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Circulating Tumour Cells and Survival in Abiraterone- and Enzalutamide-treated Patients
with Castration-Resistant Prostate Cancer

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Key words: CRPC, circulating tumour cells, abiraterone, enzalutamide, CellSearch

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Abstract

Background: The outcome to treatment administered to patients with metastatic castration-resistant prostate cancer (mCRPC) greatly differs between individuals, underlining the need for biomarkers guiding treatment decision making.

Objective: To investigate the prognostic value of circulating tumour cell (CTC) enumeration and dynamics, in the context of second-line endocrine therapies (i.e. abiraterone acetate or enzalutamide), irrespective of prior systemic therapies.

Design, settings and participants: In a prospective, multicentre study blood samples for CTC enumeration were collected from patients with mCRPC at baseline (n=174). In patients who responded for minimally 10-12 weeks a follow-up sample was collected.

Outcome measurements and statistical analysis: For baseline analysis, patients were stratified in <5 or ≥ 5 CTCs/7.5mL, whereas for the analysis of CTC dynamics at 10-12 weeks, in patients with stable, increasing or decreasing CTC counts. Progression-free survival (PFS), overall survival (OS), and PSA changes at 10-12 weeks were compared between groups.

Results: Patients demonstrating increasing CTCs on therapy had a shorter median PFS (4.03 vs. 12.98 vs. 13.67 months, HR 3.6, 95% CI 1.9–6.8; $p < 0.0001$) and OS (11.2 months vs. not reached, HR 9.5, 95% CI 3.7–24; $p < 0.0001$), compared to patients with decreasing or stable CTCs. Multivariable Cox regression showed that prior chemotherapy (HR 4.1, 95% CI 1.9–8.9; $p = 0.0003$), a high baseline CTC count (HR 1.5, 95% CI 1.2–1.9; $p = 0.002$) and increasing CTCs at follow-up (HR 3.3, 95% CI 1.4–7.6; $p = 0.005$) were independent predictors of worse PFS. Previous chemotherapy (HR 7, 95% CI 1.9–25; $p = 0.003$), high baseline CTC counts (HR 2.2, 95% CI 1.4–3.7; $p = 0.002$) and

increasing CTCs during therapy (HR 4.6, 95% CI 1.4-15; $p = 0.01$) were independently associated with shorter OS. $\geq 30\%$ and $\geq 50\%$ PSA responses less frequently occurred in patients with CTC inclines at 10-12 weeks on therapy (χ^2 test: $p < 0.01$).

Conclusions: CTC dynamics during therapy are associated with PSA response and provide independent clinical prognostication over PSA declines. Hence the study demonstrates the pharmacodynamic properties of CTCs.

Introduction

With nearly 410.000 diagnoses in Europe each year, prostate cancer remains the most commonly diagnosed cancer in men ¹. Approximately 10-20% of the patients will ultimately advance to lethal, metastatic castration-resistant prostate cancer (mCRPC) ². A continued targeting of the androgen receptor (AR) signalling pathway has proven to be useful ³. This has led to the development and clinical validation of next generation androgen deprivation therapies, encompassing the CYP17 inhibitor abiraterone acetate and the anti-androgen enzalutamide ⁴⁻⁷. Due to shifts in reimbursement criteria, novel agents and guidelines in the treatment of CRPC, a heterogeneous landscape of patients exists with respect to the current clinical practice ⁸⁻¹⁰. Circulating tumour cell (CTC) enumeration using the CellSearch System demonstrated how CTCs are an accurate and independent predictor of survival in metastatic prostate cancer ^{11,12}. Here, we reassessed the prognostic value of baseline CTC enumeration via a prospective, multicentre study in patients with mCRPC starting a new line of systemic therapy with either abiraterone acetate or enzalutamide. Next, we evaluated the association between survival and CTC changes relative to baseline CTC enumeration (i.e. CTC dynamics) in patients who responded for at least 10-12 weeks. Secondary objectives included the assessment of the relationship of CTCs with baseline prostate-specific antigen (PSA), lactate dehydrogenase (LDH) and alkaline phosphatase (AP), and PSA responses at 10-12 weeks on therapy.

Materials and Methods

Study Design

Via a prospective and multicentre clinical study, we collected blood samples from patients with mCRPC at eight clinical centres in Belgium, and one in the Netherlands. Patients had a biochemically and histologically confirmed prostate adenocarcinoma, starting a new treatment for progressive disease in the presence of castrate levels of serum testosterone (<50 ng/dl [1.73 nmol/L]), consistent with Prostate Cancer Clinical Trials Working Group guidelines ¹³. Patients were eligible for enrolment when starting a new line of endocrine therapy with abiraterone acetate or enzalutamide. The number of prior chemotherapy or AR-directed therapy regimens was not limited. The institutional review board at each centre approved the study protocol, and all patients provided written informed consent. The study was registered by the leading ethical committee from the Antwerp University Hospital (registration number: B300201524217). At treatment initiation, a peripheral blood sample was collected for enumeration of CTCs and biobanking for future research. In patients who were responding for ca. 10-12 weeks, a follow-up blood sample was collected to assess CTC dynamics (i.e. stable, in- or decreasing CTC count compared to baseline measurement).

Enumeration of circulating tumour cells

For CTC enumeration, 7.5mL blood was collected in CellSave Vacutainer tubes (Menarini Silicon Biosystems, Italy), and processed within 24 hours after collection, as described previously ¹⁴. Briefly, samples were processed on the CellPrep system (Menarini Silicon Biosystems, Italy), after which cartridges were analysed using the

CellTracks Analyzer II, a semi-automated fluorescence-based microscopy system. EpCAM-enriched CTC are defined as nucleated cells that lack CD45 and express cytokeratin. At baseline patients were stratified as having favourable (<5 CTCs/7.5mL) or unfavourable (≥ 5 CTCs/7.5mL). At 10-12 weeks follow-up patients were stratified on CTC dynamics, as having either increasing or decreasing CTC counts compared to baseline, or as having stable CTC levels.

Statistical analysis

Serum PSA, LDH and AP levels were compared between defined groups using Mann–Whitney U test. Progression-free survival (PFS) was defined as the time between treatment initiation and clinical symptomatic progression and/or radiologic progression (Response Evaluation Criteria in Solid Tumors v1.1). Overall survival (OS) was defined as the time between the baseline blood sample and the date of death or the last follow-up visit (i.e. censored event). Differences with respect to progression-free survival (PFS) and overall survival (OS) were analysed using Kaplan-Meier (KM) analysis. Significance of prognostic assessments was determined using the log-rank test and univariable Cox regression analysis. Significant variables in univariable analysis were entered into a multivariable Cox proportional hazards regression model. All PFS and OS survival analyses were performed using either the baseline or 10-12 weeks characteristics. The latter encompassed besides the baseline variables also CTC dynamics and PSA change at 10-12 weeks. For multivariable analysis including the 10-12 weeks characteristics we only used the continuous baseline CTC count variable as baseline CTC measure. The CTC dynamics and PSA change variables were dichotomized in increasing or

stable/decreasing CTC counts, and whether a 50% PSA change was obtained, respectively. Due to a positive skew in blood chemistry and CTC distributions, continuous data were log-transformed prior to Cox regression analysis. To additionally demonstrate how measurement of CTC changes enhances prognostication of patients, survival models based on baseline blood chemistry or baseline blood chemistry with CTC metrics (baseline CTC count and/or CTC changes at 10-12 weeks) were evaluated by area under the ROC curves (AUC)/receiver operating characteristic (ROC) analysis. AUCs were compared by De Long's test for median, 3rd quartile and maximum survival endpoints (PFS: 6, 12 and 26 months; OS: 11, 17 and 40 months). PSA responses ($\geq 30\%$ or $\geq 50\%$) at 10-12 weeks in defined groups were compared by χ^2 test. All tests were performed in R, with a two-sided p -value < 0.05 being considered as statistically significant.

Results

Patient and sample characteristics

From October 2013 through June 2017, peripheral blood samples for CTC enumeration ($n=276$) were collected from 174 patients with mCRPC at start (i.e. baseline) and 10-12 weeks on therapy. Patient characteristics and baseline blood chemistry are given in Table 1. At baseline 109/174 (62.6%) patients were chemotherapy-naïve and 65/174 (37.4%) were pretreated with chemotherapy. Of all patients, 124 (71%) started treatment with abiraterone acetate, whereas 50 (29%) patients started enzalutamide. At the time of analysis, 121/174 (69.5%) patients progressed on abiraterone acetate or enzalutamide with a median follow-up time for PFS of 5.7 months (range, 0.3 to 26.3 months). In total

36/174 (20.6%) patients progressed before 10-12 weeks, of whom the CTC dynamics could not be evaluated. For the remaining 138 patients who responded for 10-12 weeks or longer, we collected follow-up samples in 102/138 (73.9%) cases. The average time to follow-up sampling of the evaluable 102 patients was 2.37 ± 0.67 months (median: 2.3 months). Supplementary table 1 provides an overview of patients with and without follow-up sampling. Sixty-three percent of the patients (111/174) were still alive at the time of the analysis. The median follow-up time for OS was 11.4 months (range, 0.5 to 40.2 months).

Circulating tumour cells and blood chemistry at baseline

At baseline, CTCs were prevalent as 118/170 (69.4%) patients were CTC-positive, of whom 69/170 (40.6%) had ≥ 5 CTCs/7.5mL. For 4/174 recruited patients, baseline CTC enumeration failed during the CellSearch procedure. Having ≥ 5 CTCs/7.5mL was associated with a higher tumor stage at diagnosis, previous chemotherapy exposure and more widespread disease at start of abiraterone or enzalutamide (Table 1). At baseline we observed 5-fold higher median CTC levels in patients pretreated with chemotherapy (Mann–Whitney U: $p = 0.0012$) (Supplementary Figure 1A). Additionally, we observed that patients with ≥ 5 CTCs/7.5mL at baseline, demonstrated 5.3-, 2.6-, and 1.9-fold higher serum prostate-specific antigen (PSA), lactate dehydrogenase (LDH), and alkaline phosphatase (AP) levels, respectively (Mann–Whitney U test: all $p < 0.0001$) (Supplementary Figure 1B). The latter was irrespective of previous exposure to chemotherapeutic agents, except for serum LDH levels in patients with < 5 CTCs at

baseline (Supplementary Figure 2). Here, we observed 1.9-fold higher LDH levels in chemotherapy-pretreated patients (Mann–Whitney U test: $p < 0.001$).

Circulating tumour cells, progression-free and overall survival

Baseline CTC and time-to-event data were available in 170 patients. Before the start of a new line of endocrine therapy, patients with ≥ 5 CTCs/7.5mL ($n=69$), as compared with the group with <5 CTCs/7.5mL ($n=101$), had a shorter PFS (3.9 vs. 11.5 months, hazard ratio [HR] 2.5, 95% confidence interval [CI] 1.7–3.7; $p < 0.0001$) and OS (11.2 months vs. 30 months, HR 4.7, 95% CI 2.8–8.3; $p < 0.0001$) (Figure 1). Multivariable Cox proportional-hazards regression using baseline variables demonstrated how prior chemotherapy exposure (HR 2.2, 95% CI 1.3–3.6; $p = 0.003$) and high baseline CTC counts (HR 1.5, 95% CI 1.2–1.8; $p = 0.002$) were independently associated with a shorter PFS. Prior chemotherapy (HR 2.2, 95% CI 1.2–4; $p = 0.01$) and high baseline CTC count (HR 1.4, 95% CI 1.1–1.9; $p = 0.02$) were independent predictors for OS (Table 2).

Follow-up CTC counts at 10-12 weeks were available for 102/138 (73.9%) patients with baseline sampling and minimal 10-12 weeks response. Stratifying the patients in favourable (i.e. <5) and unfavourable (≥ 5 CTCs/7.5mL) based on their follow-up sample recapitulated the differences in both PFS and OS (Supplementary Figure 3). In 11/28 (39.3%) patients with ≥ 5 CTCs/7.5 mL at baseline a shift towards the <5 CTCs/7.5 mL group was observed, suggesting a benefit from endocrine therapy.

When stratifying patients based on their CTC dynamics at 10-12 weeks (Supplementary Figure 4), patients with a CTC increase (n=19), as compared to patients with decreasing CTCs (n=44) or a stable (n=39) CTC count, had a shorter PFS (4.03 vs. 12.98 vs. 13.67 months, HR 3.6, 95% CI 1.9–6.8; $p < 0.0001$) and OS (11.2 months vs. not reached, HR 9.5, 95% CI 3.7–24; $p < 0.0001$) (Figure 2). Additionally, focusing on PSA change at 10-12 weeks, we observed that patients who did not reach a 50% decrease in serum PSA level had a shorter PFS (6 vs. 15.3 months, HR 2.2, 95% CI 1.3–3.7; $p = 0.002$) (Supplementary Figure 5).

Multivariable Cox proportional-hazards regression using significant baseline variables plus CTC dynamics and $\geq 50\%$ PSA decline at 10-12 weeks showed that prior chemotherapy exposure (HR 4.1, 95% CI 1.9–8.9; $p = 0.0003$), high baseline CTC counts (HR 1.5, 95% CI 1.2–1.9; $p = 0.002$) and having increasing CTCs during therapy (HR 3.3, 95% CI 1.4–7.6; $p = 0.005$) were independently associated with a shorter PFS (Table 3). Prior chemotherapy (HR 7, 95% CI 1.9–25; $p = 0.003$), high baseline CTC count (HR 2.2, 95% CI 1.4–3.7; $p = 0.002$) and increasing CTCs during therapy (HR 4.6, 95% CI 1.4–15; $p = 0.01$) were independent predictors for OS (Table 3).

The added prognostic value of CTC dynamics was also demonstrated by AUC/ROC analysis by comparing survival models using conventional blood chemistry variables and models including blood chemistry and CTC variables (i.e. baseline CTC count or baseline CTC count with CTC dynamics at 10-12 weeks). For PFS, at a 6-month (i.e. median follow-up time) survival endpoint, the AUC significantly increased for the survival model

using baseline CTC counts and CTC dynamics (De Long's test, $p < 0.05$) (Supplementary Figure 6a). For OS, at 11- (median), 17- (3rd quartile) and 40-month (complete follow-up time) survival endpoints, the AUC significantly increased for the survival models using baseline CTC counts and CTC dynamics compared to survival models using conventional blood chemistry (De Long's test, all $p < 0.05$) (Supplementary Figure 6d-f).

In an exploratory analysis we inferred the prognostic value of having no detectable CTCs (i.e. CTC0) at follow-up in patients with at least 10-12 weeks response ($n=102$). At 10-12 weeks 62/102 (60.8%) were CTC0, whereas 40/102 (39.2%) patients had detectable CTCs on therapy who demonstrated inferior PFS (5.2 months vs. 13.9 months, HR 2.7, 95% CI 1.6–4.4; $p < 0.0001$) and OS (12.8 months vs. not reached, HR 17, 95% CI 5–58; $p < 0.0001$) (Supplementary Figure 7).

Circulating tumour cells and PSA response

PSA measurements at baseline and 10-12 weeks follow-up were available for 143/174 (82.2%) patients. At 10-12 weeks, a PSA response of $\geq 30\%$ and $\geq 50\%$ was achieved in 74/143 (51.7%) and 57/143 (39.9%) patients, respectively (Supplementary Figure 8). PSA responses of $\geq 30\%$ and $\geq 50\%$ less frequently occurred in chemotherapy-pretreated patients (χ^2 test: $p < 0.05$). Additionally, PSA $\geq 50\%$ responses were less frequent in patients with ≥ 5 CTCs/7.5L at baseline (χ^2 test: $p < 0.05$). We observed significant differences in $\geq 30\%$ and $\geq 50\%$ PSA responses between patients with increasing, stable, or decreasing CTCs. Patients with increasing CTCs at 10-12 weeks less frequently

demonstrated a $\geq 30\%$ and $\geq 50\%$ PSA response, compared to patients with stable/decreasing CTC counts (χ^2 test: all $p < 0.01$) (Table 4).

Discussion

We collected liquid biopsies from 174 patients with mCRPC, starting a new therapy with abiraterone acetate or enzalutamide. By associating baseline CTC levels and changes in CTC levels at 10-12 weeks with survival, we recapitulate and present four key findings: 1) CTCs are prevalent in mCRPC with 118/170 (69.4%) patients being CTC-positive at initiation of a new line of endocrine therapy, with significantly increased CTC levels in chemotherapy pretreated patients. 2) At baseline, patients with ≥ 5 CTCs/7.5mL blood have higher serum PSA, LDH and AP levels. 3) Demonstrating a CTC increase during therapy provides independent clinical prognostication over PSA declines, for both PFS and OS. 4) Measuring CTC changes during treatment is a strong prognosticator, which is associated with PSA response and can demonstrate therapy effectiveness.

In 2004, The FDA cleared the CellSearch platform for semi-automated isolation and enumeration of CTCs, and confirmed the prognostic properties of CTC enumeration in patients with metastatic breast, prostate, and colorectal cancer ^{11,14,15}. Focusing on prostate cancer, Danila *et al.* demonstrated on a cohort of 120 patients with progressive clinical castrate metastatic disease how the baseline CTC count was strongly associated with OS, and modestly correlated to serum PSA levels ¹⁶. In 2008, de Bono *et al.* showed in 231 patients starting a new line of chemotherapy the independent prognostic value of the baseline and 2-5 week CTC level (i.e. $<$ or ≥ 5 CTCs per 7.5mL). At all times CTCs

were better prognosticators than PSA declines. This study resulted in the FDA clearance of the CellSearch platform for CTC enumeration in patients with mCRPC ¹¹. Similarly, in a cohort of 119 patients Olmos *et al.* associated pre- and posttherapy CTC counts with OS and demonstrated how baseline CTC levels and changes in CTC levels during therapy were strongly associated with prognosis ¹⁷. Goodman *et al.* enumerated CTCs from 100 mCRPC patients starting a new line of chemotherapy and demonstrated a positive correlation with serum PSA levels and independent prognostic value of LDH and CTCs ¹⁸. In a reanalysis of the IMMC38 trial data, encompassing CRPC patients starting first-line chemotherapy (n=156), Scher *et al.* reported that changes in CTC counts at 4, 8, and 12 weeks on therapy were strongly associated with OS, whereas PSA changes after 12 weeks only demonstrated modest association with prognosis ¹⁹. In a small postchemotherapy cohort of 48 men with CRPC, Danila *et al.* demonstrated that patients with ≥ 5 CTCs per 7.5mL after 4 weeks of abiraterone acetate had a worse OS compared to patients with < 5 CTCs ²⁰. In chemotherapy-pretreated CRPC patients receiving cabozantinib in the COMET-1 trial with evaluable CTC counts (n=103), Scher *et al.* reported how a CTC conversion from ≥ 5 to 4 or less per 7.5 mL of blood was associated with improved OS ^{21,22}. In 55 taxane-naïve and refractory patients with metastatic CRPC Thalgott *et al.* reported a positive correlation between CTC counts and serum LDH and AP levels, and how unfavourable baseline CTC counts were associated with inferior OS ²³. In SWOG S0421, a phase III trial of docetaxel plus prednisone with or without atrasentan, Goldkorn *et al.* reported how baseline CTC levels in 263 patients with mCRPC were associated with PSA and AP levels and how ≥ 5 CTCs per 7.5mL blood were associated with shorter OS. Additionally, decreasing CTC counts were associated with

PSA and RECIST response and longer OS ²⁴. In 57 docetaxel-treated patients with mCRPC, Okegawa *et al.* reported how ≥ 5 CTCs per 7.5mL at baseline and conversion towards ≥ 5 after three cycles of therapy predicted for a worse OS ²⁵. In 258 post-chemotherapy patients with mCRPC recruited during the phase III AFFIRM trial and treated with enzalutamide, Fleisher *et al.* reported how conversions from ≥ 5 to < 5 CTCs per 7.5mL were observed in 48% of the patients and were associated with an OS benefit ²⁶. Upon CTC enumeration in 711 patients from the COU-AA-301 trial (abiraterone acetate plus prednisone versus prednisone alone in docetaxel-pretreated patients with mCRPC), Scher *et al.* demonstrated how baseline CTC counts in combination with serum LDH levels enables patient stratification in low (CTC < 5), intermediate (CTC ≥ 5 and LDH ≤ 250 U/L) and high risk (CTC ≥ 5 and LDH > 250 U/L) groups, and that CTC conversion after 12 weeks was the strongest prognostic variable for OS ¹². In 33 docetaxel-treated patients with mCRPC Thalgott *et al.* reported how CTCs were strongly associated with OS at baseline and at all time points during therapy. Additionally, CTC conversions were superior to predict OS, compared to radiology and PSA evaluations ²⁷. In 89 men with mCRPC starting a new line of systemic therapy, Bitting *et al.* demonstrated how a baseline CTC count of ≥ 5 CTCs per 7.5mL was associated with shorter OS, and that CTC counts modestly correlated with serum LDH and AP levels ²⁸. Recently, Vogelzang *et al.* reported how ≥ 5 CTCs/7.5mL at baseline was associated with shorter OS in 208 mCRPC patients treated with docetaxel and prednisone with or without lenalidomide. Increasing CTCs after three cycles associated with shorter OS, whereas CTC declines were associated with better prognosis ²⁹.

As described above, improvement of prognosis based on early CTC changes has been reported in previous studies, however data in the endocrine therapy setting is limited. In 2016, Lorente *et al.* revisited the COU-AA-301 (abiraterone acetate after chemotherapy) and IMMC-38 (chemotherapy) trials, and could demonstrate how a $\geq 30\%$ CTC decrease at 4, 8, and 12 weeks was associated with increased survival, both in uni- and multivariable analysis ³⁰. In our study, we also observed how CTC changes at 10-12 weeks are strongly and independently associated with survival, following abiraterone acetate or enzalutamide. However, our “CTC stable” patient population encompasses patients with primarily 0 or 1 CTC, who were excluded for analysis by Lorente *et al.*. Nonetheless, we provide confirmatory evidence how decreasing CTC counts (which, except for 2 patients, was at least 30%) during therapy remains an important and independent biomarker of survival benefit in the context of endocrine therapy. A limitation of our study is that the CTC follow-up blood draw was fixed at 10-12 weeks, whereas Lorente *et al.* reported that a follow-up CTC evaluation at 4 weeks can already demonstrate therapy benefit. A second limitation of this study is the unavailability of a follow-up sample for all patients. It is important to note that 36/174 (20.6%) patients progressed before reaching the 10-12 week sampling point, which emphasizes the necessity of including earlier sampling timepoints for future studies. Nonetheless, the presented prospective study, provides essential CTC data specifically in the context of abiraterone acetate and enzalutamide, as used in current clinical practice. Importantly we demonstrate how CTC dynamics at 10-12 weeks provides independent clinical prognostication over PSA declines, and how PSA responses (both $\geq 30\%$ and $\geq 50\%$) are less frequent in patients with increasing CTCs at 10-12 weeks.

Most recently Heller et al. demonstrated the superior discriminatory power of having no detectable CTC at week 13 (i.e. CTC0) for OS in a retrospective analysis of 5 randomized phase 3 registration trials, encompassing a total of 6081 patients ³¹. The CTC0 marker outperformed other measures of CTC or PSA reduction. We performed an exploratory analysis and provide supportive evidence that absence of CTCs at 10-12 weeks on therapy has a high discriminatory power for PFS and OS in patients treated with abiraterone acetate or enzalutamide. However, in contrast to Heller *et al.* the CTC0 measure in this study was defined as having no CTCs at week 10-12, instead of achieving CTC0 at week 13 after a nonzero baseline CTC count. Nonetheless, we demonstrate how CTC enumeration and measurement of CTC dynamics as such can provide independent clinical prognostication. The latter and recently proposed CTC0 endpoint emphasize the clinical utility the CTC biomarker could have in oncology, which now requires further research and implementation in mCRPC clinical trials ³².

All previous and the presented study target traditional EpCAM-enriched CTCs, defined as nucleated cells, positive for cytokeratin and lacking CD45. Even though molecular heterogeneity may exist in this CTC population ³³, it is becoming clear that e.g. EpCAM-negative, low-expressing or cytokeratin-negative CTC subpopulations may coexist next to traditional CTCs ³⁴, which remain undetected by conventional methodologies. Recently, marker-independent techniques such as the Epic Science Platform revealed the existence of non-traditional CTCs (e.g. small or cytokeratin-negative CTCs) in 98% of patients with mCRPC ^{35,36}. Hence, future studies incorporating the enumeration of both traditional and non-traditional CTCs are warranted.

Conclusions

CTC enumeration at initiation of abiraterone acetate or enzalutamide is an important prognostic biomarker. However, measuring the change in CTC counts during treatment follow-up revealed the pharmacodynamic properties of CTCs, which may guide the treating physicians in their patient management. Larger prospective phase 3 trials monitoring CTC dynamics are warranted.

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Figures and legends

Fig. 1 – Kaplan–Meier estimates of probabilities of progression-free (A) and overall survival (B) in patients with mCRPC (n = 170). Patients were stratified according to baseline CTC level (<5 or ≥ 5 CTCs/7.5mL). CTC denotes circulating tumour cell. HR denotes hazard ratio. CI denotes confidence interval. mCRPC denotes metastatic castration-resistant prostate cancer.

Fig. 2 – Kaplan–Meier estimates of probabilities of progression-free (A) and overall survival (B) in patients with mCRPC (n = 102). Patients were stratified according to CTC change (stable, increasing, or decreasing) at 10-12 weeks. CTC denotes circulating tumour cell. HR denotes hazard ratio. CI denotes confidence interval. mCRPC denotes metastatic castration-resistant prostate cancer.

Supplementary Fig. 1 – CTCs and baseline blood chemistry in patients with mCRPC. A) Level of baseline CTCs in patients with (CT pretreated) and without (CT naive) previous treatment with chemotherapy. B) Baseline serum PSA (left), LDH (mid), and AP (right) levels in patients with <5 or ≥ 5 CTCs/7.5mL. CTC denotes circulating tumour cell. PSA denotes prostate-specific antigen. LDH denotes lactate dehydrogenase. AP denotes alkaline phosphatase. mCRPC denotes metastatic castration-resistant prostate cancer.

Supplementary Fig. 2 – Baseline blood chemistry in patients with <5 or ≥5 CTCs/7.5mL, stratified by previous chemotherapy exposure. Left: Level of serum PSA. Mid: Level of serum LDH. Right: Level of serum AP. *** denotes $p = 0.0006$ (Mann–Whitney U test). CTC denotes circulating tumour cell. PSA denotes prostate-specific antigen. LDH denotes Lactate dehydrogenase. AP denotes alkaline phosphatase.

Supplementary Fig. 3 – Kaplan–Meier estimates of probabilities of progression-free (upper) and overall (lower) survival in patients with mCRPC, based on baseline (A&C) and follow-up (B&D) CTC evaluation (n=102). CTC denotes circulating tumour cell. HR denotes hazard ratio. CI denotes confidence interval. mCRPC denotes metastatic castration-resistant prostate cancer.

Supplementary Fig. 4 – CTC response at therapy follow-up in mCRPC patients with baseline and follow-up CTC enumeration. A) Absolute change in number of CTCs between baseline and follow-up in patients with mCRPC, stratified in groups demonstrating decreasing (green, n=44), increasing (red, n=19) CTC levels or remaining stable (blue, n = 39) at 0 or 1 CTC/7.5mL. B) Percentage change in number of CTC. Dashed and full lines represent 30% and 50% increases and decreases. * patients with CTC increases > 200%. CTC denotes circulating tumour cell. FU denotes follow-up. mCRPC denotes metastatic castration-resistant prostate cancer.

Supplementary Fig. 5 – Kaplan–Meier estimates of probabilities of progression-free survival in patients with mCRPC. Patients were stratified by having $\geq 50\%$ PSA decrease or not at 10-12 weeks on therapy.

Supplementary Fig. 6 – Receiver operating characteristic (ROC) curves for the PFS (A-C) and OS (D-F) survival models using conventional blood chemistry variables (blue), blood chemistry variables with baseline CTC count (green) and blood chemistry variables with baseline CTC count and dynamics at 10-12 weeks (orange). Models have been generated for median, 3rd quartile and complete follow-up survival endpoints. CTC denotes circulating tumour cell. AUC denotes area under the ROC curve. * De Long's rest test. – denotes the reference model when comparing ROC differences.

A)

B)

Supplementary Fig. 7 – Kaplan–Meier estimates of probabilities of progression-free (A) and overall (B) survival in patients with mCRPC. Patients were stratified by having no detectable CTC at week 10-12 (CTC0) or being CTC positive (CTC+)

Supplementary Fig. 8 – Waterfall plots of PSA responses after 10–12 weeks on abiraterone acetate or enzalutamide. Patients were stratified by A) Increasing, decreasing, or stable CTC dynamics, B) Increasing or decreasing/stable CTC dynamics, C) Prior chemotherapy exposure or D) Baseline CTC level. Dashed and full lines represent 30% and 50% increasing and decreases. * denotes patient with PSA increase > 150%. CT denotes chemotherapy. PSA denotes prostate-specific antigen.

Tables and legends

Table 1 – Patient characteristics and baseline blood chemistry. * Baseline CTC enumeration failed for 4 patient samples. ** *p* values are from Mann–Whitney U test (continuous), and χ^2 test (categorical). PSA denotes prostate-specific antigen. LDH denotes lactate dehydrogenase. AP denotes alkaline phosphatase. Hb denotes haemoglobin. RBC denotes red blood cells. WBC denotes white blood cells. NEU denotes neutrophils. LYM denotes lymphocytes. MON denotes monocytes. TROM denotes thrombocytes. CTC denotes circulating tumour cells. IQR denotes interquartile range.

	Number of Circulating Tumor Cells at baseline *						
	All Patients		< 5		≥ 5		<i>p</i> **
	n	%	n	%	n	%	
Patients	174	100%	101	59,41%	69	40,59%	
Age at registration, yr, mean ± SD	74±7.6		75±8		73±7.4		0.049
Tumor stage at diagnosis							
T1/2	45	25,86%	30	17,65%	14	8,24%	0.04 *
T3/4	42	24,14%	21	12,35%	21	12,35%	
M1	49	28,16%	20	11,76%	26	15,29%	
node-positive	13	7,47%	10	5,88%	3	1,76%	
Not specified	25	14,37%	20	11,76%	5	2,94%	
Gleason score at diagnosis							
≤ 7	71	40,80%	40	23,53%	31	18,24%	0.9
8 - 10	78	44,83%	43	25,29%	31	18,24%	
Not specified	25	14,37%	18	10,59%	7	4,12%	
Primary treatment							
ADT	72	41,36%	41	24,12%	28	16,47%	0.3
Radical Px	58	33,33%	34	20,00%	23	13,53%	
RT+ADT	15	8,62%	6	3,53%	9	5,29%	
RT	9	5,17%	8	4,71%	1	0,59%	
TURP/HIFU	6	3,45%	3	1,76%	3	1,76%	
Radical Px + ADT	6	3,45%	4	2,35%	2	1,18%	
Radical Px + RT	4	2,30%	3	1,76%	1	0,59%	
CT	1	0,57%	1	0,59%	0	0,00%	
watchful waiting	1	0,57%	1	0,59%	0	0,00%	
Not specified	2	1,15%	0	0,00%	2	1,18%	
Previous Chemotherapy							
Chemotherapy naïve	109	62,64%	72	42,35%	34	20,00%	0.006 **
Chemotherapy pretreated	65	37,36%	29	17,06%	35	20,59%	
Previous ARS inhibitor for CRPC							
no	152	87,36%	90	52,94%	58	34,12%	0.5
yes	22	16,18%	11	6,47%	11	6,47%	
Initiating Therapy							
Abiraterone Acetate	124	71,26%	76	44,71%	44	25,88%	0.1
Enzalutamide	50	28,74%	25	14,71%	25	14,71%	
Metastatic burden at start Therapy							
LN only	25	14,37%	22	12,94%	3	1,76%	0.001 **
Bone only	66	37,93%	40	23,53%	25	14,71%	
Bone and LN	46	26,44%	24	14,12%	19	11,18%	
Visceral and bone and/or LN	31	17,82%	11	6,47%	20	11,76%	
none	4	2,30%	4	2,35%	0	0,00%	
Not specified	2	1,15%	0	0,00%	2	1,18%	
Baseline blood chemistry ***	median	IQR	median	IQR	median	IQR	
LDH, U/L, n=133	335	212 - 632	228	180.5 - 370.5	597.5	330.5 - 789	< 0.001 ***
AP, U/L, n=137	99	72 - 157	80	61.5 - 102.2	153	107 - 243	< 0.001 ***
PSA, µg/L, n=132	28.34	11.62 - 93.41	14.72	5.84 - 35.85	79.28	29.65 - 347	< 0.001 ***
Baseline hematology ***	median	IQR	median	IQR	median	IQR	
Hb, g/dL, n=142	12.4	11.12 - 13.5	13	11.85 - 13.85	11.5	10.1 - 13	< 0.001 ***
RBC, 10e6/mm3, n=135	4.09	3.78 - 4.38	4.23	4 - 4.47	3.86	3.27 - 4.27	< 0.001 ***
WBC, 10e3/mm3, n=142	6.8	5.4 - 9	6.8	5.6 - 9	6.71	5.25 - 8.88	0.899
NEU, 10e3/mm3, n=127	4.72	3.67 - 6.75	4.3	3.6 - 6.5	5.24	3.73 - 7.21	0.232
LYM, 10e3/mm3, n=123	1.2	0.865 - 1.59	1.2	0.9 - 1.59	1.1	0.66 - 1.47	0.073
MON, 10e3/mm3, n=124	0.8	0.458 - 0.793	0.8	0.48 - 0.77	0.59	0.47 - 0.87	0.954
TROM, 10e3/mm3, n=139	220	179 - 285.5	218	180 - 253	227	173.8 - 287	0.577

Table 2 – Uni- and multivariable Cox regression analysis of progression-free (A) and overall (B) survival using baseline characteristics. PSA denotes prostate-specific antigen. LDH denotes lactate dehydrogenase. AP denotes alkaline phosphatase. HR denotes hazard ratio. CI denotes confidence interval. PFS denotes progression-free survival. OS denotes overall survival. * Variables were log-transformed. ‡ *p* value from logrank test. ¶ *p* value from Wald test of Z statistic.

A		PFS				
Variable	Categories	Univariable		Multivariable		
		Kaplan-Meier	Cox Proportional Hazard	Cox Proportional Hazard		
		<i>p</i> [‡]	HR (95% CI)	<i>p</i> [¶]	HR (95% CI)	<i>p</i> [¶]
Age	Continuous	NA	1.013 (0.992 - 1.035)	0.24	-	-
Stage at diagnosis	T1,2 / T3,4 / N+ vs M1	0.60	1.182 (0.745 - 1.875)	0.48	-	-
Gleason at diagnosis	≤ 7 vs 8-10	0.35	1.19 (0.824 - 1.719)	0.36	-	-
Chemotherapy status	naïve vs pretreated	< 0.0001	2.616 (1.825 - 3.751)	< 0.0001	2.173 (1.311 - 3.602)	0.003 **
Prior ARS inhibitor for CRPC	no vs yes	0.014	1.779 (1.117 - 2.834)	0.02	0.979 (0.515 - 1.863)	0.95
Metastases at start abiraterone/enzalutamide	non-visceral vs visceral	< 0.0001	2.374 (1.547 - 3.645)	< 0.0001	1.298 (0.776 - 2.171)	0.32
Baseline CTC level	< 5 vs ≥ 5	< 0.0001	2.475 (1.724 - 3.670)	< 0.0001	0.788 (0.357 - 1.742)	0.56
Baseline CTC (#/7.5 mL)	Continuous *	NA	1.507 (1.347 - 1.687)	< 0.0001	1.457 (1.150 - 1.844)	0.002 **
Baseline PSA (ng/mL)	Continuous *	NA	1.310 (1.175 - 1.460)	< 0.0001	1.113 (0.952 - 1.302)	0.18
Baseline LDH (U/L)	Continuous *	NA	1.972 (1.432 - 2.714)	< 0.0001	0.786 (0.496 - 1.247)	0.31
Baseline AP (U/L)	Continuous *	NA	1.957 (1.458 - 2.626)	< 0.0001	1.112 (0.743 - 1.664)	0.61
* Variables were log-transformed						
‡ <i>p</i> value from logrank test						
¶ <i>p</i> value from Wald test of Z statistic.						
B		OS				
Variable	Categories	Univariable		Multivariable		
		Kaplan-Meier	Cox Proportional Hazard	Cox Proportional Hazard		
		<i>p</i> [‡]	HR (95% CI)	<i>p</i> [¶]	HR (95% CI)	<i>p</i> [¶]
Age	Continuous	NA	1.007 (0.977 - 1.039)	0.64	-	-
Stage at diagnosis	T1,2 / T3,4 / N+ vs M1	0.08	1.564 (0.935 - 2.616)	0.09	-	-
Gleason at diagnosis	≤ 7 vs 8-10	0.74	1.089 (0.658 - 1.803)	0.74	-	-
Chemotherapy status	naïve / pretreated	< 0.0001	3.319 (1.981 - 5.56)	< 0.0001	2.181 (1.182 - 4.023)	0.01 *
Prior ARS inhibitor for CRPC	no vs yes	0.029	1.915 (1.054 - 3.477)	0.03	1.159 (0.544 - 2.469)	0.70
Metastases at start abiraterone/enzalutamide	non-visceral vs visceral	0.002	2.247 (1.32 - 3.824)	0.003	1.340 (0.738 - 2.434)	0.34
Baseline CTC level	< 5 vs ≥ 5	< 0.0001	4.751 (2.763 - 8.268)	< 0.0001	1.208 (0.425 - 3.437)	0.72
Baseline CTC (#/7.5 mL)	Continuous *	NA	1.758 (1.526 - 2.025)	< 0.0001	1.428 (1.052 - 1.939)	0.02 *
Baseline PSA (ng/mL)	Continuous *	NA	1.416 (1.211 - 1.656)	< 0.0001	0.966 (0.781 - 1.194)	0.75
Baseline LDH (U/L)	Continuous *	NA	2.619 (1.755 - 3.908)	< 0.0001	1.269 (0.727 - 2.217)	0.40
Baseline AP (U/L)	Continuous *	NA	2.378 (1.671 - 3.382)	< 0.0001	1.042 (0.619 - 1.755)	0.88

Table 3 – Uni- and multivariable Cox regression analysis of progression-free (A) and overall (B) survival using baseline characteristics including PSA change plus CTC dynamics at 10-12 weeks. PSA denotes prostate-specific antigen. LDH denotes lactate dehydrogenase. AP denotes alkaline phosphatase. HR denotes hazard ratio. CI denotes confidence interval. PFS denotes progression-free survival. OS denotes overall survival. * Variables were log-transformed. ‡ *p* value from logrank test. ¶ *p* value from Wald test of Z statistic.

A						
PFS						
Variable	Categories	Univariable			Multivariable	
		Kaplan-Meier	Cox Proportional Hazard		Cox Proportional Hazard	
		<i>p</i> [‡]	HR (95% CI)	<i>p</i> [‡]	HR (95% CI)	<i>p</i> [¶]
Age	Continuous	NA	1.011 (0.983 - 1.039)	0.45	-	-
Stage at diagnosis	T1,2 / T3,4 / N+ vs M1	0.58	1.160 (0.722 - 1.866)	0.54	-	-
Gleason at diagnosis	≤ 7 vs 8-10	0.51	0.843 (0.504 - 1.41)	0.52	-	-
Chemotherapy status	naïve vs pretreated	< 0.0001	3.479 (2.117 - 5.719)	< 0.0001	4.146 (1.923 - 8.942)	0.0003 ***
Prior ARS inhibitor for CRPC	no vs yes	0.001	2.706 (1.429 - 5.123)	0.002	0.869 (0.308 - 2.454)	0.79
Metastases at start abi/enza	non-visceral vs visceral	0.01	2.294 (1.163 - 4.527)	0.02	1.184 (0.436 - 3.191)	0.74
Baseline CTC (#/7.5 mL)	Continuous *	NA	1.463 (1.215 - 1.761)	< 0.0001	1.499 (1.154 - 1.947)	0.002 **
CTC dynamics (10-12w)	stable/decreasing vs increasing	< 0.0001	3.567 (1.886 - 6.773)	< 0.0001	3.317 (1.440 - 7.640)	0.005 **
Baseline PSA (ng/mL)	Continuous *	NA	1.342 (1.125 - 1.602)	0.001	1.180 (0.926 - 1.505)	0.18
PSA change (10-12w)	≥50% vs no PSA response	0.002	2.232 (1.333 - 3.739)	0.002	1.261 (0.584 - 2.721)	0.55
Baseline LDH (U/L)	Continuous *	NA	2.14 (1.314 - 3.487)	0.002	0.664 (0.331 - 1.334)	0.25
Baseline AP (U/L)	Continuous *	NA	2.068 (1.329 - 3.216)	0.001	1.048 (0.551 - 1.994)	0.89
* Variables were log-transformed						
‡ <i>p</i> value from logrank test						
¶ <i>p</i> value from Wald test of Z statistic.						
B						
OS						
Variable	Categories	Univariable			Multivariable	
		Kaplan-Meier	Cox Proportional Hazard		Cox Proportional Hazard	
		<i>p</i> [‡]	HR (95% CI)	<i>p</i> [‡]	HR (95% CI)	<i>p</i> [¶]
Age	Continuous	NA	1.006 (0.959 - 1.055)	0.81	-	-
Stage at diagnosis	T1,2 / T3,4 / N+ vs M1	0.48	1.365 (0.572 - 3.257)	0.48	-	-
Gleason at diagnosis	≤ 7 vs 8-10	0.20	0.575 (0.243 - 1.36)	0.21	-	-
Chemotherapy status	naïve vs pretreated	< 0.0001	5.746 (2.37 - 13.93)	0.0001	6.999 (1.935 - 25.322)	0.003 **
Prior ARS inhibitor for CRPC	no vs yes	0.001	3.836 (1.638 - 8.985)	0.002	1.457 (0.336 - 6.323)	0.61
Metastases at start abi/enza	non-visceral vs visceral	0.22	1.845 (0.682 - 4.993)	0.23	-	-
Baseline CTC (#/7.5 mL)	Continuous *	NA	2.051 (1.605 - 2.62)	< 0.0001	2.246 (1.351 - 3.733)	0.002 ***
CTC dynamics (10-12w)	stable/decreasing vs increasing	< 0.0001	9.463 (3.676 - 23.97)	< 0.0001	4.642 (1.429 - 15.068)	0.01 **
Baseline PSA (ng/mL)	Continuous *	NA	1.597 (1.168 - 2.184)	0.003	0.993 (0.583 - 1.693)	0.97
PSA change (10-12w)	≥50% vs no PSA response	0.03	2.321 (0.91 - 5.939)	0.06	0.333 (0.089 - 1.253)	0.10
Baseline LDH (U/L)	Continuous *	NA	3.298 (1.761 - 6.175)	0.0002	0.637 (0.216 - 1.875)	0.41
Baseline AP (U/L)	Continuous *	NA	3.904 (2.124 - 7.174)	< 0.0001	2.809 (0.829 - 9.523)	0.09

Table 4 – Clinical variables and CTC prognostic biomarkers versus $\geq 30\%$ (A) and $\geq 50\%$ (B) prostate-specific antigen (PSA) responses after 10-12 weeks of treatment in patients with mCRPC and PSA monitoring (n=143). CTC denotes circulating tumour cell. PSA denotes prostate-specific antigen. mCRPC denotes metastatic castration-resistant prostate cancer.

A)

PSA response	All Patients (n=143)	Chemotherapy		CTC level at baseline		CTC dynamics			CTC dynamics (alternative)	
	n (%)	Naive Pretreated		< 5	≥ 5	Increasing	Stable	Decreasing	Increasing	Stable/Decreasing
$\geq 30\%$	74 (51.7)	52	22	50	23	3	24	25	3	49
No	69 (48.3)	35	34	42	25	16	12	16	16	28
χ^2 test:		$p = 0.026$		$p = 0.59$		$p = 0.0008$			$p = 0.0005$	

B)

PSA response	All Patients (n=143)	Chemotherapy		CTC level at baseline		CTC dynamics			CTC dynamics (alternative)	
	n	Naive Pretreated		< 5	≥ 5	Increasing	Stable	Decreasing	Increasing	Stable/Decreasing
$\geq 50\%$	57 (39.9)	42	15	43	13	2	19	20	2	39
No	86 (60.1)	45	41	49	35	17	17	21	17	38
χ^2 test:		$p = 0.017$		$p = 0.038$		$p = 0.006$			$p = 0.004$	

Supplementary Table 1 – Overview of baseline characteristics from patients with (n=102) and without (n=72) follow-up sampling.

	with follow-up sampling		without follow-up sampling	
	n	%	n	%
Patients	102	100,00%	72	100,00%
Age at registration, yr, mean ± SD	74±8.6		74±6.9	
Tumor stage at diagnosis				
T1/2	28	27,45%	17	23,61%
T3/4	23	22,55%	20	27,78%
M1	26	25,49%	24	33,33%
node-positive	9	8,82%	4	5,56%
Not specified	16	15,69%	7	9,72%
Gleason score at diagnosis				
≤ 7	44	43,14%	28	38,89%
8 - 10	42	41,18%	37	51,39%
Not specified	16	15,69%	7	9,72%
Primary treatment				
ADT	44	43,14%	28	38,89%
Radical Px	35	34,31%	24	33,33%
RT+ADT	7	6,86%	9	12,50%
RT	7	6,86%	2	2,78%
TURP/HIFU	2	1,96%	4	5,56%
Radical Px + ADT	3	2,94%	3	4,17%
Radical Px + RT	2	1,96%	2	2,78%
CT	1	0,98%	0	0,00%
watchful waiting	1	0,98%	0	0,00%
Previous Chemotherapy				
Chemotherapy naïve	71	69,61%	38	52,78%
Chemotherapy pretreated	31	30,39%	34	47,22%
Previous ARS inhibitor for CRPC				
no	89	87,25%	62	86,11%
yes	13	12,75%	10	13,89%
Initiating Therapy				
Abiraterone Acetate	75	73,53%	49	68,06%
Enzalutamide	27	26,47%	23	31,94%
Metastatic burden at start Therapy				
LN only	17	16,67%	8	11,11%
Bone only	43	42,16%	23	31,94%
Bone and LN	26	25,49%	22	30,56%
Visceral and bone and/or LN	13	12,75%	18	25,00%
none	3	2,94%	1	1,39%
Baseline blood chemistry	median	IQR	median	IQR
LDH, U/L	242	198 - 505	502.5	306.2 - 689.8
AP, U/L	95	70 - 118	117	77.25 - 188.2
PSA, µg/L	21.98	9.45 - 62.75	53.4	13.04 - 215.60
Baseline hematology				
Hb, g/dL	13	11.7 - 13.7	11.6	10.7 - 13.2
RBC, 10e6/mm3	4.18	3.86 - 4.43	3.97	3.42 - 4.26
WBC, 10e3/mm3	6.8	5.7 - 9	6.7	5.3 - 9
NEU, 10e3/mm3	4.4	3.67 - 6.79	4.9	3.5 - 6.75
LYM, 10e3/mm3	1.4	0.95 - 1.9	1	0.8 - 1.3
MON, 10e3/mm3	0.6	0.46 - 0.78	0.59	0.45 - 0.79
TROM, 10e3/mm3	227	179.5 - 263	234.3	179 - 268.5
CTC, #/7.5mL	1	0 - 6	8	1 - 61