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Prognostic association of plasma cell free DNA based androgen receptor amplification and circulating tumor cells in pre-chemotherapy metastatic castration resistant prostate cancer patients

Manish Kohli, M.D.^{1,*,#}, Jian Li^{2,3,#}, Meijun Du³, David Hillman⁴, Scott M. Dehm⁵, Winston Tan⁶, Rachel Carlson⁴, Michael B. Champion⁷, Ligu Wang⁴, Liewei Wang⁸, Huijuan Zhang^{3,9}, Peng Zhang³, Deepak Kilari¹⁰, Chiang-Ching Huang¹¹, and Liang Wang^{*,3}

¹Division of Medical Oncology, Department of Oncology, Mayo Clinic, Rochester, MN, USA

²Department of Internal Medicine, People's Hospital of Zhengzhou University, Henan Provincial People's Hospital, Zhengzhou, Henan, China

³Department of Pathology and Cancer Center, Medical College of Wisconsin, Milwaukee, WI, USA

⁴Division of Biomedical Statistics and Informatics, Department of Health Sciences, Mayo Clinic, Rochester, MN, USA

⁵Masonic Cancer Center, Department of Laboratory Medicine and Pathology, and Department of Urology, University of Minnesota, Minneapolis, MN, USA

⁶Division of Hematology-Oncology, Mayo Clinic, Jacksonville, FL, USA

⁷Department of Laboratory Medicine and Pathology, Mayo Clinic, Rochester, MN, USA

⁸Department of Molecular Pharmacology and Experimental Therapeutics, Mayo Clinic, Rochester, MN, USA

⁹Physical Examination Center, Zhengzhou Seventh People Hospital, Zhengzhou, Henan, China

¹⁰Department of Medicine, Medical College of Wisconsin and Milwaukee VA Medical Center, Milwaukee, WI, USA

¹¹Joseph J. Zilber School of Public Health, University of Wisconsin, Milwaukee, WI, USA

Abstract

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Co-corresponding authors: Manish Kohli, Division of Medical Oncology, Mayo Clinic, 200 First Street SW, Rochester, MN 55905 USA, Phone: 1+507-266-9160, Fax: 1+507-284-1803, Kohli.manish@mayo.edu; Liang Wang, Department of Pathology and Cancer Center, Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, WI 53226 USA, Phone: 1+414-955-2574, ljwang@mcw.edu.

#Co-First authors. These authors contributed equally.

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Background—The prognostic significance of plasma cell-free DNA (cfDNA) androgen receptor amplification (*ARamp*) in metastatic castration resistant prostate cancer (mCRPC) compared with circulating tumor cell (CTC) counts is not known.

Methods—As part of correlative aims of a prospective study in mCRPC, concurrent and serial collections of plasma and CTCs were performed. Specimen collections were performed at baseline after progression on androgen deprivation therapy and then 12 weeks later. QuantStudio3D digital PCR system (dPCR) was used to determine plasma cfDNA *AR* copy number variations and Cell search assay for enumerating CTC counts. Association of baseline cfDNA *ARamp* status/CTC counts with overall survival (OS) (primary goal) was evaluated using Kaplan–Meier method and log-rank test ($p < 0.05$ for significance) and Receiver Operator Curves (ROC) for *ARamp* status and CTCs ≥ 5 . A multivariate analysis was performed using Cox regression models that included *ARamp*, CTC counts and other clinical factors.

Results—*ARamp* was detected in 19/70 patients at baseline. At the time of analysis, 28/70 patients had died (median follow-up 806 days (IQR: 535–966)). *ARamp* was associated with poor OS (2 year OS of 35% in *ARamp* vs. 71% in non-*ARamp*; log-rank p -value = <0.0001). Baseline CTC count ≥ 5 (vs < 5) was also associated with poor survival (2 year OS of 44% vs 74%; log-rank $p=0.001$). ROC analysis demonstrated area under the curve (AUC) of 0.66 for *ARamp*- and 0.68 for CTC counts-based prognosis ($p=0.84$ for difference). The best two variables included for multivariable analysis were *ARamp* and CTC ≥ 5 , however the two factor model was not significantly better than using *ARamp* alone for predicting survival (HR=5.25; $p=0.0002$).

Conclusions—CTCs and plasma cfDNA *ARamp* were observed to have equal prognostic value in mCRPC. Larger cohorts that incorporate molecular and clinical factors are needed to further refine prognosis in CRPC.

Keywords

Androgen receptor; cell-free DNA; prognostication; amplification

Introduction

Among men in the US prostate cancer is the second most common cause of cancer related deaths¹. Progression from advanced castration sensitive disease treated with androgen deprivation therapy (ADT) to a castrate resistant state is inevitable². Metastatic castration resistant prostate cancer (mCRPC) includes a heterogeneous group of patients for whom novel therapeutic options include drugs which target the AR axis^{3–5}. Despite therapeutic advances challenges in predicting survival or matching treatments with drug response or resistance (primary or acquired) in mCRPC remain and disease progression occurs in majority of the patients. The underlying heterogeneity of cancer biology in castrate resistance^{6–8} can impact treatment response as well as survival. At present while several non-FDA cleared clinical prognostic factor based models have been proposed⁹, no genome-based prognostic markers are used to predict survival in castrate resistance state and the only FDA cleared prognostic marker of survival in mCRPC is the circulating tumor cell (CTC) count with CTCs ≥ 5 per 7.5ml whole blood as a predictor for inferior survival¹⁰. The widespread use of CTC count-based prognostication has its limitations that prevent it from

being widely adopted in clinical practice. These include the lack of a single objective and agreeable definition for a CTC for detecting these rare cells, the inability to reliably probe molecular profiles in CTCs to evaluate underlying tumor biology during treatments, and the clinical observation that CTCs are not shed or captured universally in all advanced stage patients.

Several attempts for improving the limitations of CTC-based assays are under development¹¹ while alternative methods for prognostication or predicting treatment outcomes by molecular characterization of the metastatic prostate cancer genome are also being developed. One such novel method involves the evaluation of cell free DNA (cfDNA) in blood plasma. Such a concept of “liquid biopsies” has begun to be evaluated for use in clinical practice in many tumor types including advanced prostate cancer where prospective studies with limited patient populations have evaluated cfDNA based markers for prognosis and prediction in mCRPC^{12–16}. Since *AR* copy number variations (CNVs), particularly *AR* amplification (*ARamp*), is a key genomic aberration signature observed at the time of developing castrate resistance, *ARamp* in plasma cfDNA has been investigated in mCRPC, but after the use of chemotherapy or AR axis directed therapeutic agents for predictive and prognostic value^{12,16,17}. Since it is unclear if plasma *AR* CNVs in mCRPC immediately following progression on ADT prior to initiating any standard of care mCRPC treatments can also be used to prognosticate survival we determined the prognostic value of plasma *AR* CNVs and CTC count specimens collected concurrently as part of correlative studies in an on-going prospective trial (<https://clinicaltrials.gov/> identifier NCT # 01953640) in pre-chemotherapy mCRPC patients progressing on ADT. These correlative aims were considered exploratory and hypothesis generating in nature.

Materials and Methods

Patient recruitment and blood collection methods

Metastatic CRPC patients with progressive disease on continuous ADT were enrolled in a prospective trial in which patients underwent uniform collection and processing of blood, CTCs and urine specimens as part of the correlative aims of the study at baseline, before initiating abiraterone acetate and prednisone therapy (AA/P). Prospective collections of specimens were repeated after 12 weeks of AA/P treatment. The goal of the correlative study presented here was to determine prognostic outcomes based on CNVs observed in plasma cfDNA *AR* gene and CTC counts. The study was approved by Institutional Review Boards at Mayo Clinic and Medical College of Wisconsin and all patients signed an informed consent at the time of enrollment.

Plasma preparation and cfDNA extraction

Plasma blood collection was performed in 4 ml K2-EDTA plasma separator tubes and centrifuged at 2000 rpm for 10 minutes within 2 hours of collection for generating platelet rich plasma, followed by a second round of centrifugation of the supernatant for generating platelet poor plasma. The supernatant was fractioned into multiple 500 uL aliquots for storage at -80°C . Aliquots did not undergo any freeze-thaw cycles. QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA) was used for extracting cfDNA from 500 uL plasma. The

DNA concentrations were quantified by a Qubit 2.0 Fluorometer following the standard protocol (Life Technologies, Carlsbad, CA).

Quantification of *AR* copy number by digital PCR and CTC assays

To quantify *AR* copy numbers (CN) in plasma cfDNA, Taqman-based *AR* CN assays was utilized including FAM-*AR* assay ID: Hs04511283_cn (cat 4400291) (Life Technologies) and VIC Copy Number Reference Assay: RNase P (cat 4403326) (Life Technologies). Details of digital PCR methods used for quantitation of *AR* copy numbers are provided separately under “Supplementary Methods”.

To enumerate CTCs, 7.5 ml whole blood was collected in CELLSEARCH[®] Circulating Tumor Cell Kits as per manufacturer’s direction. CTC enumeration was performed using the FDA cleared CELLSEARCH[®] CTC Test¹⁸.

Statistical Methods

As part of the correlative aims in the study cohort enrolled, association of baseline plasma cfDNA *AR*amp and CTC counts with overall survival (OS) at 19 months was explored. OS was defined as the time between registration on study and the date of death from any cause or the date of the last follow-up visit. Kaplan-Meier (KM) method was used to show overall survival distributions by *AR*amp status and CTC counts (<5 vs ≥ 5 cells). Tests of significance for *AR* amplification status and CTC counts association with OS were performed using log-rank test. Receiver operator curves (ROC) for both variables evaluated area under the curve (AUC) for predicting survival at 19 months. For the ROC analyses AUCs for both markers were calculated and compared when used alone and in combination. A ROC sensitivity analysis was also performed where AUCs were determined at various time points (15, 18, 21, 24 months). In order to determine the effect of multiple factors on survival including volume of metastatic disease (high versus low), a multivariate Cox regression model was utilized to assess association of several covariates measured at study enrollment (baseline) with OS as detailed under “Supplementary Methods”.

A secondary analysis was performed to explore if baseline *AR*amp status was predictive for developing treatment resistance at 12 weeks (primary resistance) using Chi-square tests and with progression-free survival (PFS) using log-rank test. Further details for the secondary analysis are provided under “Supplementary Methods”. All statistical analyses were performed with SAS 9.3 software (SAS Institute, Cary, NC, USA) and all P-values were two-sided with a cutoff for significance at P = 0.05.

Results

Clinical characteristics of the study cohort

Between 5/2013 and 9/2015, 92 patients were enrolled on the main study of which 70 had plasma samples for cfDNA isolation available at baseline and 12-weeks after initiating AA/P treatment. Demographic characteristics of the enrolled population are summarized in Table 1. These were used in the analysis of *AR*amp-based prognosis. Supplementary Table 1 provides the demographics of the 22 patients not included in this analysis for lack of

specimens. We observed that 38/70 had high and 32/70 had low volume metastatic disease. The median study follow-up at the time of this analysis was 806 days (IQR: 535–966) during which time 28/70 patients had died. At twelve weeks of treatment 30/70 patients demonstrated progression using composite assessment criteria and during the follow up period at the time of this analysis a total of 55/70 patients had progressed with a median progression free period of 194 days.

Association of *AR* amplification with overall survival and other clinical factors

The median amount of cfDNA isolated from 500 μ L plasma samples at baseline and at 12 weeks was 3.7 ng and 3.5 ng (Table 1), respectively. cfDNA amounts in pre-treatment plasma specimens did not differ by volume of disease (median amount=3.3 ng for low volume, and =3.8 ng for high volume, p-value for difference between groups = 0.86) or by CTC counts (median cfDNA amount of 3.7 for CTC < 5 and 3.0 for CTC \geq 5; p-value for difference between group =0.76). The amount of cfDNA in plasma at baseline was not associated with OS after dividing the cohort into low cfDNA vs high cfDNA based on less than or greater than median (3.7 ng) amount (log-rank p-value=0.33). Using the criterion for defining *ARamp* we observed 19/70 patients at baseline and 12/70 at 12 weeks with *ARamp*, respectively (Table 1). Baseline *ARamp* status was not associated with age or Gleason score at diagnosis but associated with PSA levels at study entry (baseline) (p=0.0064) and 12-week plasma (p=0.0032). In baseline plasma samples, 16/38 (42%) patients with high volume disease showed *ARamp* while 3/32 (9%) patients with low volume disease demonstrated *ARamp*. Plasma *ARamp* status was associated with metastatic volume of disease at study enrolment (p=0.002). We did not detect baseline *ARamp* to be associated with duration of prior ADT (non-*ARamp* group =2.6 years vs 2.0 years in *ARamp* group; p=0.75). Prior exposure to anti-androgens was also not associated with baseline *ARamp* (28% in those with prior anti-androgen and 20% with no prior anti-androgen; p=0.69).

Predictive performance of prognosis using *ARamp* and CTC counts

Survival of the 19/70 (27%) patients with baseline *ARamp* at 10, 20 and 30 months follow up was 100%, 53% and 0%, respectively. Among the remaining 51 (73%) patients without *ARamp* the survival rate was 96%, 80% and 58%, respectively. Plasma *ARamp* was significantly associated with poor survival (2 year OS of 35% vs 71% in non-*ARamp*; log-rank p=<0.0001) (Figure 1A). Baseline CTC count \geq 5 (vs < 5) was also associated with poor OS (2 year OS of 44% vs 74%; log-rank p=0.001) (Figure 1B). ROC for plasma *ARamp*-based prognosis had an AUC of 0.66 (95% CI: 0.52-0.81) and for CTC had an AUC of 0.68 (95% CI: 0.54-0.83). There was no difference between AUCs for *ARamp* status (0.66) and CTC (0.68) (difference = 0.02, 95% CI: 0.21-0.18; p=0.84) (Figure 2A). By combining both *ARamp* and CTC the AUC was 0.75 (95% CI: 0.61-0.89) (Figure 2B). Compared to the AUC of CTC alone this increased AUC was not statistically significant (difference=0.07, 95% CI: 0.01-0.14). A sensitivity analysis investigating the AUCs of *ARamp* and CTC at different time points were consistent to those observed. AUCs ranged from 0.65 to 0.75 with no significant differences indicated at any time point. To determine the frequency of *ARamp* based on the number of CTCs (<5 or \geq 5) at baseline, 62.5% of patients (15/24) with CTC \geq 5 were detected with *ARamp* while only 28.5% of patients (12/42) with CTC <5 were detected to have *ARamp* (P=0.014). Four patients had no CTC

data at visit 1. For visit 2 while the number of patients with CTC data was lower than baseline collections, 57.1% of patients (4/7) with CTC ≥ 5 had AR amplification while only 16.6% of patients (8/48) with CTC <5 had AR-amp (p=0.05). Fifteen patients had no CTC data at visit 2.

Multivariate predictive model of prognosis in pre-chemotherapy mCRPC

At the univariate level using seven known and promising prognostic factors in mCRPC Cox regression analysis showed association of OS with ARamp (HR=5.25; p=0.0002), CTC ≥ 5 (HR=3.42; p=0.003), log PSA (HR=1.35; p=0.009) and high metastatic disease volume (HR=2.36; p=0.04) (Figure 3). All possible combinations of these 4 covariates were considered in the search for the most parsimonious model. No two factor or higher multivariate model was identified that was statistically better than univariate model with ARamp alone (HR=5.25; p=0.0002). The best two variable multivariable model included ARamp and CTC ≥ 5 . However, this two factor model was not significantly better than ARamp alone (p-value for difference in models of 0.06).

Association of ARamp as a predictive biomarker with treatment response to AA/P

An exploratory analysis was performed to determine the effect of ARamp status before initiating AA/P treatment and response at 12-weeks and with disease free progression. ARamp status at baseline was not associated with the composite progression at 12 weeks (p=0.49), PSA progression at 12 weeks (p=0.31), or progression free survival (p=0.27) (Supplementary Figure 3). In 30/70 patients with progression at 12-weeks, 23 patients did not have plasma ARamp at baseline (AR copy number neutral). Of these five converted to ARamp at 12 weeks while the remaining 18 did not. Of the remaining 7/30 with ARamp in the baseline specimen four patients retained AR amplification status at time of 12 week progression (Supplementary Figure 2).

Discussion

In a prospective cohort of pre-chemotherapy mCRPC patients we observed plasma cfDNA-based ARamp status and CTC counts (≥ 5 per 7.5 ml blood sample) to be independently prognostic for survival. We explored ARamp status using a limited set of seven clinical factors and CTC counts (Figure 3) for incorporating in a multivariate regression model and detected ARamp status detected at the time of ADT failure to independently predict survival. This allows for additional refinement of prognostic groups in mCRPC using emerging genomic biomarkers and for pursuing aggressive treatment interventions in poor prognosis patients more accurately. Assessing prognosis by developing validated molecular prognostic biomarkers in CRPC is an active and highly recommended area of research¹⁹. Since the cohort size is small, it limits the value of our observation and a more formal determination is needed in larger cohorts⁹.

We did not observe ARamp status to predict efficacy of pre-chemotherapy AA/P response. This result differs from a previous report in which ARamp was predictive of post chemotherapy efficacy for AA/P¹². The difference could be because our study was performed in mCRPC patients immediately following progression on ADT from a hormone

sensitive stage, at which time the full repertoire of *AR* axis-based genomic aberrations including focal amplifications in *AR* gene has not fully emerged. In fact the detected rate of *AR* amplification status in our study was slightly lower (27%), than the 40% incidence reported from mCRPC patients who have undergone several chemotherapy treatments¹².

Serially obtained specimens allowed us to determine changes in plasma cfDNA *AR* copy numbers. Interestingly we did not detect *ARamp* status to change in the paired samples after 12 weeks of treatment using our pre-defined cut off ratio (of 2.0) to classify *AR* amplification status. At the same time while *AR* amplification did not change after 12-weeks of treatment *AR* copy number changes were observed based on absolute numerical values of *AR* copy numbers in the two serial samples. The numerical increase in *AR* copy numbers was found in 20 (67%) of the 30 patients who were observed to have a 3-month progression while in only 13 (33%) of 40 patients without 3-month progression. Shifts in *AR* copy numbers post treatment in advanced prostate cancer have been previously reported in plasma and may represent rapid adaptations to selection pressures under treatment¹⁷. Not all studies in advanced prostate cancer evaluating plasma cfDNA *AR* have reported consistently similar shifts in copy numbers in mCRPC patients for prediction of treatments outcomes. Results from only one large study suggest that plasma *ARamp* serially captured may have predictive value for determining AA/P efficacy¹². At present it is not clear if the shift in *AR* copy numbers from a time-dependent clonal evolution process represents a steady accumulation of *AR*-based aberrations/amplifications over time as mCRPC progresses or if this represents a treatment effect.

The PCR-based cfDNA “liquid biopsy” assay for directly monitoring specific tumor-associated molecular changes has shown several advantages.^{15,20} In prostate cancer specifically it offers a chance to monitor putative tumor-specific genomic aberrations like chromosomal rearrangements and CN gains and losses such as the ETS gene family fusions, *PTEN* loss and *ARamp*. Studies show that cfDNA “liquid biopsy” clinical applications are promising and increasingly used in the clinic such as the *EGFR* gene-based T790M in non-small cell lung cancer.^{12,17,21}

A limitation of this study is the relatively small sample size and the lack of comparison with known RNA-based biomarkers such as *AR-V7* in CTCs which have been shown to be predictive biomarker for treatment response^{22,23}. The value of *AR-V7* CTC assay in mCRPC patients continues to evolve as a predictive and prognostic biomarker as was recently evaluated in a prospective cohort of 202 men of which at least forty percent men had previously received some form of mCRPC therapy²³. It is unclear if the incorporation of this assay with cfDNA candidates and clinical prognostic factors including quality of life and pain scores will yield superior prognostic models than any one factor alone and definitive studies remain on-going. Another limitation is the use of one single reference gene. Since mCRPC is a genetically unstable disease, it will be more robust if using several reference genes to correct for possible genetic alterations in the targeted regions. Therefore, additional validation is needed in prospective and larger patient cohorts to confirm the prognostic value of circulating *AR* CNVs as a prognostic and predictive biomarker, preferably by comparing it to CTCs, existing clinical models and *AR* splice variants.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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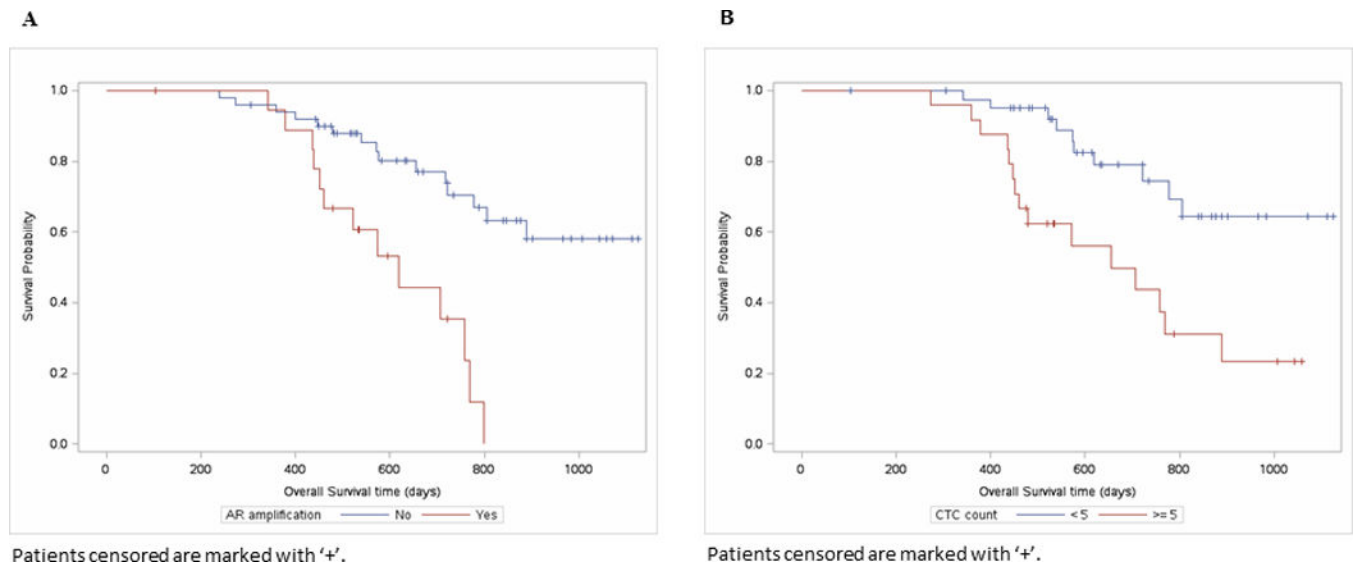


Figure 1.
A: Kaplan Meier curves for baseline plasma cfDNA *AR* amplification with overall survival.
B: Overall Survival by CTC Count at baseline. Patients with CTC counts greater or equal to 5 cells are at higher risk of death than those with < 5 cells (log-rank p-value=0.001).

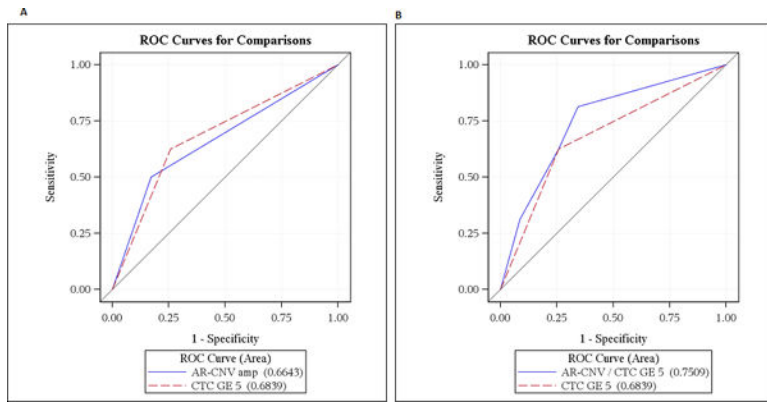


Figure 2.

A: ROC curves comparing AR amplification and CTC based overall survival prognostication. No difference between AUCs for AR amp (0.66) and CTC (0.68), $p=0.84$
B: No difference between AUCs for AR amp + CTC (0.75) vs. CTC alone (0.68), $p=0.08$

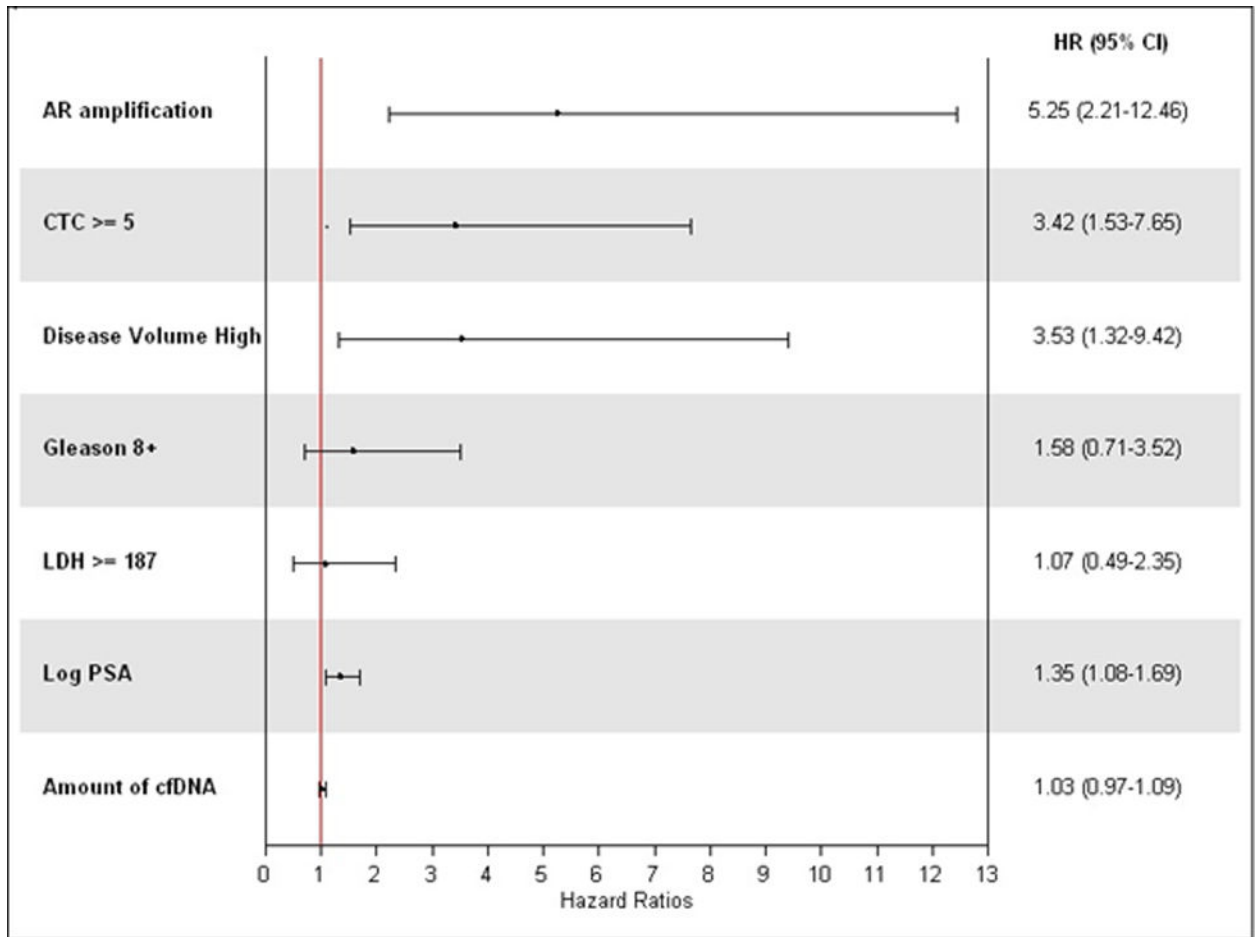


Figure 3.
 Forrest plot for univariate analysis of baseline covariates with survival

Table 1Baseline Patient Characteristics and cfDNA, *AR* amplification and CTC results

Characteristic	Total (N=70)
Race (N=69) – no. (%)	
White	66 (96)
Black or African American	1 (1)
Asian	1 (1)
American Indian or Alaska Native	1 (1)
Age - yr	
Median	71.5
Range	39-91
Gleason score at initial diagnosis – no. (%)	
2 – 6	12 (17)
7	19 (27)
8 – 10	39 (56)
Primary radiation therapy at initial diagnosis – no. (%)	
Yes	41 (59)
No	29 (41)
Primary radical prostatectomy at initial diagnosis – no. (%)	
Yes	33 (47)
No	37 (53)
Volume of metastatic disease – no. (%)	
Low	32 (46)
High	38 (54)
Time from starting ADT to CPRC - yr	
Median	2.5
Interquartile range	1.1–4.6
Metastatic Biopsy site at study enrollment – no. (%)	
Bone	49 (70)
Lymph nodes	13 (19)
Liver/lung	3 (4)
Others	5 (7)
PSA at study enrollment – ng/ml	
Median	16.2
Interquartile range	8.0-38.9
Serum Chromogranin levels at study enrollment (N=68) – ng/ml	
Median	91.0
Interquartile range	55.0–235.5
Testosterone at study enrollment (N=68) – ng/dl	
Median	7.0
Interquartile range	6.9–10.0
LDH at study enrollment (N=66) – U/L	

Characteristic	Total (N=70)
Median	187
Interquartile range	170–209
FACT-P: Physical Well Being score at study enrollment	
Median	23.5
Interquartile range	20–26
FACT-P: Total Score at study enrollment	
Median	118
Interquartile range	106–131
Opiate/pain medication use (N=59) – no. (%)	
Yes	43 (73%)
No	16 (27%)
Study follow-up	
Median days of follow-up (IQR)	806 (535-966)
Number of deaths (%)	28 (40)
Median time to death	805
Baseline and 12 week cfDNA, AR amplification and CTC Levels	
cfDNA (N=70)/AR amp (N=70)/CTC (N=66)	Value
Baseline cfDNA yield (ng)	
Mean (SD)	5.7 (6.3)
Median (IQR)	3.7 (1.5–6.8)
Baseline AR amplification (%)	
Yes	27
No	73
Baseline CTC count	
Mean (SD)	16.3 (53.4)
Median (IQR)	2 (1–9)
Baseline CTC >= 5 cells (%)	
Yes	36
No	64
12-week cfDNA purified amount	
Mean (SD)	5.4 (5.6)
Median (IQR)	3.5 (1.8-7.5)
12-week AR amplification (%)	
Yes	17
No	83
12-week CTC count (N=55)	
Mean (SD)	5.4 (17.3)
Median (IQR)	0 (0-2)
12-week CTC >= 5 cells (N=55) (%)	
Yes	13
No	87