤600 and AR-amp≤1.26
Score 1: Total cfDNA >600 or AR-amp >1.26
Score 2: Total cfDNA >600 and AR-amp >1.26

Fig. S1

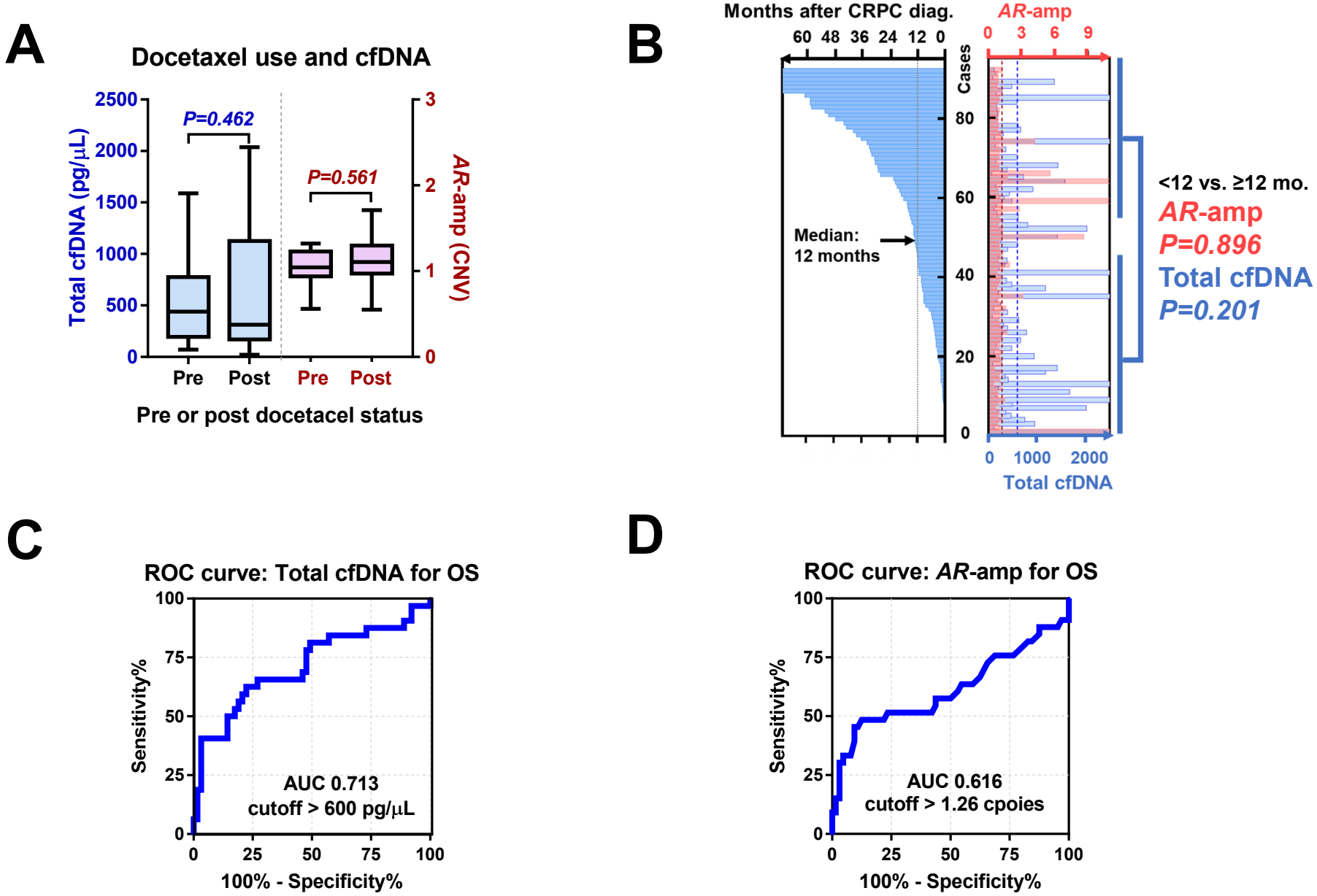
A
Docetaxel use and cfDNA
P=0.462
P=0.561
Total cfDNA (pg/μL)
AR-amp (CNV)
Pre
Post
Pre
Post
Pre or post docetacel status
B
Months after CRPC diag.
AR-amp
Cases
Median:
12 months
<12 vs. ≥12 mo.
AR-amp
P=0.896
Total cfDNA
P=0.201
Total cfDNA
C
ROC curve: Total cfDNA for OS
Sensitivity%
AUC 0.713
cutoff > 600 pg/μL
100% - Specificity%
D
ROC curve: AR-amp for OS
Sensitivity%
AUC 0.616
cutoff > 1.26 cpoies
100% - Specificity%

Table 1 Background of patients

	Healthy individuals	Localized PC without ADT	CSPC-ADT	CRPC
Number of patients, n	42	57	97	97
Median Age, years (IQR)	51 (39-51)	76 (70-80)	74 (69-80)	74 (60-80)
Median initial PSA, ng/mL (IQR)		8.2 (5.4-12)	21 (11-259)	97 (17-826)
Gleason score at diagnosis (IQR)		7 (7-9)	9 (8-9)	9 (9-10)
Median PSA at cfDNA, ng/mL (IQR)		0.02 (0.02-0.15)	0.21 (0.02-1.21)	5.37 (0.47-48.3)
Metastatic disease, n		0 (0%)	41 (42%)	78 (80%)
CHAARTED high-volume, n		0 (0%)	30 (31%)	46 (47%)
Post docetaxel, n		0 (0%)	11 (11%)	43 (44%)
Therapy line after CRPC (IQR)				1 (1-2)
Months from CRPC diagnosis to cfDNA evaluation (IQR)				12 (4-12)
Deceased, n		0 (0%)	1 (1%)	33 (34%)

PC: prostate cancer, ADT: androgen deprivation therapy, PSA: prostate-specific antigen, CSPC: castration-sensitive prostate cancer, CRPC: castration-resistant prostate cancer, CHAARTED high-volume criteria: the presence of visceral metastases, or ≥4 bone lesions with ≥1 beyond the vertebral body and pelvis.