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Original Research

Development and validation of circulating tumour cell enumeration (Epic Sciences) as a prognostic biomarker in men with metastatic castration-resistant prostate cancer

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Abstract ***Purpose:*** To evaluate the prognostic significance of circulating tumour cell (CTC) number determined on the Epic Sciences platform in men with metastatic castration-resistant prostate cancer (mCRPC) treated with an androgen receptor signalling inhibitor (ARSI).
Patients and methods: A pre-treatment blood sample was collected from men with progressing mCRPC starting either abiraterone or enzalutamide as a first-, second- or third-line systemic therapy at Memorial Sloan Kettering Cancer Center (Discovery cohort, N = 171) or as a first- or second-line therapy as part of the multicenter PROPHECY trial (NCT02269982) (Validation cohort, N = 107). The measured CTC number was then associated with overall survival (OS) in the Discovery cohort, and progression-free survival (PFS) and OS in the Validation cohort. CTC enumeration was also performed on a concurrently obtained blood sample using the CellSearch® Circulating Tumor Cell Kit.

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Results: In the MSKCC Discovery cohort, CTC count was a statistically significant prognostic factor of OS as a dichotomous (<3 CTCs/mL versus ≥ 3 CTCs/mL; hazard ratio [HR] = 1.8 [95% confidence interval {CI} 1.3–3.0]) and a continuous variable when adjusting for line of therapy, presence of visceral metastases, prostate-specific antigen, lactate dehydrogenase and alkaline phosphatase. The findings were validated in an independent datas et from PROPHECY (HR [95% CI] = 1.8 [1.1–3.0] for OS and 1.7 [1.1–2.9] for PFS). A strong correlation was also observed between CTC counts determined in matched samples on the CellSearch® and Epic platforms (r = 0.84).

Conclusion: The findings validate the prognostic significance of pretreatment CTC number determined on the Epic Sciences platform for predicting OS in men with progressing mCRPC starting an ARSI.

1. Introduction

Circulating tumour cells (CTCs) that are shed into the bloodstream from the primary tumour or metastatic sites play a key role in the development of metastases [1]. It follows both intuitively and biologically that the detection of CTCs in a patient's blood would predict for a worse outcome relative to those in whom CTCs are not detected prior to or following a therapeutic intervention. Such is the case for every tumour type studied [2–6] independent of the assay used to determine the presence or absence of CTCs in a pre-treatment blood sample, or as a quantitative measure of the number of CTCs in the blood determined both pre- and post-therapy, thereby providing a non-invasive method to monitor disease status longitudinally over time [7].

Doing so is not straightforward because defining, detecting and characterising a CTC has its own set of challenges in that they are rare (1 in 10^5 to 1 in 10^7 nucleated cells typically) and morphologically, phenotypically and biologically diverse [1]. It is therefore essential that what is determined to be a 'CTC' is both rigorously and reproducibly defined, and that the device/assay utilised to determine the number of CTCs present in a patient blood sample is analytically valid or at a minimum, achieved the level of performance to justify its use for this context. Presently there are a wide range of technologies to isolate and capture CTCs many of which are based on cell size or affinity capture [8]. Only one, the CellSearch® Circulating Tumor Cell kit and corresponding device [9] has achieved an FDA clearance as an aid to monitoring breast, colorectal and prostate cancers. With this platform, a CTC is defined as a cell in the blood captured by an EpCAM ferrofluid that is CK^+CD45^- with an intact DAPI stained nucleus and was shown to strongly associate with overall survival (OS) both pre- and post-therapy using a cutoff of 4 or fewer (favourable) or 5 or more CTCs/7.5 mL of blood (unfavourable) [10]. Further, a separate analysis of 5 phase III registration trials in prostate cancer further validated the prognostic significance of the conversion from unfavourable pre-therapy to favourable counts post-therapy, and separately, a change from present (1 or more) to absent (none), CTC0) both of which serve as an indicator of favourable response to therapy that reflects patient benefit [11].

In contrast, the Epic Sciences platform is a non-selection based method in which all nucleated cells from a tube of blood are deposited on glass pathology slides (Fig. 1A) [12], stained and imaged on a cell-by-cell basis to identify cells of interest *in silico* using computer vision. Those malignant, CTCs, and non-malignant cells, myeloid/lymphoid cells can be evaluated independently. The slides can be stored long term at −80 °C and analysed at a later date. A typical assay images and analyzes between 10^6-10^8 individual nucleated cells in a blood sample depending on the analytic requirements of the test being performed.

Clinically, the platform was used to analytically and clinically validate the nuclear-localised AR-V7 protein biomarker in CTCs and show the clinical utility of the defined biomarker to inform the selection of a taxane versus an androgen receptor signalling inhibitor in the second line or greater mCRPC treatment setting: level IIA evidence in the 2019 NCCN guidelines (v1.0), which lead to coverage by Center for Medicare Services and New York State approval as a Laboratory Developed Test [13–16]. The relationship of CTC number to clinical outcomes using the platform has not been established.

The primary focus of this analysis was to clinically validate CTC number, enumerated on the Epic Sciences platform as a prognostic biomarker for OS in men with progressing mCRPC about to start treatment with second-generation ARSI, such as abiraterone or enzalutamide [17–22]. Similar to CellSearch®, a CTC was defined as any CK+, CD45- cell with an intact DAPI-

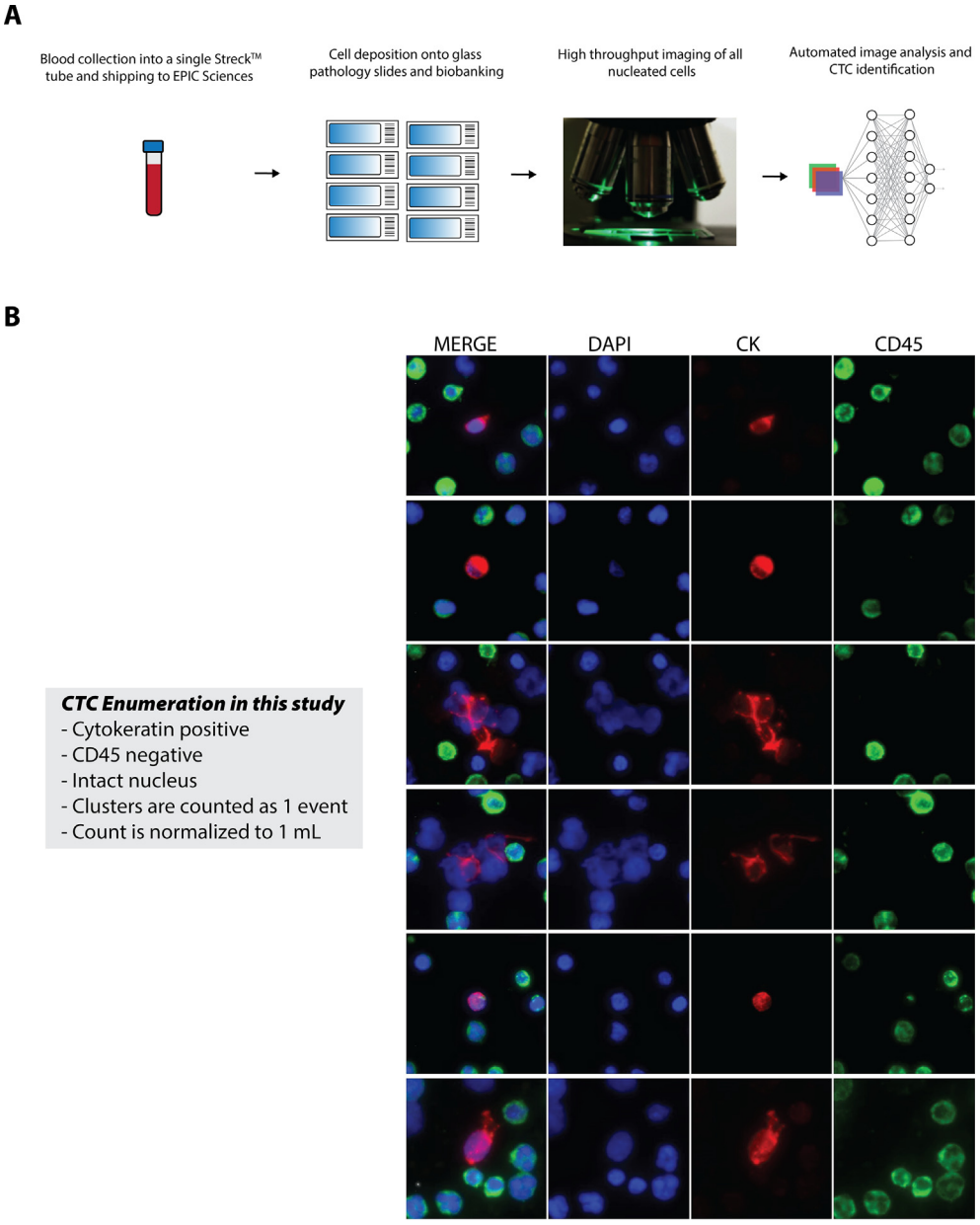

Fig. 1. **The Epic Sciences platform for CTC detection and enumeration**. A) Schematic of blood collection, shipping, bio-banking and CTC analysis and detection. B) Example CTC images. A CTC is defined in this study as any CK + CD45-cell detected in circulation with an intact nucleus. A cluster of CTCs is counted as one event.

stained nucleus with a cluster of CTCs considered as 1 count or event (Fig. 1A and B).

2. Methods

2.1. Patient selection

All studies were performed with respect to the ethical guidelines outlined in the Declaration of Helsinki.

2.1.1. Discovery Cohort

Blood samples were collected from patients with progressing mCRPC treated between December 2012 and September 2016 at MSKCC about to start first, second or third line of therapy. All patients provided written informed consent to an Institutional Review Board (IRB)–approved biospecimen protocol and had histologic confirmation of prostate cancer. The evaluation included a physical examination, recording the Karnofsky performance status and laboratory studies that included a complete blood count with haemoglobin (Hgb), chemistry panel (albumin [ALB], alkaline phosphatase [ALK], lactate dehydrogenase [LDH], prostate-specific antigen [PSA]) and serum testosterone to confirm castrate status (<50 ng/dl). Blood draws taken more than 30 days prior to therapy initiation were excluded.

2.1.2. Validation Cohort

Similarly, blood samples were collected from men with progressing mCRPC in either the first or second line setting prior to starting an ARSI collected as part of the multicenter IRB approved PROPHECY trial (NCT02269982) [13,16]. Eligibility here included 2 or more poor prognostic factors [23,24], and all provided written informed consent. Additional details regarding patient population, eligibility criteria and design have been described elsewhere [25].

2.2. Epic Sciences CTC collection, enumeration and analysis

A single tube of blood (Streck™ Cell-Free DNA BCT®) was collected from each patient and after red cell lysis, all nucleated cells were deposited onto glass slides at MSKCC or shipped to Epic Sciences as whole blood and processed within 96 h of the blood draw as previously described [12,14] (full details are available in the Supplementary Materials). Both sites handled and processed all samples identically using established Standard Operating Procedures. Any cell that was CK + CD45- with an intact nucleus was classified as a CTC, and CTC clusters, defined as at least two adjacent cells, were classified as one event in the final count. CTC counts were normalised to blood volume and expressed as the number detected per 1 mL. In the case of the PROPHECY Validation cohort, time-matched blood samples were sent to a CAP/CLIA approved laboratory at MSKCC for analysis using the CellSearch® Circulating Tumor Cell kit [9]. All blood samples were collected within 30 days prior to the start of ARSI, and all enumeration results were blinded to the treating physicians and to patients. In both cohorts, Epic Sciences laboratory personnel were blinded to the clinical outcomes and clinical investigators were blinded to all CTC biomarker results.

2.3. Statistical analyses

The primary end-point of this retrospective analysis was OS, defined as the date that therapy was initiated until the date of death from any cause or of last follow-up in the MSKCC Discovery and Validation cohorts. In addition, in the Validation cohort, PFS was defined as date of therapy initiation to date of radiographic progression defined by the Prostate Cancer Working Group 2 soft tissue and bone scan criteria, clinical progression, or death, and excluded PSA progression [13,26]. The analysis of the Discovery cohort was performed by the Epic and MSKCC statistical teams and the results used to inform the writing of a Statistical Analysis Plan for the Validation cohort (Supplementary Materials). The biomarker data were then sent to the study statistician for the Validation cohort (SH) who unblinded the data and performed the analysis. Datalocks for the Discovery and Validation cohorts were July 29, 2020 and February 4, 2020 respectively.

In the Discovery cohort, the proportional hazards model was used to explore if CTC count (as a continuous and dichotomized variable) is prognostic of OS and the Kaplan–Meier product-limit approach to estimate the OS distribution dichotomized by CTC cut-point. To determine a poor prognosis cut-point in the Discovery cohort, the univariate hazard ratio (HR) was plotted for each unit increase in CTC/mL value and a cut-point defined qualitatively based on the overall trend in HR and the number of patients in the high CTC group. In the multivariable analysis, covariates included line of therapy, presence of visceral metastases, LDH levels, PSA levels, haemoglobin (Hgb) levels, ALK levels, white blood cell (WBC) counts, albumin levels, and CTC counts as either a continuous or dichotomized covariate. Covariates were selected based on the best subset selection method using the global χ^2 statistic and WBC, ALB, Hgb and patient age were excluded. In the Validation cohort, the proportional hazards model was utilised to confirm the prognostic significance of CTC dichotomized at 3 or greater level and as continuous variable (modelled as log2(CTC+1), adjusting for the validated baseline risk (Halabi prognostic risk-score) as previously described [13,24] that includes Eastern Cooperative Oncology Group performance status, site of spread, LDH, opioid analgesic use, albumin, haemoglobin, PSA, and ALK.

The cutoff for poor prognosis was pre-specified in the Statistical Analysis Plan for the Validation cohort prior to unblinding and analysis (Supplementary Materials). The full details for analysis of the association between OS and PFS with CTC counts, as well as a method agreement analysis between Epic Sciences CTC counts and CellSearch® CTC counts in time matched samples in the Validation cohort are listed in the Statistical Analysis plan (Supplementary Materials).

3. Results

3.1. Patient demographics and clinical baseline

Between March 30, 2013 and August 8, 2018, 218 unique samples were collected from men with progressing mCRPC prior to starting either abiraterone acetate or enzalutamide as standard of care at MSKCC in which 171 were considered evaluable (Discovery cohort, Fig. 2A). Samples were excluded if the blood draw was taken prior to 30 days of therapy initiation, or if the patient was starting a therapy beyond the third-line setting. Patient demographics and clinical baseline characteristics are presented in Table 1. Among the 171 patients, the median age was 68 years (range 45–87). Sixty percent were about to start first-line therapy for mCRPC, 29% and 11% of the samples were taken prior to starting second- and third-line therapy, respectively. Sixty patients (35%) had received a prior ARSi and 14

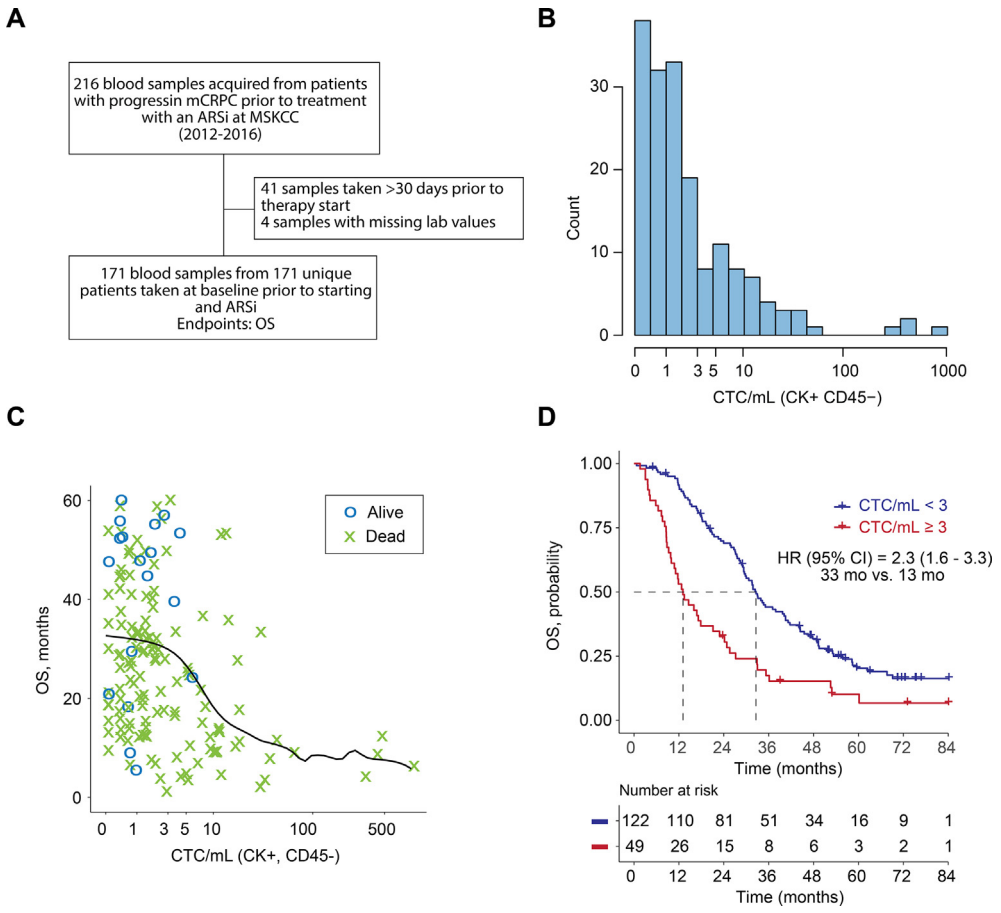

Fig. 2. **CTC detection frequency and prognostic associations with OS in the MSKCC Discovery cohort**. A) Patient selection. B) Histogram of CTC/mL values in the cohort. C) Plot of survival times versus CTC/mL. An estimate of the median survival using a Gaussian kernel density estimate (KDE) shown. D) Kaplan–Meier estimate dichotomized at the 3 CTC/mL cutoff.

(8%) a prior taxane chemotherapy. Eighty-three (48.5%) had bone only or lymph node only disease while 88 (51.5%) had multiple sites of metastases. The median follow-up time among surviving patients was 56.5 months, ranging from 5.0 months to 84.2 months and 138 had died as of July 29, 2020.

In the PROPHECY Validation cohort, 118 patients were enrolled from May 2015 until January 2017 of whom EPIC data were available from 107 patients. The median age was 73 years; of these men, 71% were first-line mCRPC, and 29% were second-line mCRPC after progression on abiraterone or enzalutamide. The median PSA, LDH and ALK were 22.1 ng/mL, 110 and 200 U/L, respectively and demographics have been previously published [13]. The majority of patients had multiple sites of metastases and 22% had bone only disease. The median follow-up time among surviving patients was 31 months (range 3.4–42.3) and 83 patients had died.

3.2. CTC detection rate and survival analysis in the discovery cohort

At least one CTC, defined as any CK+, CD45-cell with an intact nucleus, was detected in 91.8%(157 of 171) of patients in whom $\geq$ 3 CTC/mL, $\geq$ 5 CTC/mL and $\geq$10 CTC/mL were detected in 28.7%, 21.6% and 14.0% of patients, respectively. A histogram of pre-treatment CTC count by patient sample in the Discovery cohort is presented in Fig. 2B and was numerically higher in patients with multiple sites of metastases (Supplementary Fig. 1).

Qualitatively, the survival times decreased significantly after 3 or more CTCs/mL were detected as shown in a plot of OS times versus CTC/mL along with an estimate (solid line) of median survival per unit increase in CTC/mL value Fig. 2C. This was also visualised in a plot of the univariate HR versus CTC/mL dichotomization cutoff point (Supplementary Fig. 2) in which a plateau in the HR was observed after approximately the 3/mL cutoff point. Kaplan–Meier analysis is presented in Fig. 2D in which patients were dichotomized at $<$ 3 CTCs/mL (CTC-low), and those with $\geq$3 (CTC-high), and longer median survival times were observed in the CTC-low group (33 versus 13 months, respectively). A demographic comparison between the CTC $\geq$3 and $<$ 3 is presented in Supplementary Table 1.

The proportional hazards model was utilised to test for CTC number adjusting for line of therapy, presence of visceral metastases and known blood-based

Table 1
Patient demographics.

	MSKCC Discovery set	PROPHECY Validation set
Unique patients, no. (%)	171	107
Unique blood samples, no. (%)	171	107
Median age in years (range)	68 (45, 87)	73 (44, 92)
Death events, no. (%)	138 (80.7%)	83 (77.6%)
Median follow-up of survivors in months (range)	56.5 (5.0, 84.2)	31 (3.4, 42.3)
Therapy line, no. (%)		
pre-1st	103 (60.2%)	76 (71%)
pre-2nd	49 (28.7%)	31 (29%)
pre-3rd	19 (11.1%)	0 (0%)
Sites of metastases, no. (%)		
Lymph node only	24 (14.0%	3 (2.8%)
Bone only	59 (34.5%)	23 (21.5%)
Lung only	1 (0.6%)	0 (0%)
Multiple sites	88 (51.5%)	79 (73.8%)
Prior taxane chemotherapy, no. (%)	14 (8.2%)	20 (18.7%)
Prior ARSi, no. (%)	60 (35.1%)	40 (37.4%)
Baseline lab values, median (range)		
PSA ng/mL	18.1 (0.09, 2010)	22.1 (0.1, 4195)
ALB g/L	4.2 (3.3, 4.9)	4.0 (2.7, 4.9)
ALK U/L	96 (42, 2170)	110 (91, 150)
HGB g/dL	12.6 (8.2, 15.7)	12.8 (8.7, 15.9)
LDH U/L	208 (124, 2120)	200 (100, 618)
WBC x 10^9/L	5.9 (2.6, 12.1)	6.4 (3.7, 22.3)
CellSearch® CTC count/7.5 mL	n/a	4 (0, 12,972)

Abbreviations: PSA, prostate-specific antigen; ALB, albumin; ALK, alkaline phosphatase; HGB, haemoglobin; LDH, lactate dehydrogenase; WBC, white blood cell.

prognostic factors including LDH and PSA [13,24]. Application of the model confirmed the prognostic significance of the CTC ≥3 CTCs/mL threshold with OS (HR [95% confidence interval [CI] = 2.0 [1.3–3.0]; $P = 0.001$) (Table 2) and this threshold was chosen for external validation based on the prognostic significance and the prevalence of patients above this threshold. Patients in the CTC ≥3 group also had higher PSA and LDH levels, and a higher proportion had multiple sites of metastatic spread relative to those with lymph node or bone only (41% versus 61%), explained in part by the presence of a higher burden of disease (Supplementary Table 1). CTC counts were also strongly adversely prognostic on a continuous scale when other baseline prognostic factors were considered, further validating the relationship of higher CTCs to an inferior survival outcome (Table 2).

3.3. Validation of CTC count as a prognostic biomarker in the PROPHECY cohort

A blinded and independent analysis was performed to validate the aforementioned associations with OS and to assess the significance of CTC in predicting PFS in the PROPHECY Validation cohort [13] (Fig. 3A). Here, CTCs were detected in 83.2% (89 of 107) of baseline pre-treatment samples of which 36% (39 of 107) had ≥ 3 CTCs/mL (histogram in Fig. 3B and boxplot of CTC counts by site of spread is shown in Supplementary Fig. 3). In the univariate analysis, the median OS was 12.1 mo (95% CI = 10.4–20.4) for CTC ≥ 3/mL versus 25.0 mo (95% CI = 19.2–30.4) for CTC < 3/mL, respectively. The univariate HR for death was 2.5 (95% CI 1.6–3.9). The median PFS times on abiraterone or enzalutamide were 3.7 (95% CI = 2.9–6.0) and 7.5 months (95% CI = 5.5–9.5) in patients with CTC ≥ 3 and < 3 respectively. The univariate HR for PFS was 2.2 (95% CI 1.4–3.3) (Table 3, Fig. 3C and D). In the multivariable analysis, adjusting for clinical prognostic factors (prognostic risk-score [27]), CTC counts dichotomized at the ≥3 cutpoint were again statistically significantly associated with poor OS (HR = 1.8 (95% CI = 1.1–3.0); $P = 0.03$) and poor PFS (HR = 1.7 (95% CI 1.1–2.9); $P = 0.03$) (Table 3). CTC count as a continuous variable was also significantly associated with OS (HR = 1.3 (95% CI 1.1–1.6), $P = 0.002$) and PFS (HR = 1.3 (95% CI 1.1–1.5), $P = 0.01$, Table 3).

Thirty-six patients (33.6%) had received prior abiraterone or enzalutamide, and the HR for survival CTC counts were similar as both a continuous (HR = 2.3 [95% CI 1.4–3.6]) and dichotomized variable (HR = 1.4 [95% CI 1.2–1.6]) when adjusting for this factor, as well as when the risk-score was included in this model (HR = 1.7 [95% CI 1.0–2.8] for ≥ 3 and < 3 CTC/mL and HR = 1.7 [95% CI 1.0–2.8] as a $\log_2+1$ transform) (Supplementary Table 3).

Finally, 106 patients were evaluable for a post-therapy PSA50 response, and 53 patients were evaluable for soft tissue response. Of the patients in the ≥3 CTC/mL group, 4/38 (11%) had confirmed PSA declines, and 2/20 (10%) had RECIST response, while in the <3 CTC/mL group 21/68 (31%) had confirmed PSA declines while 4/33 (12%) had RECIST response.

3.4. Comparison of CTC counts between Epic Sciences and the CellSearch® platforms

Time matched samples from a single blood draw taken at baseline in the PROPHECY cohort (n = 102) were analysed after overnight shipping within 48 h for CellSearch® CTC and Epic counts in independent blinded laboratories and a method agreement analysis was performed as described in the Statistical Analysis Plan (Supplementary Materials). CTCs were detected in 75% of samples on the CellSearch® platform and in 85% of samples on the Epic Platform with the counts on the two platforms strongly correlated (r = 0.84; Supplementary Fig. 4). As a dichotomized variable (Epic ≥ 3/mL and CellSearch® ≥ 5/7.5 mL), 73% concordance was observed and of the 37 samples in the Epic ≥ 3/mL group, 81% (30) had unfavourable, 5 or more cells/7.5 of blood, CellSearch® counts.

Table 2
Proportional hazards models of overall survival (OS) with Epic Sciences CTC count represented continuously and dichotomized at 3 CTC/mL in the Discovery cohort.

	Model with dichotomized CTC counts (≥3/mL vs < 3)		Model with continuous CTC counts[a]	
	HR (95% CI)	P	HR (95% CI)	P
Overall survival				
Univariate analysis				
CTC	2.3 (1.6, 3.3)		1.3 (1.2, 1.5)	
Multivariable analysis				
Presence of visceral metastases	1.7 (1.1, 3.1)	0.02	1.8 (1.1, 3.1)	0.02
More than one line of therapy (Yes vs. No)	2.5 (1.8, 3.6)	<0.001	2.6 (1.8, 3.8)	<0.001
ALK[a]	1.3 (1.0, 1.6)	0.05	1.2 (1.0, 1.5)	0.10
LDH[a]	1.8 (1.3, 2.4)	0.001	1.7 (1.1, 2.4)	0.008
PSA[a]	1.1 (1.0, 1.3)	0.03	1.1 (1.0, 1.3)	0.03
CTC	2.0 (1.3, 3.0)	0.001	1.2 (1.1, 1.4)	0.001

CTC, circulating tumour cell; ALK, alkaline-phosphatase; LDH, lactate dehydrogenase; PSA, prostate-specific antigen.
[a] log2 (x+1) transformed.

Survival analyses for CellSearch® CTC counts were also performed (Supplementary Fig. 5 & Supplementary Table 4), and the HR for CellSearch® CTC counts as a dichotomized variable (≥5 versus 4 or fewer) was comparable in the same multivariable model for OS (HR = 1.7 (1.0–2.9), p = 0.03) and PFS (HR = 1.5 [0.9–2.3]; p = 0.11). HR adjusting for prior abiraterone or enzalutamide were also comparable and are presented in Supplementary Table 5:

3.5. CTC nuclear localised AR-V7 in the context of total counts on the Epic Sciences platform

In an exploratory analysis, we examined the association of the Epic nuclear AR-V7 protein detection in CTCs with OS after adjustment for Epic CTC enumeration in both cohorts. In the MSKCC Discovery and PROPHECY Validation cohorts, 9.9% (17 of 171) and 10.4% (11 of 107) were positive by the nuclear localised CTC AR-V7 Epic Sciences assay at baseline and of the positive cases, 71% (12 of 17) and 73% (8 of 11) also had ≥ 3 CTC/mL, respectively. In the multivariable analysis of OS of the Discovery cohort, CTC AR-V7 remained associated with OS (HR [95% CI] = 2.21 [1.24, 3.93]) along with Epic CTC enumeration (HR [95% CI] = 1.83 [1.20, 2.79]). In the Validation cohort, nuclear localised AR-V7 also remained associated with OS in multivariable modelling (HR 2.30 95% CI 1.16–4.55) and a similar HR for Epic CTC enumeration was observed (HR 1.63 95% CI 0.96–2.78) (Supplementary Table 6). These data indicate that while CTC enumeration is strongly prognostic of OS, CTC AR-V7 nuclear detection remained prognostic for poor AR therapy outcomes even after adjusting for CTC burden.

4. Discussion

The presence of CTCs in blood reflects the ability of cancer cell to detach from the primary or metastatic focus to develop new sites of spread that results a worsening prognosis. In this study, we show that the number of CTCs, defined as any nucleated cell that CK+, CD45-, enumerated with Epic Sciences platform as continuous and dichotomized variables is independently prognostic for survival in univariate and multivariable modelling in men with progressing mCRPC about to start a second-generation ARSI such as abiraterone or enzalutamide. The findings were validated using an independent cohort of men with high risk mCRPC treated similarly where an additional finding was the comparable association with PFS. Separately, CTC counts measured on the Epic Sciences platform were shown to correlate strongly to CTC counts obtained using the CellSearch® Circulating Tumor Cell kit, an FDA cleared predicate device/assay that applies similar criteria to define a CTC.

Much of the success in drug discovery in advanced prostate cancer can be attributed to the availability of biomarkers reported using analytically and clinically valid devices and assays for the context of use being studied. Pre-treatment contexts to inform treatment selection include an understanding of a patient's prognosis and predicting and selecting a treatment that is most likely to provide benefit and avoiding those which will not. Validated pre-treatment nomograms are available to determine patient risk, while changes in disease manifestations present at the start of therapy relative to post-treatment to determine efficacy include the measured level of PSA and those assessed by imaging [17,24,27,28]. Each has limitations and additional genomic biomarkers, AR splice variant detection such as AR-V7 and measures of disease burden such as CTC enumeration or ctDNA quantification may provide improved discrimination of outcomes as well as better monitoring biomarkers [17,29–32]. Importantly, our data show that CTC nuclear AR-V7 protein detection was strongly associated with worse survival in this mCRPC AR therapy context after adjusting for CTC enumeration although a larger cohort will be needed to assess the additive value of both biomarkers.

CTC counts measured using the FDA cleared CellSearch® Circulating Tumor Cell kit is a validated pre- and post-treatment biomarker of prognosis and response in breast, colorectal and prostate cancers

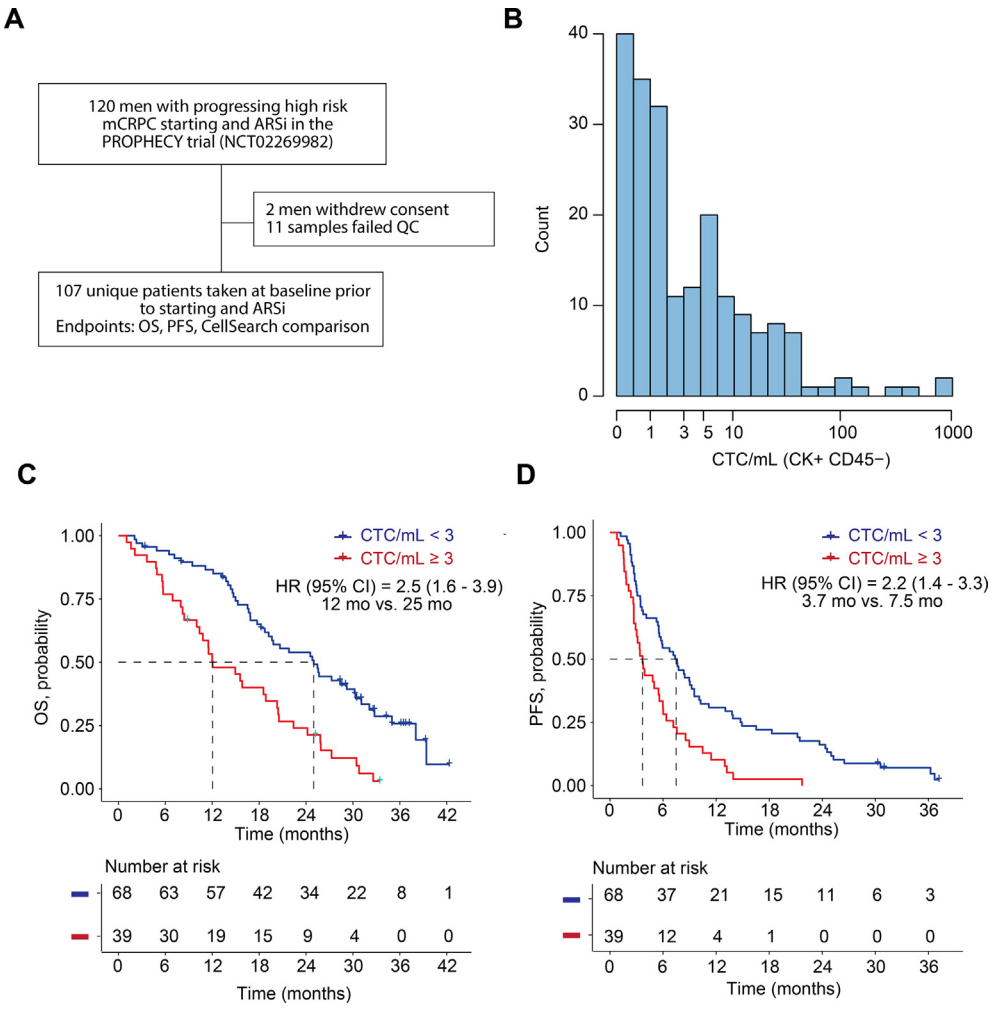

Fig. 3. **CTC detection frequency and prognostic associations with OS in the PROPHECY Validation cohort**. A) Patient selection. B) Histogram of CTC/mL values in the cohort. C) Kaplan–Meier estimate OS dichotomized at the 3 CTC/mL cutoff point, and PFS (D).

[2–7]. In this case, a CTC was defined as an intact nucleated cell captured from blood by and EpCAM ferrofluid that stained positive for markers of epithelial origin (cytokeratins), and negative for CD45, a marker of leucocyte lineage [9]. Further study in progressing mCRPC patients showed the "added value" of the CTC test result to understand the prognosis of patients specifically predicted to have a favourable outcome using standard measures [33], and that changes in count at 12-week combination with LDH, shown to meet the Prentice criteria as a surrogate for OS in a phase 3 registration trial in the post-chemotherapy mCRPC setting [34]. Also shown, was that both a post-treatment CTC conversion from unfavourable to favourable ($\geq$5 to $<$ 5 cells/7.5 mL of blood), the FDA cleared outcome measure, and a newly developed outcome measure, CTC0, representing change from any (1 or more) pre-treatment to none post-treatment were shown to have higher concordance with survival than PSA [11]. CTCs also serve as a source of tumour material for the biologic characterisation of an individual patient's disease for a predictive biomarker to guide treatment choice.

Significant here as well is that concordance of the predicted OS of patients with mCRPC determined with CTC counts obtained with the non-selection based Epic Sciences Platform and the FDA cleared predicate CellSearch® platform when a similar definition of a CTC was applied. In this context, both the CellSearch® and Epic Sciences platforms define a CTC as any circulating cell of epithelial lineage, cytokeratin positive, without leucocyte lineage, CD45 negative. At the same time, it should be noted that this definition of a CTC identifies and enumerates only a subset of the intact malignant cells that be present in blood while excluding those undergoing an epithelial to mesenchymal transition, or lineage plasticity that results in a transition to a neuroendocrine/stem cell like phenotype that grow independent of AR signalling [35,36]. While the results between the two platforms for the CTC definition used here were similar, the Epic platform has the additional advantages of bio-banking unstained sample at −80 °C allowing the immunofluorescence or genomic analysis to be completed at a later date (years), and the ability to isolate plasma for cell-free, proteomic or metabolomic analysis from the same sample.

Table 3
Proportional hazards models of overall survival (OS) and progression-free survival (PFS) with Epic Sciences CTC count represented continuously and dichotomized at 3 CTC/mL in the Validation cohort.

	Model with dichotomized CTC counts (≥3/mL vs. <3 mL)		Model with continuous CTC counts[a]	
	HR (95% CI)	P	HR (95% CI)	P
Overall survival				
Univariate analysis				
CTC	2.5 (1.6, 3.9)		1.4 (1.2, 1.6)	
Multivariable analysis				
CTC	1.8 (1.1, 3.0)	0.03	1.3 (1.1, 1.6)	0.002
Prognostic risk-score [22] (continuous)	1.01 (1.00, 1.02)	0.01	1.00 (0.99, 1.01)	0.48
Progression-free survival				
Univariate analysis				
CTC	2.2 (1.4, 3.3)		1.3 (1.2, 1.5)	
Multivariable analysis				
CTC	1.7 (1.1, 2.9)	0.03	1.3 (1.1, 1.5)	0.01
Prognostic risk-score [22] (continuous)	1.01 (1.00, 1.01)	0.07	1.00 (0.99, 1.01)	0.67

CTC, circulating tumour cell; ALK, alkaline phosphatase; LDH, lactate dehydrogenase; PSA, prostate-specific antigen.
[a] log2(x+1) transformed.

Recognised as well is that non-malignant cells with epithelial lineage can also disseminate into the blood through other mechanisms that may affect prognosis in addition to those derived from the cancer itself. They include those from cardiovascular-related events or viral infection [37–39] considered in the same context as applied here [40]. On the Epic Sciences platform, CK+, CD45-cells have been observed in a small fraction of healthy donor blood samples, albeit at a lower frequency than from mCRPC patient blood samples [41], and the true tissue origin of each CTC detected without deeper characterisation is unknown, such as through methylation analysis or transcriptomics. In prior sequencing analysis of CTCs detected in mCRPC patient blood samples on the platform, the majority of CK + CD45- cells were found to have some level of cancer related genomic copy-number alterations (CNAs), while other cells were found to be absent of CNAs [42] perhaps owing to the fact that a fraction of these cells are not of tumour origin, or owing to low coverage and the technical challenges of sequencing each individual cell.

In conclusion, we observed that CTC counts, defined as any CK + CD45-cell detected on the Epic Sciences platform is a statistically significant and independently validated prognostic factor for OS in men with progressing mCRPC about to start either abiraterone or enzalutamide. Future studies of CTC counts while on therapy relative to baseline are needed to determine the significance of changes in counts to patient outcomes.

Author contributions

Conception and design: Scher HI, Armstrong AJ, Schonhoft J, Wenstrup R, Gonen, M, Halabi, S; Development of methodology: Schonhoft J, Gill A, Wenstrup R, Lu J; Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Armstrong AJ, Scher HI, Barnett, E, Carbone, E, Schonhoft J, Gill A, Wenstrup R, Lu J, Luo J., Antonarakis ES; Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Halabi S, Gill A, Schonhoft J, Gonen M, Lu J; Writing, review and/or revision of the manuscript: All authors; Administrative, technical or material support (i.e., reporting or organizing data, constructing databases): Armstrong AJ, Halabi S, Scher HI, Barnett, E, Carbone, E, Schonhoft J, Gill A, Wenstrup R; Lu J, Study supervision: Scher HI, Armstrong AJ, Wenstrup R, Schonhoft J, Halabi S; Other (obtaining funding): Scher HI, Armstrong AJ.

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Conflict of interest statement

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Appendix A. Supplementary data

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Supplementary Data for:

Development and Validation of Circulating Tumor Cell Enumeration (Epic Sciences) as a Prognostic Biomarker in Men with Metastatic Castration Resistant Prostate Cancer

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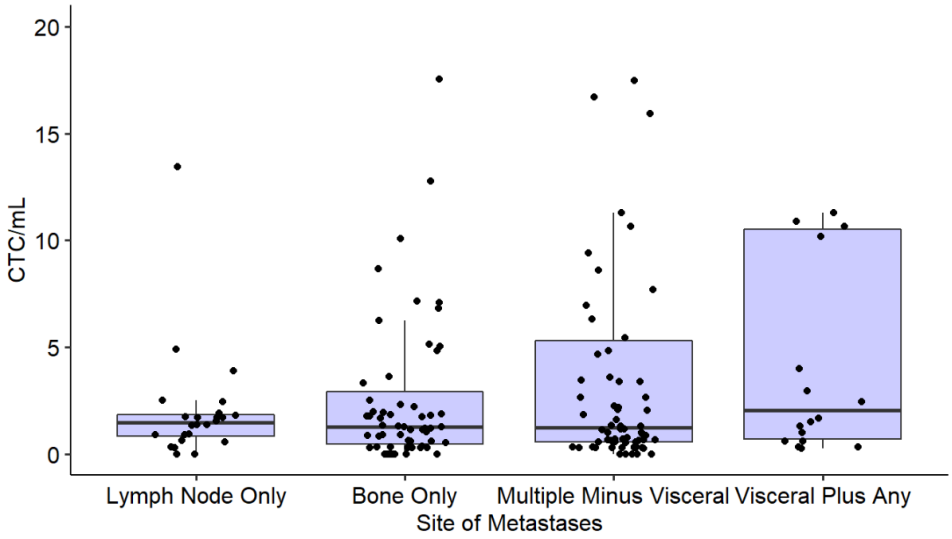
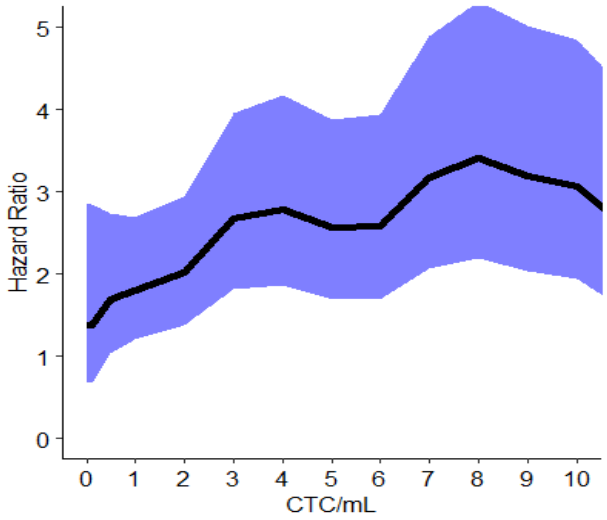


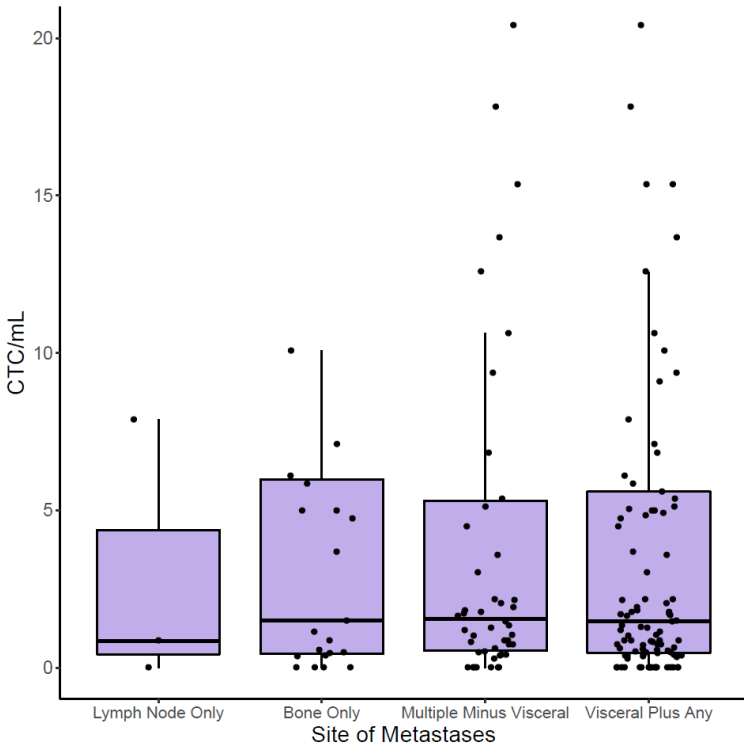
Supplementary Figure 1: Boxplot of CTC/mL values by sites of spread in the Discovery cohort.



Supplementary Figure 2: Plot of univariate HR versus CTC/mL cutoff (greater than or equal to) in the MSKCC Discovery cohort. 95% confidence intervals are shown in shaded blue.

Supplementary Table 1: Patient demographics by high and low CTC/ml values in the MSKCC Discovery cohort		
	CTC/mL < 3	**CTC/mL ≥ 3**
Therapy Line - no. (%)		
pre-1st	75 (61.5%)	28 (57.1%)
pre-2nd	38 (31.1%)	11 (22.4%)
pre-3rd	9 (7.4%)	10 (20.4%)
Sites of Metastases – no. (%)		
Lymph Node Only	21 (17.2%)	3 (6.0%)
Bone Only	44 (36.0%)	15 (30.6%)
Lung Only	1 (0.8)	0 (0)
Multiple Sites	56 (45.9)	31 (63.3)
Baseline lab values - median (range)		
PSA ng/mL	15.4 (0.510, 1190)	42.0 (0.0900, 2010)
ALB g/L	4.20 (3.40, 4.90)	4.10 (3.30, 4.60)
ALK U/L	89.0 (44.0, 342)	137 (42.0, 2170)
HGB g/dL	12.9 (8.20, 15.7)	11.7 (9.20, 14.2)
LDH U/L	202 (124, 427)	259 (139, 2120)
WBC x 10^9/L	5.80 (2.60, 10.8)	5.80 (3.00, 12.1)
Abbreviations: PSA - prostate specific antigen, ALB - albumin, ALK - alkaline phosphatase, HGB - hemoglobin, LDH - lactate dehydrogenase, WBC - white blood cell		

Supplementary Table 2: Patient demographics by high and low CTC/ml values in the PROPHECY validation cohort.		
	CTC/mL < 3	**CTC/mL ≥ 3**
Therapy Line - no. (%)		
No Prior Abi/Enza	45 (42.1%)	22 (20.6%)
Prior Abi/Enza	23 (21.5%)	17 (15.9%)
Sites of Metastases – no. (%)		
Lymph Node Only	2 (2.9%)	1 (2.6%)
Bone Only	12 (17.6%)	11 (28.2%)
Lung Only	0 (0%)	0 (0%)
Multiple Sites	52 (76.5%)	27 (69.2%)
Baseline lab values - median (range)		
PSA ng/mL	15.3 (0.1, 1104.8)	38.0 (0.3, 4194.9)
ALB g/L	4.1 (3.3, 4.7)	3.9 (2.7, 4.9)
ALK U/L	110 (91, 150)	110 (91, 136)
HGB g/dL	12.9 (8.9, 15.9)	12.6 (8.7, 15.4)
LDH U/L	200 (192, 618)	200 (100, 273)
WBC x 10^9/L	6.2 (4.5, 22.3)	6.4 (3.7, 13.9)
Abbreviations: PSA - prostate specific antigen, ALB - albumin, ALK - alkaline phosphatase, HGB - hemoglobin, LDH - lactate dehydrogenase, WBC - white blood cell		

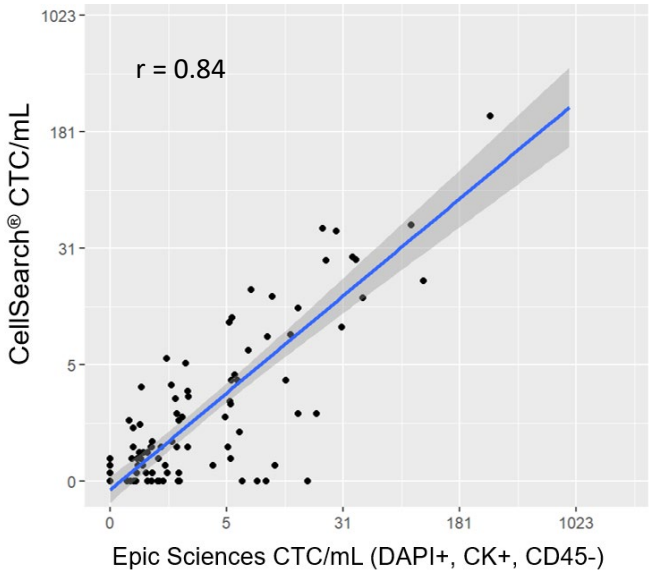


Supplementary Figure 3: Boxplot of CTC/mL values by sites of spread in the Discovery cohort.

Supplementary Table 3: Frequency and association of EPIC CTC counts with OS and PFS in the Validation cohort adjusting for prior treatment with Abi/Enza.

Biomarker Overlap			
	Epic CTC Counts ≥ 3/mL	Epic CTC Counts <3ml	Total
Prior Abi/Enza: Yes	17	23	40
Prior Abi/Enza: No	22	45	67
Total	39	68	107

	Cox PH Model with Dichotomized CTC Counts (≥ 3/mL vs. <3ml)	**Cox PH Model with Continuous CTC Counts***
	HR (95% CI)	HR (95% CI)
OS		
Multivariable Analysis: OS ~ CTC + prior abi/enza		
EPIC CTC	2.3 (1.4-3.6)	1.4 (1.2-1.6)
Prior abi/enza	1.1 (0.7-1.8)	1.3 (0.8-2.0)
Multivariable Analysis: OS ~ CTC + prior abi/enza + riskscore		
EPIC CTC	1.7 (1.0-2.8)	1.3 (1.1-1.5)
Prior abi/enza	1.5 (0.9-2.4)	1.4 (0.9-2.4)
riskscore	1.01 (1.00-1.02)	1.01 (1.00-1.02)
PFS		
Multivariable Analysis: PFS~CTC+prior abi/enza		
EPIC CTC	1.9 (1.2-3.0)	1.3 (1.2-1.5)
Prior abi/enza	1.9 (1.2-3.0)	2.2 (1.4-3.4)
Multivariable Analysis: PFS~CTC+prior abi/enza+riskscore		
EPIC CTC	1.4 (0.8-2.3)	1.2 (1.0-1.4)
Prior abi/enza	2.5 (1.6-4.0)	2.5 (1.6-4.0)
riskscore	1.01 (1.00-1.02)	1.01 (1.00-1.02)

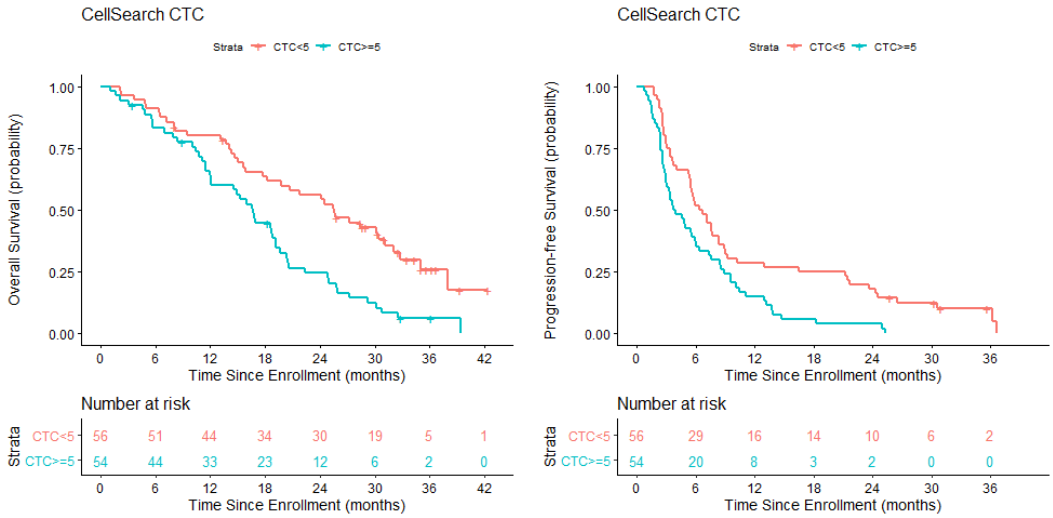


Supplementary Figure 4: Correlation of CTC/mL values in time-matched blood samples in the PROPHECY validation cohort analyzed on the Epic Sciences and CellSearch® platforms.

Supplementary Table 4: Proportional hazards models of overall survival (OS) and progression free survival (PFS) with CellSearch CTC count represented continuously and dichotomized from 0 to 4 or 5 or greater.

	Cox PH Model with Dichotomized CTC Counts (< 5 or ≥ 5)		Cox PH Model with Continuous CTC Counts*	
	HR (95% CI)	**P**	**HR (95% CI)**	**P**
Overall Survival				
Univariate Analysis				
Cell Search CTC count	2.1 (1.4-3.3)		1.2 (1.1-1.3)	
Multivariable Analysis				
Cell Search CTC count	1.7 (1.0-2.9)	0.03	1.2 (1.1-1.4)	0.002
Prognostic risk-score [22] (continuous)	1.01 (1.00-1.02)	0.006	1.00 (0.99-1.01)	0.60
Progression Free Survival				
Univariate Analysis				
Cell Search CTC count	1.8 (1.2-2.7)		1.2 (1.1-1.3)	
Multivariable Analysis				
Cell Search CTC count	1.5 (0.9-2.3)	0.11	1.2 (1.0-1.3)	0.007
Prognostic risk-score[22] (continuous)	1.01 (1.00-1.02)	0.02	1.00 (0.99-1.01)	0.63

CTC – Circulating Tumor Cell; ALK – alkaline-phosphatase; LDH – lactate-dehydrogenase; PSA – prostate specific antigen; *log2(x+1) transformed



Supplementary Figure 5: Prognostic associations with OS and PFS in the PROPHECY Validation cohort by CellSearch CTC < 5 cut-off point. (Left) Kaplan-Meier estimate OS dichotomized at the < 5 CellSearch® CTC cut-off point, and PFS (Right).

Supplementary Table 5: Frequency and association of CELLSEARCH CTC counts with OS and PFS in the Validation cohort adjusting for prior treatment with Abi/Enza.

Biomarker Overlap			
	Cellsearch CTC Counts ≥ 5/mL	Cellsearch CTC Counts <5ml	Total
Prior Abi/Enza: Yes	15	22	37
Prior Abi/Enza: No	36	29	65
Total	51	51	102*

* 102 out of 107 patients have cellsearch ctc counts

	Cox PH Model with Dichotomized CTC Counts (≥ 5 vs. < 5)	**Cox PH Model with Continuous CTC Counts***
	HR (95% CI)	HR (95% CI)
OS		
Multivariable Analysis: OS ~ CTC + prior abi/enza		
CELLSEARCH CTC	2.3 (1.4-3.8)	1.3 (1.2-1.4)
Prior abi/enza	1.7 (1.0-2.8)	1.9 (1.1-3.1)
Multivariable Analysis: OS ~ CTC + prior abi/enza + riskscore		
CELLSEARCH CTC	1.9 (1.1-3.2)	1.2 (1.1-1.4)
Prior abi/enza	2.1 (1.2-3.5)	2.0 (1.2-3.3)
riskscore	1.01 (1.01-1.02)	1.01 (1.00-1.02)
PFS		
Multivariable Analysis: PFS~CTC+prior abi/enza		
CELLSEARCH CTC	2.1 (1.3-3.3)	1.2 (1.1-1.4)
Prior abi/enza	2.8 (1.7-4.4)	3.1 (1.9-5.1)
Multivariable Analysis: PFS~CTC+prior abi/enza+riskscore		
CELLSEARCH CTC	1.7 (1.0-2.8)	1.2 (1.1-1.3)
Prior abi/enza	3.2 (2.0-5.2)	3.3 (2.0-5.3)
riskscore	1.01 (1.00-1.02)	1.01 (1.00-1.01)

Supplementary Table 6: Biomarker overlap and proportional hazards model of overall survival (OS) with CTC AR-V7 status in the Discovery and Validation cohort

DISCOVERY COHORT

Biomarker Overlap of AR-V7 status and CTC counts ≥ 3 or < 3:			
	EPIC CTC < 3	EPIC CTC ≥ 3	Total
CTC AR-V7 Negative	117	37	154
CTC AR-V7 Positive	5	12	17
Total	122	49	171

Factor	**HR (95% CI)**
OS	
Presence of visceral metastases	1.73 (1.03, 2.91)
More than one line of therapy (Yes vs. No)	2.56 (1.80, 3.65)
ALK (log2+1) Fold Change	1.22 (0.97, 1.54)
LDH (log2+1) Fold Change	1.89 (1.33, 2.69)
PSA (log2+1) Fold Change	1.10 (0.98, 1.22)
Epic CTC (>=3 vs. <3)	1.83 (1.20, 2.79)
AR-V7 (positive vs. negative)	2.21 (1.24, 3.93)

Validation Cohort

Biomarker Overlap of AR-V7 status and CTC counts ≥ 3 or < 3:			
	EPIC CTC < 3	EPIC CTC ≥ 3	Total
CTC AR-V7 Negative	65	31	96
CTC AR-V7 Positive	3	8	11
Total	68	39	107

Factor	**HR (95% CI)**
OS	
AR-V7 (positive vs. negative)	2.30 (1.16 - 4.55)
Epic CTC (≥ 3 vs. < 3)	1.63 (0.96 - 2.78)
Risk score (continuous)	1.01 (1.01-1.02)

Epic Sciences, INC.
9381 Judicial Drive
Suite 200
San Diego, CA 92121

Epic Sciences CTC Sample Logistics

CTC Sample Collection Instructions:

Whole blood samples should be collected by investigator sites in 10mL Streck Cell-Free DNA blood collection tubes (RUO, IVD, or CE-marked tubes). These tubes are commercially available through Streck (Omaha, NE).

IMPORTANT: The first 5 mL of blood collected from the fresh venipuncture cannot be used for the collection into the Streck tubes due to possibility of contaminating epithelial cells during venipuncture. **Please ensure that at least one blood tube of 5 mL or more is collected prior to collection of the CTC sample to avoid adversely affecting the test results.**

1. Confirm blood tube is not expired. Expired tubes should not be used for blood collection.
2. Draw whole blood sample into 10 mL Streck Cell-Free DNA BCT tube.
    - Since Streck Cell-Free DNA BCT tubes contain chemical additives, it is important to avoid possible backflow from the tube. To guard against backflow, observe the following precautions:
        - Keep patient's arm in the downward position during the collection procedure.
        - Hold the tube with the stopper uppermost.
        - Release tourniquet once the blood starts to flow into the tube, or within 2 minutes of application.
        - Tube contents should not touch stopper or the end of the needle during the collection procedure.
3. Fill tube until blood flow stops. Epic requires a minimum of 4 mL blood per sample for CTCs and 5 mL of blood per sample for plasma isolation, but a full 10 mL tube of blood should be provided when possible.
4. Remove tube from adapter and immediately mix by gentle inversion 8 to 10 times. Tube inversion prevents clotting. Inadequate or delayed mixing may result in inaccurate test results.
5. Label the tube with subject's identification and date and time of blood draw. Unlabeled blood tubes may not be processed.

CTC Sample Shipment Instructions:

Prior to collection, schedule courier for same-day pick-up prior to collection.

Whole blood samples should be kept at **room temperature** until time of shipment. Whenever possible**, samples should be sent on the day of collection for overnight delivery** to the appropriate Epic Sciences processing facility (see **CTC Specimen Shipment Reference Table***). Do not refrigerate or freeze specimens. Infectious Substance labels should not be placed on the shipment, as this can result in delay of shipment.

Epic Sciences, INC.
9381 Judicial Drive
Suite 200
San Diego, CA 92121

All shipments should include requisition forms containing the following information:

Sponsor Protocol ID / Epic Sciences Project ID, Collection Site, Patient ID, Time Point, Collection Date and Collection Time (If not provided, Epic Sciences will assume the sample was collected at 8:00AM local time on the date of collection).

Sample Disposal Criteria

- **Blood sample for CTC samples will be discarded for any of the following reasons:**
  - Out of stability; blood age >96 hours from time of collection
  - Sample <4 mL total volume
  - Sample clotted or hemolyzed
  - Broken or expired blood collection tube
  - Incorrect blood collection tube type
  - Sample received at incorrect temperature (frozen)
  - Failure during processing
- **Blood samples for plasma isolation will be discarded for any of the following reasons:**
  - Out of stability; blood age >6 days from time of collection
  - Sample <5 mL total volume
  - Sample clotted or hemolyzed
  - Broken or expired blood collection tube
  - Incorrect blood collection tube type
  - Sample received at incorrect temperature (frozen)

Development and Validation of Circulating Tumor Cell Enumeration (Epic Sciences) as a Prognostic Biomarker in Men with Metastatic Castration Resistant Prostate Cancer

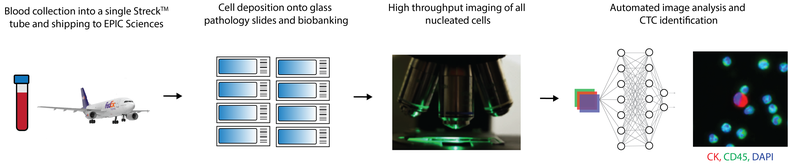
Scher H.I.*[1,2], Armstrong A.J.*[3], Schonhoft J.D.[4], Gill A.[4], Zhao, J.L.[1], Barnett E.[1], Carbone, E.[1], Lu J.[4], Antonarakis E.S.[5], Luo, J. [5], Tagawa S.[2], dos Anjos C.H. [1], Yang. Q. [6], George D.[3] Szmulewitz R.[6], Danila D.C. [1,2], Wenstrup R.[4], Gonen M. ‡[1], Halabi S. ‡[3,6]

*co-first; ‡co-last; 1. Genitourinary Oncology Service, Department of Medicine, Memorial Sloan Kettering Cancer Center, New York, NY; 2. Department of Medicine, Weill Cornell Medical College, New York, NY; 3. Duke Cancer Institute Center for Prostate and Urologic Cancers, Duke University, Durham NC; 4. Epic Sciences, San Diego, CA; 5. Johns Hopkins University, Baltimore MD; 6. Department of Biostatistics and Bioinformatics, Duke University, Durham NC; 7. University of Chicago, Chicago IL

PRIMARY OBJECTIVE OF THIS STUDY

The **primary objective** of this pre-specified retrospective analysis is to assess CTC counts (Epic Sciences platform) as a biomarker of prognosis in patients treated with AR signalling inhibitors (ARSi) and compare to counts measured on the FDA approved predicate CellSearch™ Circulating Tumor Cell kit

THE EPIC SCIENCES NON-ENRICHMENT CTC DETECTION AND ENUMERATION TECHNOLOGY

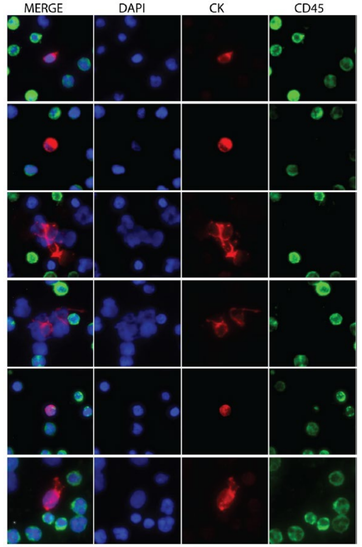


Summary of CTC detection technologies and available large clinical validations/studies

CTC Detection Technology	Method	CTC Definition (Epithelial)	Clinical Validation/Studies (CTC counts only) (Studies of > 100 patients only)
CellSearch™ Circulating Tumor Cell Kit	Affinity Capture by EpCAM ferrofluid	EpCAM captured, CK+, CD45-	Validation in multiple ph III trials
Epic Sciences	Non-enrichment, all nucleated cells plated onto slides and IF imaged. CTCs detected *in silico*.	CK+, CD45-, DAPI+ (this study)	Scher HI et al. ASCOGU 2021 (This study) de Bono J et al. ASCOGU 2021 (abstract #161)
Other	Affinity capture, microfluidics, size based	Variable depending on platform	Limited or none

ENUMERATING CTCS IN THIS STUDY

CTC Enumeration in This Study
- Cytokeratin positive (epithelial)
- CD45 negative (leukocyte)
- Intact nucleus
- Clusters of CTCs are counted as 1 event
- Count is normalized to volume



PATIENT SELECTION

MSKCC Discovery Cohort

216 mCRPC blood samples from patients with progressing mCRPC starting prior to starting an ARSi (2012-2016)

→ 45 samples taken >30 days prior to therapy start

171 unique patients with baseline blood-draws
End Pts: Overall Survival

91.4% had detectable CTCs

PROPHECY Trial Validation Cohort

120 men with progressing high risk mCRPC starting an ARSi in the PROPHECY trial

→ 2 men withdrew consent
11 failed QC

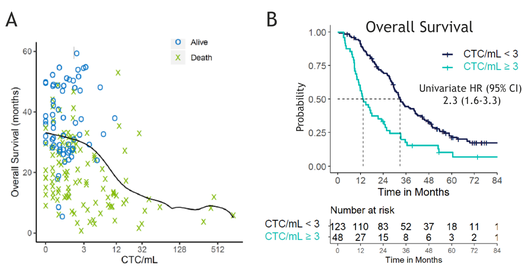
107 unique patients with baseline blood-draws
End Pts: OS, PFS, CellSearch method comparison

83.2% had detectable CTCs

Analysis Methods: The Kaplan-Meier estimate and Cox proportional hazards models adjusting for known prognostic features were used to assess the prognostic significance of CTC number. Analyses were completed by independent statisticians and the analysis of the Validation cohort was pre-specified based on the findings in the Discovery cohort.

CTC COUNTS ARE A PRE-TREATMENT PROGNOSTIC BIOMARKER IN THE DISCOVERY AND VALIDATION COHORTS

MSKCC Discovery Cohort



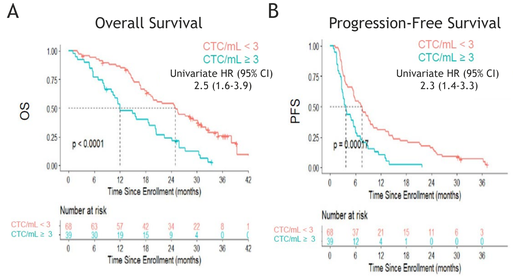
Multivariable Risk Adjusted Hazard Ratios in the Discovery Cohort (Epic Sciences)

	CTC/mL ≥ 3 HR** (95% CI); P	Fold Increase CTC (log2 + 1) HR** (95% CI); P
MSKCC Discovery Cohort	1.9 (1.3, 2.9); P = 0.002	1.2 (1.1, 1.4); P = 0.001

**adjusted for therapy line, sites of spread, LDH, PSA, Alkaline phosphatase.

A) Plot of survival times versus CTC/mL value. **B)** Kaplan-Meier estimate for OS. A natural cutoff of approximately 3 CTCs/mL is identified from (A). **C)** Multivariable Cox Proportional Hazards model of OS adjusting for standard baseline prognostic features as a dichotomized (< 3 versus ≥ 3 CTC/mL) and continuous variable (Fold change or log2 + 1 transform).

PROPHECY Validation Cohort



Multivariable Risk Adjusted Hazard Ratios in the Validation Cohort (Epic Sciences)

Endpoint	CTC/mL ≥ 3 HR** (95% CI); P	Fold Change CTC HR** (95% CI); P
OS	1.8 (1.1, 3.0): P = 0.03	1.3 (1.1, 1.6); P = 0.002
PFS	1.7 (1.1, 2.9); P = 0.03	1.3 (1.1, 1.5); P = 0.01

**HR adjusted for Halabi Prognostic Risk score (includes; sites of metastases, opioid use, LDH, ECOG, Albumin, Hemoglobin, Alkaline Phosphatase, PSA) See Halabi et al. JCO 2014; PMID24449231

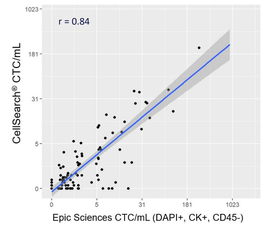
A&B) Kaplan-Meier estimate for OS and PFS using the 3 CTCs/mL cutoff identified above **C)** Multivariable Cox Proportional Hazards model of OS and PFS adjusting for standard baseline prognostic features as a dichotomized (< 3 versus ≥ 3 CTC/mL) and continuous variable (Fold change or log2 + 1 transform).

CONCLUSIONS

- The findings validate CTC number determined on the Epic Sciences platform as a prognostic biomarker prior to treatment with Androgen Receptor signaling inhibitors in two independent cohorts.
- CTC counts showed strong method agreement and correlation with counts determined using the FDA cleared CellSearch Circulating Tumor Cell kit which is approved for use as an aid to monitoring.
- In univariate and multivariable analyses, the associations with OS and PFS of CTC counts on both platforms were comparable in the Validation cohort.

COMPARISON WITH THE FDA CLEARED CELLSEARCH DEVICE

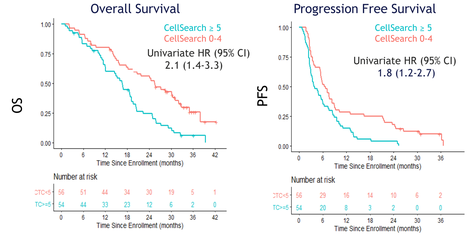
Epic Sciences CTC Counts in the PROPHECY Validation Cohort Show Strong Correlation and Agreement to the CellSearch Predicate With the Base CTC Definition



CTC Definition By Platform	
Epic Sciences	CK+, CD45- intact cell
CellSearch	EpCAM captured CK+, CD45- intact cell

Summary of Comparisons Between Platforms
- Strong correlation observed between platforms (r = 0.84)
- 94% of CTC counts within Bland-Altman limits
- Lin's concordance correlation coefficient (CCC) of 0.81 observed

Epic Sciences and CellSearch CTC Counts (CK+, CD45-) are Comparably Prognostic for OS/PFS in the Validation Cohort



Multivariable Risk Adjusted Hazard Ratios in the Validation Cohort

Technology	Endpoint	CTC ≥ 5 vs < 5 HR (95% CI); P	Fold Change CTC (continuous log2 +1) HR (95% CI); P
CellSearch	OS	1.7 (1.0, 2.9): P = 0.03	1.2 (1.1, 1.4); P = 0.002
CellSearch	PFS	1.5 (0.9, 2.3); P = 0.11	1.2 (1.0, 1.3); P = 0.007

**HR adjusted for Halabi Prognostic Risk score (includes; sites of metastases, opioid use, LDH, ECOG, Albumin, Hemoglobin, Alkaline Phosphatase, PSA) See Halabi et al. JCO 2014; PMID24449231

PATIENT DEMOGRAPHICS

Patient Demographics	MSKCC Discovery Set	PROPHECY Validation set
Unique Patients, no. (%)	171	107
Unique Blood Samples, no. (%)	171	107
Median Age in years (range)	68 (45,87)	73 (44,92)
Death events, no. (%)	137 (80.0%)	83 (77.6%)
Median Follow Up of Survivors in months (range)	60.3 (5.0, 84.8)	31 (3.4, 42.3)
Therapy Line - no. (%)		
pre-1st	103 (60.2%)	76 (71%)
pre-2nd	48 (28.1%)	31 (29%)
pre-3rd	20 (11.7%)	0 (0%)
Sites of Metastases – no. (%)		
Lymph Node Only	29 (17.0%)	4 (3.7%)
Bone Only	59 (34.5%)	25 (23.4%)
Lung Only	1 (0.6%)	0 (0%)
Multiple Sites	82 (48.0%)	76 (71.0%)
Prior Taxane Chemotherapy – no. (%)	12 (7.0%)	20 (18.7%)
Prior ARSi – no. (%)	56 (33.1%)	40 (37.4%)
Baseline lab values – median (range)		
PSA ng/mL	18.1 (0.1, 2006.1)	22.1 (0.1, 4194.9)
ALB g/L	4.2 (3.3, 4.9)	4.0 (2.7, 4.9)
ALK U/L	96 (42, 2170)	110 (91, 150)
HGB g/dL	12.6 (8.2, 15.7)	12.8 (8.7, 15.9)
LDH U/L	208 (124, 2115)	200 (100, 618)
WBC x 10^9/L	5.9 (2.6, 12.1)	6.4 (3.7, 22.3)
CellSearch® CTC count/7.5mL	n/a	4 (0, 12,972)
EPIC Sciences CTC count/mL	1.3 (0.0, 906.3)	1.3 (0.0, 916.2)

Abbreviations: PSA - prostate specific antigen, ALB - albumin, ALK - alkaline phosphatase, HGB - hemoglobin, LDH - lactate dehydrogenase, WBC - white blood cell

FUNDING AND DISCLOSURE STATEMENTS

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Statistical Analysis Plan to Confirm Circulating Tumor Cell Enumeration as a Prognostic Biomarker at Baseline in the PROPHECY trial (NCT02269982)

Version 1.0

ACRONYMS AND DEFINITIONS

Term or Abbreviation	Description
ALK	Alkaline-phosphatase
ARSi	Androgen Receptor Signaling inhibitor
CD45	Cluster of Differentiation 45; protein marker of leukocyte lineage
CK	pan-Cytokeratin, protein marker of epithelial lineage composed of Cytokeratins 1,4,5,6,8,10,13,18, and 19
CTC	Circulating Tumor Cells; Epic Sciences defines CTCs as any CK+, CD45- cell in the blood stream with an intact nucleus. A cluster of adjacent cells is considered one event or count.
DAPI	4′,6-diamidino-2-phenylindole
EpCAM	Epithelial Cell Adhesion Molecule
LDH	Lactate-dehydrogenase
mCRPC	Metastatic Castration Resistant Prostate Cancer
NCCN	National Comprehensive Cancer Network
OS	Overall Survival
PFS	Progression Free Survival
PSA	Prostate-specific antigen
PROPHECY	**P**rospective Ci**R**culating pr**O**state Cancer **P**redictors in **H**igh**E**r Risk m**C**RPC stud**Y**; NCT02269982

1. PURPOSE

This statistical analysis plan (SAP) describes the planned analysis for confirming the prognostic value of CTC counts as a biomarker in higher risk Metastatic Castration Resistant Prostate Cancer (mCRPC) patients about to start an Androgen Receptor Signaling inhibitor (ARSi) using outcome data generated under the clinical trial NCT02269982 (named as PROPHECY). This SAP focuses on the CTC count biomarker as part of the secondary objectives of the PROPHECY trial and describes the related analysis including statistical methodology, data handling, analyzing, and reporting. The clinical trial protocol and clinical trial SAP for the PROPHECY trial are published in the following link and as an attachment to this SAP.

Protocol and SAP for the PROPHECY trial (NCT02269982) entitled "*Development of Circulating Molecular Predictors of Chemotherapy and Novel Hormonal Therapy Benefit in Men with Metastatic Castration Resistant Prostate Cancer (mCRPC)*" can be downloaded from the following link:

https://ascopubs.org/doi/suppl/10.1200/JCO.18.01731/suppl_file/protocol_JCO.18.01731.doc

2. BACKGROUND

2.1. General Information

Tumor cells that shed into the bloodstream from the primary or metastatic tumors are known as Circulating Tumor Cells (CTCs) and a small proportion have the ability to colonize distal tissue and are hypothesized to drive or initiate spread of metastatic disease[5]. It follows both intuitively and biologically that the presence of CTCs in the blood of a patient would be associated with shorter survival times relative to those with fewer or no CTCs, and could provide a more accessible means of monitoring disease.

However, defining, detecting, and characterizing a CTC provides its own set of challenges and requires specialized technology, as i) they are rare in the blood (1 in 10^5 to 1 in 10^7 nucleated cells typically)[5] and ii) they are genomically and morphologically heterogenous[6]. Therefore, it is critical to clearly define the precise definition of a "CTC" in each study and what technology was used to detect and count them. The FDA approved, and analytically valid CellSearch device defines any "CTC" as a cell in the blood captured by epithelial cell adhesion molecules (EpCAM) that is CK^+CD45^- with an intact DAPI stained nucleus[7]. CTCs captured and enumerated on this platform, prior to starting life prolonging therapies, have been shown be strongly prognostic and changes in CTCs from baseline to those measured on-therapy have been shown to associate strongly with overall survival and drug response as measured by imaging[8–11]. Further recent analysis of 5 phase III trials in prostate cancer have demonstrated that CTC based change metrics, CTC conversion and CTC0 on the CellSearch platform, have strong concordance with overall survival and meet criteria for a "reasonably likely surrogate endpoint" by the FDA Biomarkers EndpointS and Tools (BEST) definitions[12,13]. Additionally, several other platforms exist for CTC detection based primarily on cell size or affinity capture, however for enumeration of CTCs, based on the above definition or by any other definition, only the CellSearch platform has gone through extensive and rigorous clinical validation to assess the clinical validity of CTC count.

The Epic Sciences platform, alternatively, is a non-enrichment based method in which all nucleated cells from a tube of blood are deposited onto glass pathology slides and fixed using aldehyde based cross-linking [14]. The slides can be stored long-term and then later analyzed and imaged by immunofluoresence and small-molecule DNA staining. After staining, each cell is individually imaged and rare cells of interest are identified *in silico*. A typical assay on this platform images and analyzes anywhere from 10^6-10^8 individual cells depending on the analytic requirements. In the clinical setting, the platform to date has been used to measure nuclear-localized AR-V7 protein in CTCs, and the resulting biomarker has demonstrated clinical utility informing the selection of a taxane or androgen receptor signaling inhibitor in the 2nd line or greater mCRPC treatment setting[15], reaching level IIA evidence in the 2019 NCCN guidelines (v1.0), and is now covered by Center for Medicare Services and is a New York State approved Laboratory Developed Test (LDT).

In prior analysis of baseline samples obtained from MSKCC, we observed CTC counts to be prognostic of overall survival as both a continuous variable and dichotomized variable ($\geqslant$ 3 or < 3 CTCs/mL) in a cohort of mCRPC patients prior to starting an ARSi in the 1st, 2nd, or 3rd line settings. This work was presented as a poster in the ASCO 2020 general meeting. Here our primary objective is to validate the prognostic value of CTC counts using data generated under the PROPHECY trial (NCT02269982). Epic Sciences defines CTCs as any CK+, CD45- negative cell with an intact DAPI stained nucleus, and clusters of CTCs are defined as 1 count or event. ***Epic Sciences is currently blinded to outcome data and all analyses will be conducted by Professor Susan Halabi Ph.D. at Duke University.***

2.2. The PROPHECY Trial

PROPHECY is a multicenter prospective study that validated the use of AR-V7 status in CTCs as a biomarker of futility on abiraterone or enzalutamide. CTC enumeration was collected via the CellSearch and Epic Sciences platforms. AR-V7 status was determined by the Epic nlAR-V7 test and by the Johns Hopkins University modified-AdnaTest CTC AR-V7 mRNA assay as previously described in the parent protocol. The trial enrolled 118 mCRPC high-risk patients at five clinical sites, of which 107 had evaluable baseline blood draws sent to Epic Sciences. All men subsequently started abiraterone or enzaltumide treatment. Laboratory personnel were blinded to clinical results, and the patients' physicians were blinded to AR-V7 status at baseline. The primary outcome was progression-free survival (PFS), and secondary outcome measures were overall survival (OS), 50% PSA decline, and radiographic response assessed by RECIST v1.1 criteria . The trial demonstrated that AR-V7 positive status by the Epic Sciences test was independently associated with shorter PFS and OS and that zero patients who were AR-V7 positive at baseline by the Epic Sciences test received clinical benefit on an ARSi.

3. STUDY OBJECTIVES

3.1. Primary Analysis:

3.1.1. Confirm CTC count at baseline (both as a continuous variable and dichotomized at $\geqslant$ 3 CTCs/mL or < 3 CTC/mL) using the Epic Sciences enumeration test as a prognostic biomarker of OS in patients treated with an ARSi.

3.2. Secondary Analysis:

3.2.1. Explore CTC counts at baseline (both as a continuous variable and dichotomized at $\geqslant$ 3 CTCs/mL or < 3 CTC/mL) using the Epic Sciences enumeration test as a response biomarker with radiographic or clinical progression-free survival (PFS), confirmed 50% PSA decline, and radiographic response assessed by RECIST v1.1 in patients treated with an ARSi.

3.2.2. Compare CTC counts by the Epic Platform to counts determined on the CellSearch platform on the basis of detection frequencies, agreement, and association with clinical outcomes.

4. STUDY DESIGN

4.1. Clinical Trial Design

The PROPHECY trial is a multicenter, prospective-blinded study of men of with high risk mCRPC about to start either abiraterone or enzalutamide treatment (NCT02269982). The primary objective of the study was to validate pre-treatment AR-V7 status in CTCs as a predictor of resistance to enzalutamide, abiraterone acetate, or taxane-based therapy in men with mCRPC, as determined by radiographic or clinical progression-free survival. Secondary objectives included the analysis of other CTC based biomarkers of futility to abiraterone or enzalutamide.

4.2. Schedule of Assessments

As part of the PROPHECY trial, 107 baseline blood samples were evaluable for the Epic Sciences test and 61 samples were evaluable at progression on Abiraterone or Enzalutamide.

4.3. Epic Sciences IF Assays and Determination of Total CTC Counts

Each blood sample will be analyzed with the Epic Sciences CTC platform. A CTC is defined as a CK+, CD45- cell in the blood stream with an intact DAPI+ nucleus. Clusters of CTCs are classified as 1 event in the total count. Counts will be normalized to blood volume and expressed as CTC per mL of blood in the final report.

4.4. Assay Design and Methods

Blood (7.5 mL) from each participant was collected in Streck tubes and shipped to Epic Sciences and processed within a required 72-hour time limit during the course of the PROPHECY trial. Upon receipt, red blood cells were first lysed, and 3 million nucleated blood cells were dispensed onto 10-16 glass microscope slides (25.3 mm × 75.3 mm) and placed at −80°C for long-term storage until analysis.

5. ANALYSIS ENDPOINTS

CTC counts will be analyzed with respect to the following endpoints as defined in the parent study (PROPHECY trial):

1. Overall Survival (OS)
2. Progression Free Survival (PFS). Defined from date of registration to clinical/radiographic progression or death, whichever occurred first.
    a. Radiographic progression was assessed using Prostate Cancer Working Group 2 soft tissue and bone scan criteria
    b. Clinical progression was defined by death, pain, or other symptomatic progression; initiation of new systemic therapy; or a skeletal-related event
3. Confirmed 50% or greater prostate-specific antigen (PSA) declines
4. Radiographic response per RECIST version 1.1

6. ANALYSIS POPULATION

6.1. Intent to Use (ITU) Population

The biomarker study will utilize Epic Sciences platform to detect CTCs by using all evaluable patient samples from the PROPHECY trial. The intent to use (ITU) population will include all subjects for which biomarker assessment was attempted and receiving treatment following inclusion /exclusion criteria. All biomarker related analysis will be on ITU population.

6.2. Inclusion Criteria

All blood samples sent to Epic Sciences from the PROPHECY trial which were taken at baseline and at progression will be analyzed.

All patients enrolled in PROPHECY with evaluable clinical outcomes will be included.

6.3. Exclusion Criteria

Patients without evaluable blood samples at baseline due to failed quality control (QC) will be excluded.

Samples that do not pass QC will be excluded with a specific reason for failure. Example Reasons for QC Fail:

- Blood sample clots and is therefore not usable
- Blood sample arrives from site greater than 96 hours after blood draw
- Error on part of lab technician, e.g. the blood tube was spilled.

7. STATISTICAL ANALYSIS

All analyses will be applied to ITU population.

7.1. Analysis with endpoints

The primary analysis will focus on confirming CTC count (continuous variable and dichotomized at $\geqslant$ 3 CTCs/mL or < 3 CTC/mL) at baseline as a prognostic biomarker in patients treated with an ARSi using OS. PFS will also be analyzed to explore the association of CTC count with drug response. Statistical method of Cox Proportional Hazard (PH) model will be applied to estimate the risk for time-to-event endpoints. In detail, the Cox PH model will be:

$$h(t) = h_0(t)\exp\,(b_1 X)$$

where

- t represents the survival time
- $h(t)$ is the hazard function determined by covariate CTC counts (continuous or dichotomized)
- the coefficients b_1 measure the effect size of CTC counts.
- $h_0(t)$ is the baseline hazard.

Two Cox PH model will be constructed one for continuous CTC counts (X in model) and one for dichotomized CTC with CTC/mL $\geqslant$ 3 or < 3. Hazard ratio along with 95% confidence interval, p-value by Wald method will be reported. Kaplan-Meier (KM) estimates with plot along with log-rank testing result will be conducted for dichotomized CTC counts. Hazard ratio (with 95% CI) versus CTC count scatter plot will be constructed from Cox PH model with CTC counts as continuous variable.

Additional analysis will investigate the association of CTC counts with PFS and OS in Cox PH models adjusting for known prognostic factors at baseline using the validated CALGB nomogram (see Halabi et al. JCO 2014[16]). Dichotomized CTC counts will also be compared to 50% PSA decline (yes vs. no), and radiographic response criteria by RECIST v1.1 (*i.e.* Complete Response, Stable Disease, Progressive Disease) using contingency tables and appropriate hypothesis tests (chi-square or fisher's exact).

7.2. Comparison between the two platforms

This analysis will be applied to ITU population with both blood samples passing Epic Sciences and CellSearch quality control metrics. The comparison analysis will focus on the comparison of CTC counts by the Epic Platform and the CTC counts by the CellSearch platform on the basis of detection frequencies, agreement, and association with outcome. Statistical analysis will include

- Summary statistics of CTC counts at baseline for both Epic and CellSearch
- Correlation analysis including scatter plot of log2 transformed CTC counts plus one (log2(CTC+1)) on the Epic and CellSearch platforms. Spearmen correlation coefficient and Lin's Concordance Correlation Coefficient will be reported as well.
- Bland-Altman agreement analysis will be conducted comparing CTC counts (using log2(CTC +1)) between the two platforms.
- Primary and exploratory analyses will be applied to CellSearch based CTC counts. Cox PH models on CTC by EPIC and by CellSearch will be compared based on likelihood ratio test. Akaike's Information Criteria (AIC) will be reported.

7.3. Disposition Analysis

Disposition: demographic tables for CellSearch CTCs greater than and less than 5/7.5mL and Epic CTC/mL counts by median, $\geqslant$ 3 CTC/mL, or similar cutoff.

Disposition: Describe the CTC/mL counts on Epic and CellSearch platforms at Baseline to Progression

8. GENERAL ANALYSIS COMMENTS

All statistical software and related packages used for the analyses, with version numbers, will be included in the statistical analysis report. All calculated statistics will be rounded to the nearest 2 digits other than hypotheses testing p values which will be round to nearest 4 digits .

Confidence intervals for all statistics will be calculated using the asymptotic method and rounded to the nearest 2 digits. Missing data will not be imputed for any analysis.

9. CHANGE OF PLANNED ANALYSIS

Should any of the planned statistical methods proposed in this SAP prove unsuitable during the final analysis, more appropriate methods will be used, and any changes, including the rationale for use, will be documented in the final report.

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