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EDITED BY

Yafeng Ma,
Ingham Institute of Applied Medical
Research, Australia

REVIEWED BY

Haitao Liu,
Shanghai General Hospital, China
Wytse Van Weerden,
Erasmus Medical Center, Netherlands

*CORRESPONDENCE

Yong-Jie Lu
✉ y.j.lu@qmul.ac.uk

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The potential of using circulating tumour cells and their gene expression to predict docetaxel response in metastatic prostate cancer

Caitlin R. Davies¹, Tianyu Guo^{1,2}, Edwina Burke¹,
Elzbieta Stankiewicz^{1,3}, Lei Xu^{1,4}, Xueying Mao¹,
Glenda Scandura¹, Prabhakar Rajan^{5,6,7,8}, Karen Tipples⁶,
Constantine Alifrangis^{8,9}, Akhila Ganeshi Wimalasingham⁹,
Myria Galazi⁹, Shanthini Crusz⁹, Thomas Powles^{6,10},
Alistair Grey^{6,7,8}, Tim Oliver¹, Sakunthala Kudahetti¹,
Greg Shaw^{6,7,8}, Daniel Berney¹, Jonathan Shamash⁹
and Yong-Jie Lu^{1*}

¹Centre for Cancer Biomarkers and Biotherapeutics, Barts Cancer Institute, Queen Mary University of London, London, United Kingdom, ²Department of Cell Biology and the Second Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou, China, ³Central Biobank, Medical University of Gdansk, Gdansk, Poland, ⁴Department of Urology, Zhongshan Hospital, Fudan University, Shanghai, China, ⁵Centre for Cancer Cell and Molecular Biology, Barts Cancer Institute, Queen Mary University of London, London, United Kingdom, ⁶Department of Urology, Barts Health National Health Service Trust (NHS), London, United Kingdom, ⁷Division of Surgery and Interventional Sciences, University College London, London, United Kingdom, ⁸University College London Hospitals, National Health Service (NHS) Foundation Trust, London, United Kingdom, ⁹Department of Medical Oncology, Barts Health National Health Service (NHS) Trust, London, United Kingdom, ¹⁰Centre for Experimental Cancer Medicine, Barts Cancer Institute, Queen Mary University of London, London, United Kingdom

Background: Docetaxel improves overall survival (OS) in castration-resistant prostate cancer (PCa) (CRPC) and metastatic hormone-sensitive PCa (mHSPC). However, not all patients respond due to inherent and/or acquired resistance. There remains an unmet clinical need for a robust predictive test to stratify patients for treatment. Liquid biopsy of circulating tumour cell (CTCs) is minimally invasive, can provide real-time information of the heterogeneous tumour and therefore may be a potentially ideal docetaxel response prediction biomarker.

Objective: In this study we investigate the potential of using CTCs and their gene expression to predict post-docetaxel tumour response, OS and progression free survival (PFS).

Methods: Peripheral blood was sampled from 18 mCRPC and 43 mHSPC patients, pre-docetaxel treatment, for CTC investigation. CTCs were isolated using the epitope independent Parsortix[®] system and gene expression was determined by multiplex RT-qPCR. We evaluated CTC measurements for post-

docetaxel outcome prediction using receiver operating characteristics and Kaplan Meier analysis.

Results: Detection of CTCs pre-docetaxel was associated with poor patient outcome post-docetaxel treatment. Combining total-CTC number with PSA and ALP predicted lack of partial response (PR) with an AUC of 0.90, $p=0.037$ in mCRPC. A significantly shorter median OS was seen in mCRPC patients with positive CTC-score (12.80 vs. 37.33 months, HR= 5.08, $p=0.0005$), ≥ 3 total-CTCs/7.5mL (12.80 vs. 37.33 months, HR= 3.84, $p=0.0053$), ≥ 1 epithelial-CTCs/7.5mL (14.30 vs. 37.33 months, HR= 3.89, $p=0.0041$) or epithelial to mesenchymal transitioning (EMTing)-CTCs/7.5mL (11.32 vs. 32.37 months, HR= 6.73, $p=0.0001$). Significantly shorter PFS was observed in patients with ≥ 2 epithelial-CTCs/7.5mL (7.52 vs. 18.83 months, HR= 3.93, $p=0.0058$). mHSPC patients with ≥ 5 CTCs/7.5mL had significantly shorter median OS (24.57 vs. undefined months, HR= 4.14, $p=0.0097$). In mHSPC patients, expression of *KLK2*, *KLK4*, *ADAMTS1*, *ZEB1* and *SNAI1* was significantly associated with shorter OS and/or PFS. Importantly, combining CTC measurements with clinical biomarkers increased sensitivity and specificity for prediction of patient outcome.

Conclusion: While it is clear that CTC numbers and gene expression were prognostic for PCa post-docetaxel treatment, and CTC subtype analysis may have additional value, their potential predictive value for docetaxel chemotherapy response needs to be further investigated in large patient cohorts.

KEYWORDS

prostate cancer, circulating tumour cells, docetaxel, response prediction, biomarker, liquid biopsy, prognosis

Introduction

Prostate cancer (PCa) is the most frequently diagnosed cancer in Western males, accounting for 24% of all new cancers in 2018 (1). The effective first-line treatment for metastatic disease is androgen deprivation therapy (ADT), although after an initial response, progression to castration-resistant PCa (CRPC) occurs within 1-3 years (2). Adding docetaxel to ADT improves overall survival (OS) in metastatic (m)CRPC (3) and since 2014 as a result of the CHAARTED (4) and STAMPEDE (5) phase III trials, docetaxel has been used in combination with ADT as a first-line treatment for metastatic hormone-sensitive PCa (mHSPC) (3, 6). However, response to docetaxel is not universal due to inherent and/or acquired resistance. While numerous studies have investigated the underlying mechanisms and pharmacogenomic biomarkers of docetaxel resistance (7–11), there remains an unmet clinical need for new surrogate markers and a robust predictive test to

stratify patients for treatment and develop personalised therapeutic approaches (12).

Tissue biomarkers representing cancer characteristics may help predict treatment outcome, but serial biopsies add morbidity and delay, and sampling of bone metastases is practically difficult. Furthermore, tissue biopsy fails to represent the entire cancer population due to intra-tumoural heterogeneity. In the case of therapy response prediction, markers that were detectable within the initial tissue biopsy sample are unlikely to truly represent the patient's disease due to continuous tumour evolution at the molecular level. This is particularly important when considering second line therapies and beyond. As an alternative to tissue biopsy, liquid biopsy refers to the analysis of tumour biomarkers such as circulating tumour cells (CTCs), circulating tumour DNA (ctDNA), microRNA (miRNA) and extracellular vesicles (EVs) in peripheral blood or other body fluids. Liquid biopsies are minimally invasive, easily repeatable and can provide real time information of the heterogeneous

tumour, providing a promising tool to overcome the limitations posed by tissue biopsy. Prostate-specific antigen (PSA) remains the standard serum biomarker for PCa diagnosis and progression, however with limited ability to predict therapeutic response (13).

CTCs are malignant cells that have gained an invasive phenotype, allowing them to shed from the tumour mass into the circulation where they travel to distant sites and form metastases (14). CTCs are unique amongst cancer biomarkers, as they provide a source of live tumour cells that carry molecular and biological information that may represent overall tumour burden and phenotypic characteristics present in both primary and metastatic sites. In addition to CTC enumeration, molecular profiling of enriched CTC populations or single CTCs provides a plethora of potentially clinically valuable markers of the metastatic process, disease status and predictors of patient individualised therapeutic response (15, 16). Therefore, CTC analysis may be a potentially ideal docetaxel response prediction biomarker.

Numerous studies have investigated various clinical applications of CTC enumeration and characterisation in PCa (17–22). Baseline CTCs have been shown to predict poor OS in patients with mCRPC (19, 23), which led to the FDA approval of CellSearch[®] detected CTCs for advanced PCa prognosis (24). The MAINSAIL phase III trial of mCRPC patients treated with docetaxel found a significant association between baseline ≥ 5 CTCs/7.5ml of peripheral blood and poor OS, but not PSA response or Response Evaluation Criteria in Solid Tumors (RECIST) (25–27). The recent PROPHECY prospective multicentre study in patients with mCRPC undergoing treatment with enzalutamide or abiraterone followed by taxane chemotherapy, focused on the detection of CellSearch[®] isolated CTCs expressing the androgen receptor splice variant, AR-V7. The study demonstrated that pre-treatment CTC AR-V7 status was independently associated with shorter progression free survival (PFS) and OS with abiraterone or enzalutamide, however men with AR-V7-positive disease still experienced clinical benefit from taxane chemotherapy (28). CTCs have also been investigated as predictive and prognostic biomarkers of clinical outcome, including mCRPC onset, in patients with mHSPC (17, 29–31), however to date there is limited information regarding their clinical utility in predicting docetaxel response in this patient cohort. Furthermore, the majority of studies to date have used epithelial epitope dependent isolation, missing a potentially important subpopulation of CTCs with epithelial negative phenotypes following epithelial-mesenchymal transition (EMT) during cancer cell invasion and metastatic spread. Previous research from ourselves and others has demonstrated that CTCs that are undergoing EMT, or those that have a fully mesenchymal phenotype have significant value as biomarkers of increased metastatic tumour burden (32), and disease progression (33, 34). Moreover, EMT is increasingly recognised as an important mechanism that drives inherent and acquired resistance to

chemotherapies (35), including docetaxel (36). As such, exclusion of CTCs with epithelial negative phenotypes limits the detection of genes which might be developed into novel predictive biomarkers of docetaxel response, which may facilitate patient personalised treatment stratification.

We previously used the cell size and deformability based Parsortix[®] CTC isolation system, which was recently FDA approved, to detect CTCs with epithelial, mesenchymal and intermediate phenotypes, and demonstrated their biomarker potential in different clinical scenarios (32, 37, 38). In this study we used Parsortix[®] to capture pre-docetaxel treatment CTCs, evaluating CTC subtypes and their gene expression as biomarkers of docetaxel response in order to identify CTC markers with clinical value for the management of advanced PCa patients. Our strategy combined epitope independent CTC isolation for enumeration and molecular characterisation using multiplex RT-qPCR for a targeted panel of genes. We demonstrate the potential of analysing multiple CTC subtypes and their gene expression as predictive and prognostic biomarkers in both mCRPC and mHSPC patients.

Methods

Patients

Between January 2015 and January 2020, 18 mCRPC and 43 mHSPC patients were recruited with informed consented at St Bartholomew's Hospital, Barts Health NHS, London, UK. Clinical characteristics for individual patients are shown in [Supplementary Table 1](#). Peripheral blood samples were collected into EDTA tubes ≤ 2 months before commencing 6 cycles of docetaxel. Patients with mHSPC had started initial hormone-therapy < 3 months before blood collection. Patients received CT and bone scans before and after treatment. Serum PSA, ALP, and LDH were measured together with CTC sampling. Radiological response assessment was based on RECIST criteria (25): (1) complete response (CR): disappearance of all target lesions; (2) partial response (PR): at least 30% decrease in the sum of the longest diameter of target lesions, taking as reference the baseline since treatment started; (3) progressive disease (PD): at least 20% increase in the sum of the longest diameter of target lesions, taking as reference the baseline since treatment started; (4) stable disease (SD): neither sufficient shrinkage to qualify for PR nor sufficient increase to qualify for PD. Assessments of response by bone scan were classified as follows: (1) CR: disappearance of all bone metastasis; (2) PR: a decrease in number, extent or intensity of bone lesions was detected; (3) PD: appearance of new bone lesion(s) and/or apparent enlargement of the bone metastases; (4) SD: little or no change in the number, extent or intensity of bone metastases was observed. PSA progression was defined as two consecutive rises above PSA nadir at least two weeks apart. CTC measurements

were also investigated for their ability to prognose OS and PFS outcomes.

Cell lines

The docetaxel-resistant human PCa cell line PC3-D12 and the sensitive counterpart PC3-Ag, were gifted by A.J. O'Neill (10), University College Dublin. PC3-D12 cells were treated every 4 weeks with 12 nM docetaxel in order to maintain resistance. The cells were maintained in RPMI-1640 medium supplemented with 10% foetal bovine serum and 2 nM L-glutamine (Invitrogen, Waltham, Massachusetts, Unites States).

CTC isolation, enumeration and characterisation

CTCs were isolated from 7.5 mL of whole blood using the Parsortix[®] (Angle Plc, Guildford, UK) isolation system and identified for CTC enumeration using four-colour immunofluorescence as previously described (32, 37). Briefly, 7 mL of blood was transferred to 50 mL LeucoSep tubes (Greiner Bio-One, Frickenhausen, Germany) with 15.3 mL of Ficoll-Paque Plus (GE Healthcare, Chicago, Illinois, Unites States) and centrifuged at 1000 g for 15 min with the break off at room temperature to recover the peripheral blood mononuclear cell (PBMC) fraction. The PBMC fraction along with the plasma above the fit of the LeucoSep tube was removed into a new 50 mL falcon tube and pelleted at 200 g for 8 min at room temperature. The pellet was then re-suspended in 4.5 mL of isolation buffer (PBS containing 1% BSA and 2 nM EDTA) and added back to the remaining 0.5 mL of whole blood and loaded onto the Parsortix[®] for CTC isolation. Once samples are loaded, cells are separated based on cell size and deformability according to a pre-set programme PX-S99F that uses 6.5 μm -gap cassette and 99 mbar pressure for isolation. Cells were then harvested using a pre-set programme and transferred onto glass slides for downstream analysis. All blood samples were processed within 4 hrs of collection. Slides were stained using mouse monoclonal PE-conjugated anti-CD45 (Miltenyi Biotec, Bergisch Gladbach, Germany), mouse monoclonal FITC-conjugated anti-Cytokeratin (Miltenyi Biotec, Bergisch Gladbach, Germany), Alexa Fluor 647-conjugated anti-Vimentin (Abcam, Cambridge, UK), and counterstained using SlowFade gold antifade mountant with DAPI (Life Technologies, Carlsbad, California, United States). CTCs were identified as Cytokeratin (CK)+/Vimentin (VIM)-/CD45- (epithelial-CTCs), CK+/VIM+/CD45- (EMTing-CTCs) and CK-/VIM+/CD45- (mesenchymal-CTCs). Patients with ≥ 1 epithelial-CTC and/or ≥ 1 EMTing-CTC and/or ≥ 4 mesenchymal-CTCs were defined as CTC-score 'positive' using our previously established definition based on the analysis of healthy control blood samples (32)

CTC RNA extraction and gene expression analysis

CTCs were isolated from a separate 7.5 mL of whole blood using the Parsortix[®] and collected into a 1.5 mL low-retention eppendorf. Total RNA was extracted using miRNeasy micro kit (Qiagen, Hilden, Germany) following manufacturer's instructions but eluted with a final volume of 11.5 μL . The total 11.5 μL of RNA extracted from CTCs was mixed with 0.5 μL of random primers and denatured at 65 °C for 5 min. After incubation for 5 min on ice, 4 μL of first strand buffer, 2 μL of 0.1 M DTT, 1 μL of 10 mM dNTPs (Roche, Basel, Switzerland), 0.5 μL of water and 0.5 μL of Superscript II (Thermo Fisher Scientific, Waltham, Massachusetts, Unites States) were added and cDNA synthesis was performed at 42 °C for 2 hrs, followed by enzyme inactivation by heating at 70 °C for 15 mins. Multiplex RT-qPCR was performed by Barts and the London Genome Centre using BioMark HD system (Fluidigm Corporation, South San Francisco, California, United States). 96.96 Dynamic Array Integrated fluidic circuit (IFC) was used to test expression levels of 32 assays in triplicates within one reaction plate. A list of TaqMan probes (Applied Biosystems, Massachusetts, Unites States) used are shown in [Supplementary Table 2](#). The brief workflows were as follows: (1) pooling the TaqMan assays. Combine equal volumes of each 20X TaqMan Gene Expression assays in a 0.5 mL microcentrifuge tube, up to 100 μL in total. Dilute the pooled assays using DNA Suspension Buffer (10mM Tris, pH 8.0, 0.1 mM EDTA) so that each assay is at a final concentration of 0.2X. (2) Combine 2.5 μL of TaqMan[®] PreAmp MasterMix (Life Technologies, Carlsbad, California, Unites States), 1.25 μL of pooled assay mix and 1.25 μL of cDNA to make the final sample mixture in each aliquot. (3) Place reaction tubes in the thermal cycler and cycle as (95 °C for 10 minutes followed by 14 cycles of 95 °C for 15 secs and then 60 °C for 4 mins). Only the targets of interests are amplified and this results in small amount of cDNA being amplified equally without introducing bias. Following pre-amplification, the samples were diluted 1:5 (v/v) in DNA suspension buffer. Reactions were then assayed using Dynamic Arrays prepared as instructed by the manufacturer. PCR was performed with 40 cycles of reactions.

Gene panel selection

Two microarray expression profile data sets (GSE36135 (39), GSE33455 (11)) were downloaded from the Gene Expression Omnibus (GEO) database (<http://www.ncbi.nlm.nih.gov/geo>), which are based on the GPL571 Affymetrix Human Genome U133A 2.0 Array [HG_U133A_2] and GPL570 Affymetrix Human Genome U133 Plus 2.0 Array [HG-U133_Plus_2], respectively. The original Series Matrix data files were analysed with GEO2R (using the GEOquery and limma R packages from Bioconductor (<http://www.bioconductor.org/>)) to identify

differentially expressed genes (DEGs) in each of the paired docetaxel-resistant and docetaxel-sensitive cell lines in the datasets. A DEG was considered to be significant according to the following criteria: Fold-change (FC) >2 and false discovery rate (FDR) <0.05. Genes that were not upregulated in ≥ 2 DOC resistant cell lines were excluded to control for random variance in gene expression. Additionally, we identified reported PCa-specific and/or docetaxel-resistance related genes by literature search, and the two gene lists were combined to form a test gene panel. To select for genes suitable for CTC analysis, the genes were searched in The Genotype-Tissue Expression Portal V7 database for their expression in prostate and whole blood. Genes were selected based on their relative high expression in the prostate and low/no expression in whole blood. Candidate gene expression was subsequently validated in a panel of PCa cell lines and PBMC samples from five PCa biopsy negative males.

Statistical analysis

Mann-Whitney U test was applied to assess differences in clinical characteristics between patient groups. Data were shown as median (interquartile range [IQR]). Spearman's rank correlation was used to assess associations of CTC counts and gene expression with concurrent PSA, ALP, LDH levels and OS/PFS. A combined risk score (CRS) was computed as the linear predictor of the fitted bivariate logistic model with PSA, ALP, CTC-score, total-CTC number and KLK2 count as only predictors (as $CRS = a * Y + b * X...$, where the values of 'a' and 'b' are the estimated log odds ratios). Survival curves were generated using the Kaplan-Meier method and compared using the logrank test. Bonferroni correction method was applied to adjust p values (p_{adj}) for multiple testing. RT-qPCR $2^{-\Delta\Delta Ct}$ was used to compare mRNA expression levels in patient samples. Receiver operating characteristic (ROC) curve analysis was used to evaluate prediction values. Follow-up time started on the date of administration of the first docetaxel dose. Observations were censored on the date of last follow-up.

Statistical analyses were performed using GraphPad Prism 9. All tests were two-sided with p values of <0.05 considered statistically significant.

Results

Patient characteristics and association of pre-docetaxel CTC measurements with clinicopathological data

Clinical characteristics and CTC counts are summarised in [Table 1](#). At least one CTC was detected in 12/18 (67%) of mCRPC patients, 50% of patients had a positive CTC-score (≥ 1

epithelial-CTC and/or ≥ 1 EMTing-CTC and/or ≥ 4 mesenchymal-CTCs) and the median total CTC count was 1.5 (interquartile range= 0-5.8). In mHSPC patients, ≥ 1 CTC was detected in 26/43 (60%), 51% of patients had a positive CTC-score and the median total CTC count was 1 (interquartile range = 0-4.3). [Figure 1A](#) shows an example of immunofluorescence staining for three CTC subtypes. [Figure 1B](#) shows individual CTC subtype counts in mHSPC and mCRPC patients. No significant differences were found between CTC subtype numbers.

We subsequently investigated the relationship between CTCs and clinicopathological data. Spearman's correlation was performed between CTCs and serum PSA and ALP, results are shown in [Table 2](#). In mCRPC patients, serum PSA was significantly correlated with total-CTC ($\rho = 0.51$, $p = 0.032$), epithelial-CTC ($\rho = 0.51$, $p = 0.030$), EMTing-CTC numbers ($\rho = 0.53$, $p = 0.024$) and positive CTC-score ($\rho = 0.68$, $p = 0.0021$). Serum ALP was significantly correlated with total-CTC ($\rho = 0.51$, $p = 0.046$) and epithelial-CTC numbers ($\rho = 0.62$, $p = 0.012$). In mHSPC patients, serum ALP was significantly correlated with mesenchymal-CTC numbers ($\rho = 0.34$, $p = 0.044$), however no other significant correlations were observed.

Correlation of pre-docetaxel CTCs with RECIST response post docetaxel treatment

To assess if CTCs could predict radiological response to docetaxel following treatment cycles, we compared CTC numbers between partial response (PR), stable disease (SD) or progressive disease (PD) groups ([Supplementary Table 1](#)), and performed ROC analysis. Due to the limited sample size, we combined patients who had SD or PD at the end of docetaxel treatment into one group. While there were no significant differences in CTC numbers between patients with PR and SD/PD in this small cohort, trends were observed. In mCRPC patients with PR, limited mesenchymal-CTCs and no epithelial- and EMTing-CTCs were detected. Total-CTCs trended towards a significantly lower number in patients with a PR ($p = 0.073$) compared to those with SD/PD ([Figure 2A](#)) with an AUC of 0.80, $p = 0.071$ in predicting SD/PD ([Figure 2C](#)). In comparison, serum PSA had an AUC of 0.78, $p = 0.089$, and ALP had an AUC of 0.74, $p = 0.20$ ([Figure 2C](#)). In order to improve our ability to predict radiological response to docetaxel using blood-based biomarkers, we generated a combined risk score (CRS) combining the total number of CTCs, serum PSA and ALP levels for SD/PD prediction as $CRS-TPA = 0.7414 * Total-CTC \text{ number} + 0.02909 * PSA + 0.01423 * ALP$, which resulted in an AUC of 0.90, $p = 0.037$, with a sensitivity of 84.62% and

TABLE 1 Summary of clinical characteristics and CTC enumeration for metastatic PCa patients.

	n	mCRPC	n	mHSPC
Age at pre-docetaxel, y				
Mean \pm SD	18	73 (66.5-75.8)	43	68 (63-73)
PSA at diagnosis, ng/mL				
Median (IQR)	14	21 (14.5-61.5)	39	54 (18.5-344.6)
Biopsy GS, n (%)				
7		8 (44)		6 (14)
>7		9 (50)		28 (65)
unknown		1 (6)		9 (21)
Pre-docetaxel PSA, ng/mL				
Median (IQR)	18	54.1 (12.9-111.8)	42	15.6 (3.6-50.1)
Pre-docetaxel ALP, U/L				
Median (IQR)	16	88 (74.8-387.5)	36	104 (70-328)
Pre-docetaxel CTC-score, n (%)				
Positive		9 (50)		22 (51)
Negative		9 (50)		21 (49)
Pre-docetaxel total CTC, n				
Median (IQR)	18	1.5 (0-5.8)	43	1 (0-4.3)
Pre-docetaxel Epithelial-CTC, n				
Median (IQR)	18	0 (0-2)	43	0 (0-1)
Pre-docetaxel EMTing-CTC, n				
Median (IQR)	18	0 (0-0)	43	0 (0-0)
Pre-docetaxel Mesenchymal-CTC, n				
Median (IQR)	18	1 (0-2.25)	43	0 (0-2)

mCRPC, metastatic castration-resistant prostate cancer; mHSPC, metastatic hormone-sensitive prostate cancer; IQR, interquartile range; PSA, prostate specific antigen; GS, Gleason score; SD, standard deviation.

specificity of 100% when the cut-off was set at >3.45 (Figure 2C). The CRS-TPA performed better than serum PSA and ALP alone, although the difference was not statistically significant ($p=0.66$, and 0.23 respectively), likely due to the limited sample size.

In mHSPC, epithelial- and EMTing-CTC numbers did not differentiate patients based on radiological response to docetaxel. Conversely, high mesenchymal-CTC numbers trended towards ($p=0.079$) higher chance of SD/PD (Figure 2B) with an AUC of 0.65 , $p=0.099$ (Figure 2D). Serum ALP levels were best able to predict SD/PD, with an AUC of 0.68 , $p=0.077$, compared to that of serum PSA which had an AUC of 0.52 , $p=0.82$ (Figure 2D). A CRS comprised of mesenchymal-CTC number and serum ALP levels as $\text{CRS-MA} = 0.3599 * \text{mesenchymal-CTC number} + 0.002037 * \text{ALP}$, increased the AUC to 0.69 , however with only a trend towards significance ($p=0.064$) (Figure 2D).

CTCs were significantly associated with PFS and OS in mCRPC and mHSPC patients

To assess the prognostic value of CTCs, we correlated CTC measurements with patient OS and PFS. Long term follow-up data was available for 18 mCRPC and 42 mHSPC patients. The median follow-up time for mCRPC patients was 22.7 months (range 8.0-53.1 months), during which time 13/18 (72%) patients progressed and/or died. Spearman's correlation (Table 3) showed that OS significantly inversely correlated with total- ($\rho=-0.66$, $p=0.0027$), epithelial- ($\rho=-0.62$, $p=0.0057$) and EMTing-CTC ($\rho=-0.65$, $p=0.0034$) numbers and a positive CTC-score ($\rho=-0.80$, $p<0.0001$) in the mCRPC cohort. Additionally, PFS was significantly inversely correlated

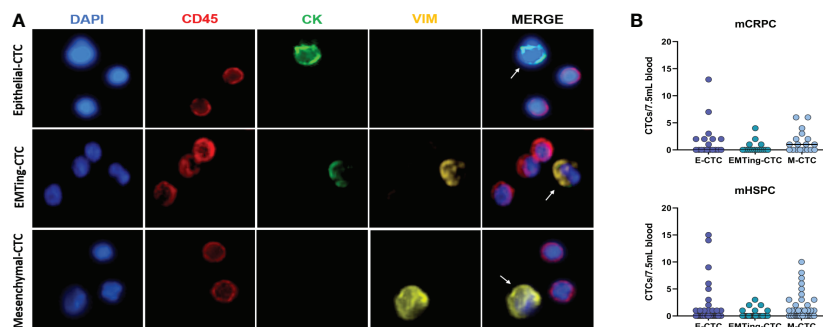


FIGURE 1

Detection of three subtypes of CTCs in PCa patient samples. (A) Three distinct CTC subtypes were identified by immunofluorescence in patient blood samples. Top: One CK+/VIM-/CD45- epithelial-CTC adjacent to two CD45+ leucocytes. Middle: One CK+/VIM+/CD45- EMting-CTC adjacent to three CD45+ leucocytes. Bottom: One CK-/VIM+/CD45- mesenchymal-CTC adjacent to two CD45+ leucocytes. (B) Individual CTC numbers in each mCRPC and mHSPC patient sample, respectively. Median CTCs number per 7.5mL of blood is shown. Abbreviations: CK, Cytokeratin; VIM, Vimentin.

with epithelial-CTC numbers ($\rho = -0.63$, $p = 0.0049$) and a positive CTC-score ($\rho = -0.65$, $p = 0.0033$). We also evaluated the performance of serum biomarkers PSA and ALP. We found that serum PSA levels significantly inversely correlated with OS ($\rho = -0.72$, $p = 0.0008$) and PFS ($\rho = -0.50$, $p = 0.035$). With a view to improve the sensitivity and specificity of pre-docetaxel

biomarkers for the prediction of OS, we generated a combined risk score using both PSA and CTC-score data. The AUC of a CRS comprised of PSA (AUC= 0.93) and CTC-score (AUC= 0.89) (CRS-PS= $0.08127 * PSA + 4.159 * CTC\text{-score}$) to discriminate mCRPC patients with <24 months OS from those with ≥ 24 months OS reached 0.96, $p = 0.0009$, with a sensitivity

TABLE 2 Spearman's correlation between CTCs and serum PSA and ALP.

	PSA	ALP
	Spearman's ρ (p-value)	
mCRPC patients		
Total-CTCs	0.51 (0.032)	0.51 (0.046)
Epithelial-CTCs	0.51 (0.030)	0.62 (0.012)
EMting-CTCs	0.53 (0.024)	0.38 (0.15)
Mesenchymal-CTCs	0.22 (0.37)	0.27 (0.31)
CTC-score	0.68 (0.0021)	0.45 (0.091)
PSA	-	-
ALP	0.35 (0.19)	-
mHSPC patients		
Total-CTCs	-0.017 (0.92)	0.27 (0.11)
Epithelial-CTCs	0.14 (0.37)	0.080 (0.65)
EMting-CTCs	0.066 (0.68)	-0.032 (0.86)
Mesenchymal-CTCs	-0.047 (0.77)	0.34 (0.044)
CTC-score	-0.006 (0.97)	0.21 (0.23)
PSA	-	-
ALP	0.35 (0.044)	-
bold black numbers, significant results		

of 88.9% and a specificity of 100% when the cut-off was set to <5.96 (Figure 3). This made an improvement on the AUC of PSA alone but without significance, $p=0.60$.

We then performed Kaplan Meier survival analysis using total and subtype CTC numbers to predict OS and PFS with optimal CTC number cut-offs evaluated (Table 4). In mCRPC patients, each total-CTC number cut-off that was explored (<2 vs. ≥ 2 to <6 vs. ≥ 6 CTCs) was significantly associated with patients with short median OS. The detection of a positive CTC-score (12.80 vs. 37.33 months, HR= 5.08, $p=0.0005$) (Figure 4A), ≥ 3 total-CTCs (12.80 vs. 37.33 months, HR= 3.84, $p=0.0053$) (Figure 4B), ≥ 1 epithelial-CTC (14.30 vs. 37.33 months, HR= 3.89, $p=0.0041$) (Figure 4C) and ≥ 1 EMTing-CTC (11.32 vs. 32.37 months, HR= 6.73, $p=0.0001$) (Figure 4D) were most significantly associated with shorter median OS. Importantly,

when the Bonferroni correction method was applied to adjust p values for multiple testing, a positive CTC-score ($p_{adj}=0.0055$), ≥ 1 epithelial-CTC ($p_{adj}=0.045$) and ≥ 1 EMTing-CTC ($p_{adj}=0.0011$) remained significantly associated with shorter median OS. The detection of ≥ 2 epithelial-CTCs was most significantly associated with shorter median PFS (7.52 vs. 18.83 months, HR= 3.93, $p=0.0058$) (Figure 4E).

The median follow-up time for mHSPC patients was 29.5 months (range 5.9-48.8 months), during which time 25/42 (60%) patients progressed and 11/42 (26%) died. Spearman's correlation did not show associations of CTC measurements with PFS and OS, however, ALP was significantly inversely correlated with OS ($\rho=-0.45$, $p=0.0068$) and PFS ($\rho=-0.62$, $p<0.0001$) (Table 3). Kaplan Meier analysis revealed that patients with ≥ 5 CTCs experienced the most significantly

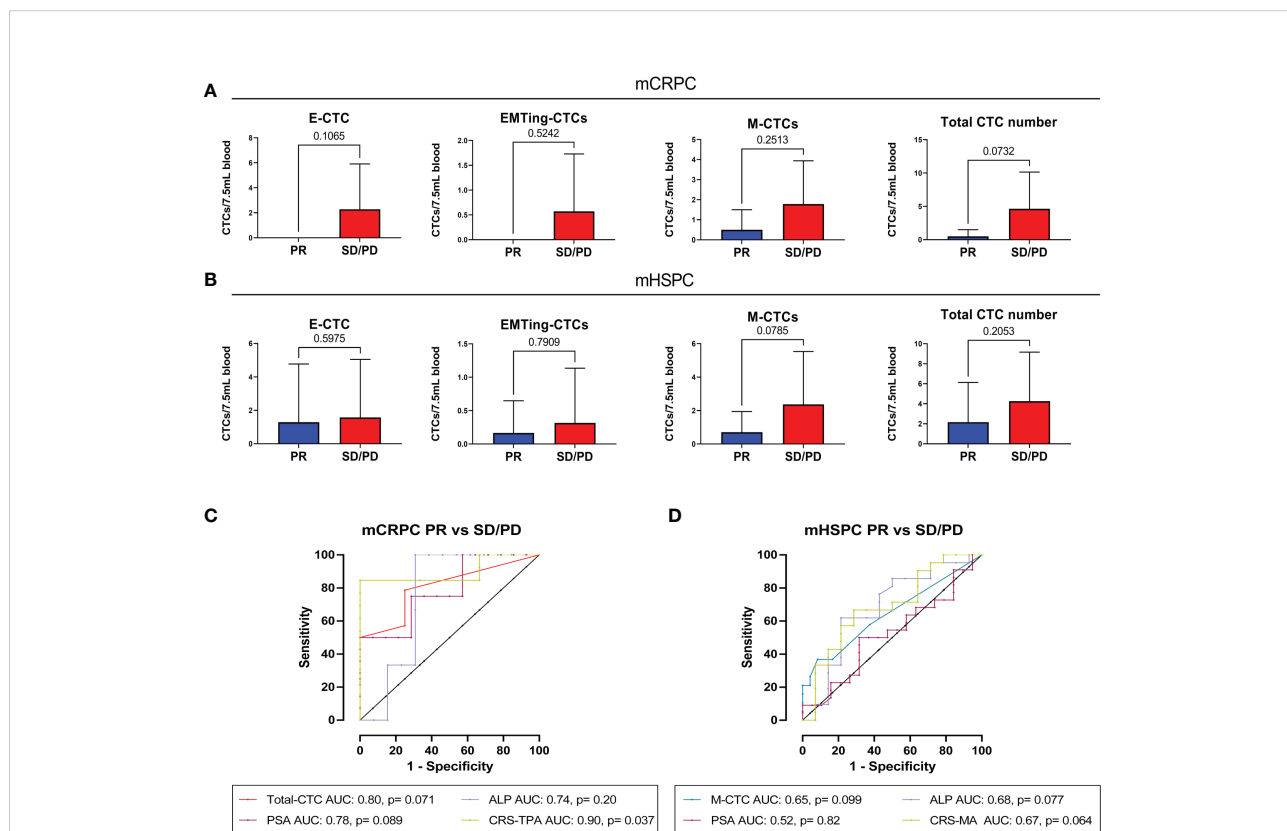


FIGURE 2

The relationship between pre-docetaxel CTCs and initial RECIST response. (A) Number of CTC subtypes and total-CTC numbers in mCRPC patients with PR or, SD and PD combined. Epithelial and EMTing-CTCs were not detected in patients with PR, although they did not significantly differentiate from patients with SD/PD, $p=0.1065$ and $p=0.7907$, respectively. Mesenchymal CTCs were detected in a small number of patients with PR but the majority were detected in patients with SD/PD ($p=0.2513$). Total CTC number trended towards a significant difference between patients with PR and SD/PD $p=0.0732$. (B) Number of CTC subtypes and total-CTC numbers in mHSPC patients with PR or, SD and PD combined. There was no significant difference in the numbers of epithelial-CTCs ($p=0.5975$), EMTing-CTCs ($p=0.7909$) and total-CTCs ($p=0.2053$) detected between patients with PR and SD/PD. However, mesenchymal-CTC numbers trended towards a significant difference between the patient outcome groups ($p=0.0785$). (C) For prediction of immediate tumour response in mCRPC patients, total-CTCs had an area under the curve (AUC) of 0.80, $p=0.071$, PSA had an AUC of 0.78, $p=0.089$ and ALP had an AUC of 0.74, $p=0.20$. A combined risk score combining all three variables was calculated as $CRS-TPA=0.7414 \times \text{Total-CTC number} + 0.02909 \times \text{PSA} + 0.01423 \times \text{ALP}$, which increased the AUC to 0.90, $p=0.037$. (D) For prediction of immediate tumour response in mHSPC patients, mesenchymal-CTCs had that highest AUC of 0.65, $p=0.099$, PSA had an AUC of 0.52, $p=0.82$ and ALP an AUC of 0.68, $p=0.077$. A combined risk score combining all mesenchymal CTC numbers and ALP was calculated as $CRS-MA=0.3599 \times \text{mesenchymal-CTC number} + 0.002037 \times \text{ALP}$, resulted in an AUC of 0.69, $p=0.064$.

TABLE 3 Spearman's correlation of CTCs and serum biomarkers with OS and PFS.

	OS	PFS
	Spearman's ρ (p-value)	
mCRPC patients		
Total-CTCs	-0.66 (0.0027)	-0.52 (0.075)
Epithelial-CTCs	-0.62 (0.0057)	-0.63 (0.0049)
EMTing-CTCs	-0.65 (0.0034)	-0.14 (0.57)
Mesenchymal-CTCs	-0.40 (0.10)	0.34 (0.17)
CTC-score	-0.80 (<0.0001)	-0.65 (0.0033)
PSA	-0.72 (0.0008)	-0.50 (0.035)
ALP	-0.41 (0.13)	-0.16 (0.55)
mHSPC patients		
Total-CTCs	0.16 (0.31)	-0.087 (0.58)
Epithelial-CTCs	-0.084 (0.60)	-0.16 (0.30)
EMTing-CTCs	0.13 (0.41)	0.11 (0.43)
Mesenchymal-CTCs	0.20 (0.20)	-0.076 (0.63)
CTC-score	0.012 (0.94)	-0.18 (0.26)
PSA	-0.21 (0.19)	-0.16 (0.33)
ALP	-0.45 (0.0068)	-0.62 (<0.0001)

PFS, progression-free survival; OS, overall survival; bold black numbers, significant results

shorter median OS (24.57 vs undefined months, HR= 4.14, p= 0.0097) (Table 4, Figure 4F).

CTC gene expression predicted PFS and OS in mHSPC patients

Subsequently, we interrogated CTC mRNA expression to enhance the efficiency of CTCs as predictive biomarkers beyond CTC enumeration in the mHSPC cohort. Up-regulated differentially expressed genes in docetaxel-resistant cell lines from two microarray datasets were considered for docetaxel-resistant CTC detection. There were 162 genes commonly up-regulated in docetaxel-resistant cell lines and considered for further validation. Additionally, we identified 75 reported docetaxel-resistance related genes by literature search of relevant publications regarding the mechanisms of docetaxel resistance. These panels were combined to form a 237-candidate gene panel (Supplementary Table 3). As the enriched CTC fraction that is harvested from the Parsortix[®] is not pure, it was necessary to account for white blood cell contamination in the sample. Therefore, we performed two steps of gene expression analysis to exclude any of the 237 candidate genes that were expressed in leucocytes, 1. *in silico* analysis and 2. *in vitro* experiments using PCA cell lines and patient derived

leucocyte samples. Firstly, the 237 genes were searched in The Genotype-Tissue Expression Portal V7 database for their expression in prostate and whole blood. Genes were selected based on their relatively high expression in prostate tissue and

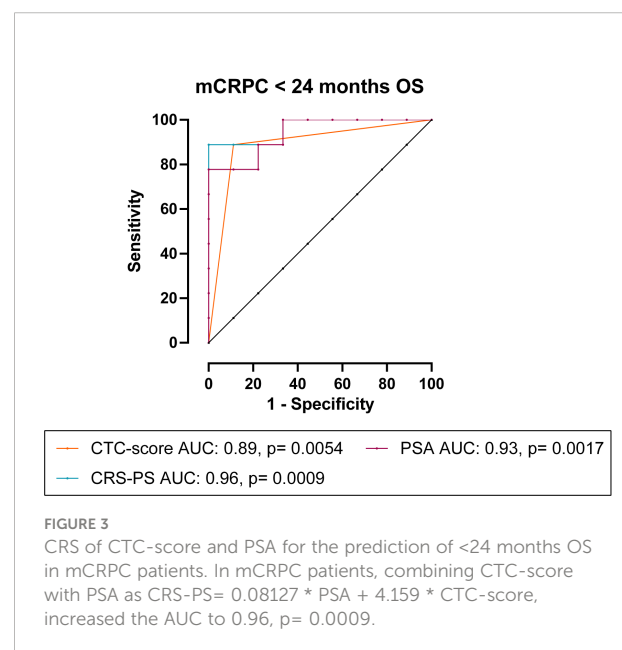


TABLE 4 Kaplan Meier analysis of CTC enumeration cut-offs for OS and PFS in mCRPC and mHSPC.

CTC parameters	Patients per group (n)	OS			PFS		
		Median survival (months)	HR (95% CI)	p-value	Median survival (months)	HR (95% CI)	p-value
mCRPC patients							
CTC-score negative vs. CTC-score positive	9 vs. 9	37.33 vs. 12.80	5.08 (3.41 to 43.57)	0.0005	18.83 vs. 8.0	2.70 (1.18 to 15.03)	0.042
< 2 vs. ≥ 2 CTCs	9 vs. 9	34.80 vs. 12.80	3.13 (1.37 to 15.82)	0.021	16.43 vs. 8.0	1.54 (0.50 to 5.50)	0.43
< 3 vs. ≥ 3 CTCs	11 vs. 7	37.33 vs. 12.80	3.84 (2.08 to 34.72)	0.0053	16.43 vs. 8.0	2.03 (0.66 to 11.16)	0.20
< 4 vs. ≥ 4 CTCs	12 vs. 6	32.27 vs. 12.53	3.66 (1.80 to 39.08)	0.010	13.82 vs. 8.30	1.57 (0.41 to 7.95)	0.46
< 5 vs. ≥ 5 CTCs	13 vs. 5	32.27 vs. 12.27	3.39 (1.38 to 41.25)	0.025	11.20 vs. 9.80	1.25 (0.25 to 6.61)	0.77
< 6 vs. ≥ 6 CTCs	14 vs. 4	28.23 vs. 12.53	2.50 (0.68 to 22.90)	0.014	11.30 vs. 7.33	2.10 (0.38 to 21.12)	0.32
0 vs. ≥ 1 E-CTC	10 vs. 8	37.33 vs. 14.30	3.89 (2.17 to 28.45)	0.0041	18.83 vs. 7.70	3.57 (1.82 to 28.47)	0.0088
< 2 vs. ≥ 2 E-CTC	11 vs. 7	37.33 vs. 12.80	3.72 (1.99 to 32.21)	0.0065	18.83 vs. 7.52	3.93 (2.20 to 51.88)	0.0058
0 vs. ≥ 1 EMTing-CTC	14 vs. 4	32.37 vs. 11.32	6.73 (11.47 to 1043)	0.0001	11.20 vs. 10.28	1.03 (0.23 to 4.72)	0.97
0 vs. ≥ 1 M-CTC	10 vs. 8	32.37 vs. 16.07	1.39 (0.60 to 5.29)	0.30	13.82 vs. 8.3	0.89 (0.30 to 2.61)	0.83
< 2 vs. ≥ 2 M-CTC	12 vs. 6	28.23 vs. 12.53	1.88 (0.56 to 8.89)	0.27	10.57 vs. Undefined	0.60 (0.18 to 2.31)	0.50
mHSPC patients							
CTC-score negative vs. CTC-score positive	20 vs. 22	Undefined vs. Undefined	2.53 (0.72 to 7.74)	0.15	36.40 vs. 12.52	1.90 (0.89 to 4.01)	0.10
< 2 vs. ≥ 2 CTCs	23 vs. 19	Undefined vs. 40.30	1.73 (0.53 to 5.60)	0.37	26.57 vs. 16.83	1.42 (0.67 to 3.02)	0.37
< 3 vs. ≥ 3 CTCs	27 vs. 15	Undefined vs. 40.30	2.74 (0.86 to 9.71)	0.090	21.80 vs. 13.33	1.58 (0.74 to 3.66)	0.23
< 4 vs. ≥ 4 CTCs	29 vs. 13	Undefined vs. 31.07	3.91 (1.31 to 16.89)	0.018	26.57 vs. 11.13	1.62 (0.74 to 4.06)	0.22
< 5 vs. ≥ 5 CTCs	32 vs. 10	Undefined vs. 24.57	4.14 (1.61 to 27.36)	0.0097	26.57 vs. 9.63	1.79 (0.77 to 5.27)	0.16
< 6 vs. ≥ 6 CTCs	34 vs. 8	Undefined vs. 24.57	3.98 (1.54 to 35.11)	0.013	26.57 vs. 9.63	2.18 (0.94 to 8.33)	0.067
0 vs. ≥ 1 E-CTC	28 vs. 14	Undefined vs. Undefined	2.86 (0.92 to 12.25)	0.068	29.37 vs. 11.42	2.07 (0.99 to 5.33)	0.098
< 2 vs. ≥ 2 E-CTC	35 vs. 7	Undefined vs. Undefined	2.07 (0.49 to 12.93)	0.27	21.80 vs. 11.13	1.54 (0.58 to 4.65)	0.39
0 vs. ≥ 1 EMTing-CTC	36 vs. 6	Undefined vs. Undefined	0.50 (0.12 to 2.81)	0.50	15.90 vs. 28.97	0.85 (0.31 to 2.34)	0.76
0 vs. ≥ 1 M-CTC	22 vs. 20	Undefined vs. 40.30	2.13 (0.65 to 7.0)	0.23	26.57 vs. 15.88	1.22 (0.57 to 2.61)	0.60

(Continued)

TABLE 4 Continued

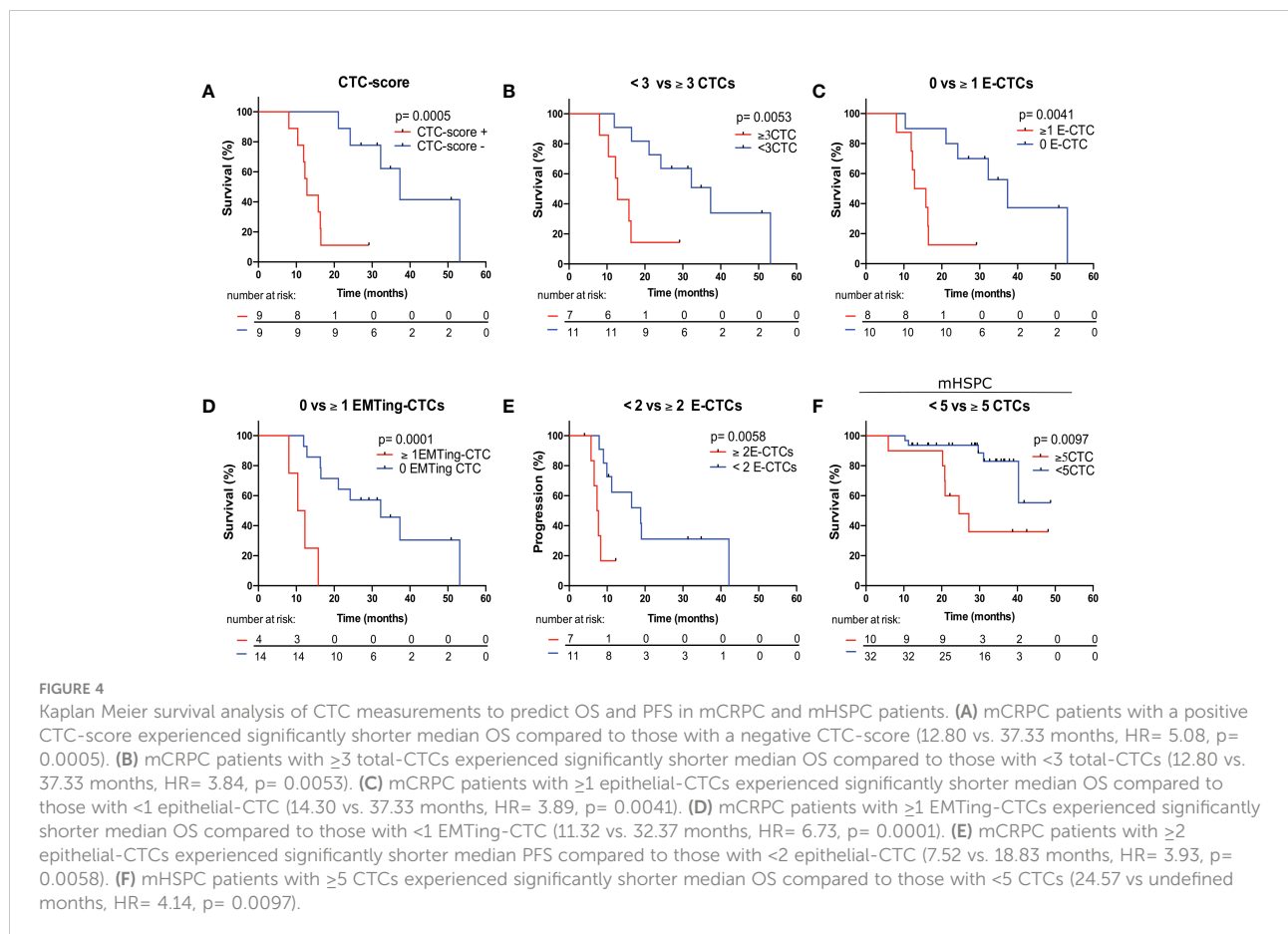
CTC parameters	Patients per group (n)	OS			PFS		
		Median survival (months)	HR (95% CI)	p-value	Median survival (months)	HR (95% CI)	p-value
< 2 vs. ≥ 2 M-CTC	31 vs. 11	40.30 vs 31.07	1.86 (0.57 to 7.28)	0.29	26.57 vs. 13.33	1.56 (0.68 to 3.97)	0.27

PFS, progression-free survival; OS, overall survival; E-CTC, Epithelial CTC; M-CTC, Mesenchymal CTC; Undefined, The probability of survival exceeds 50% at the longest time point; bold black numbers, significant results; bold grey numbers, results with a trend towards significance.

low/no expression in whole blood. This resulted in a candidate panel of 39 genes. Secondly, using RT-qPCR in paired docetaxel-sensitive (PC3-AG) and docetaxel-resistant (PC3-D12) PCa cell lines, and five PBMC samples from biopsy-negative patients, we experimentally validated 23/39 genes for lack of expression in leucocytes. A further seven genes, including EMT and stem cell markers (*PTPRC (CD45)*, *SNAI1*, *ZEB1*, *NANOG*, *POU5F1*, *PROM1* and *SOX2*) were included due to their potential prognostic value in clinical samples and lack of expression in leucocytes, along with housekeeping genes *GAPDH* and *MRFAP1*. Unfortunately, the mCRPC sample size available at this time point was limited and gene expression analysis was not performed. However, the relationship between candidate gene

expression and OS/PFS was investigated in 33 mHSPC patient samples.

Survival analysis was performed by separating patients into the following groups: 1) Expression vs. no expression 2) 50% highest expression vs. 50% lowest expression. Kaplan Meier analysis revealed that *KLK2* expression was significantly associated with shorter median OS (27.17 vs. undefined months, HR= 3.87, p= 0.037) (Figure 5A) and PFS (8.13 vs. 26.57 months, HR= 5.15, p= 0.0002) (Figure 5B). *KLK4* expression was significantly associated with shorter median PFS (10.17 vs. 26.57, HR= 3.01, p= 0.034) (Figure 5C), while *KLK3* (PSA) expression had only a trend towards shorter median OS (30.13 vs. undefined months, HR= 3.18, p= 0.068)



(Figure 5D) and did not separate patients based on PFS. Patients with 50% highest *SNAI1* and/or *ADAMTS1* expression experienced significantly shorter median OS (31.07 vs. undefined months, HR= 9.51, p= 0.0090 (Figure 5E); 31.07 vs undefined months, HR= 4.30, p= 0.047 (Figure 5F), respectively) and patients with the 50% highest *ZEB1* expression experienced significantly shorter median PFS (11.50 vs. 24.40 months, HR= 2.5, p= 0.036) (Figure 5G). Hazard ratios for each gene for OS and PFS are presented in Figure 5H.

We then investigated the clinical outcomes of patients who had CTCs expressing more than one high-risk gene. The median PFS for patients with *KLK2*+*ZEB1*^{hi} (8.13 months Logrank p= 0.0004) (Figure 6A) and/or *KLK2*+*SNAI1*^{hi} (5.9 months, Logrank p= 0.0019) (Figure 6B) and/or *KLK2*+*ADAMTS1*^{hi} (5.9 months, Logrank p= 0.0004) (Figure 6C) was significantly shorter than for patients with no *KLK2* expression and low expression of each gene. Patients expressing both genes also had high total CTC numbers detected in paired samples (*KLK2* +*ZEB1*^{hi}: ≥8 CTCs, *KLK2*+*SNAI1*^{hi}: ≥5 CTCs, *KLK2* +*ADAMTS1*^{hi}: 0, 5 and 9 CTCs).

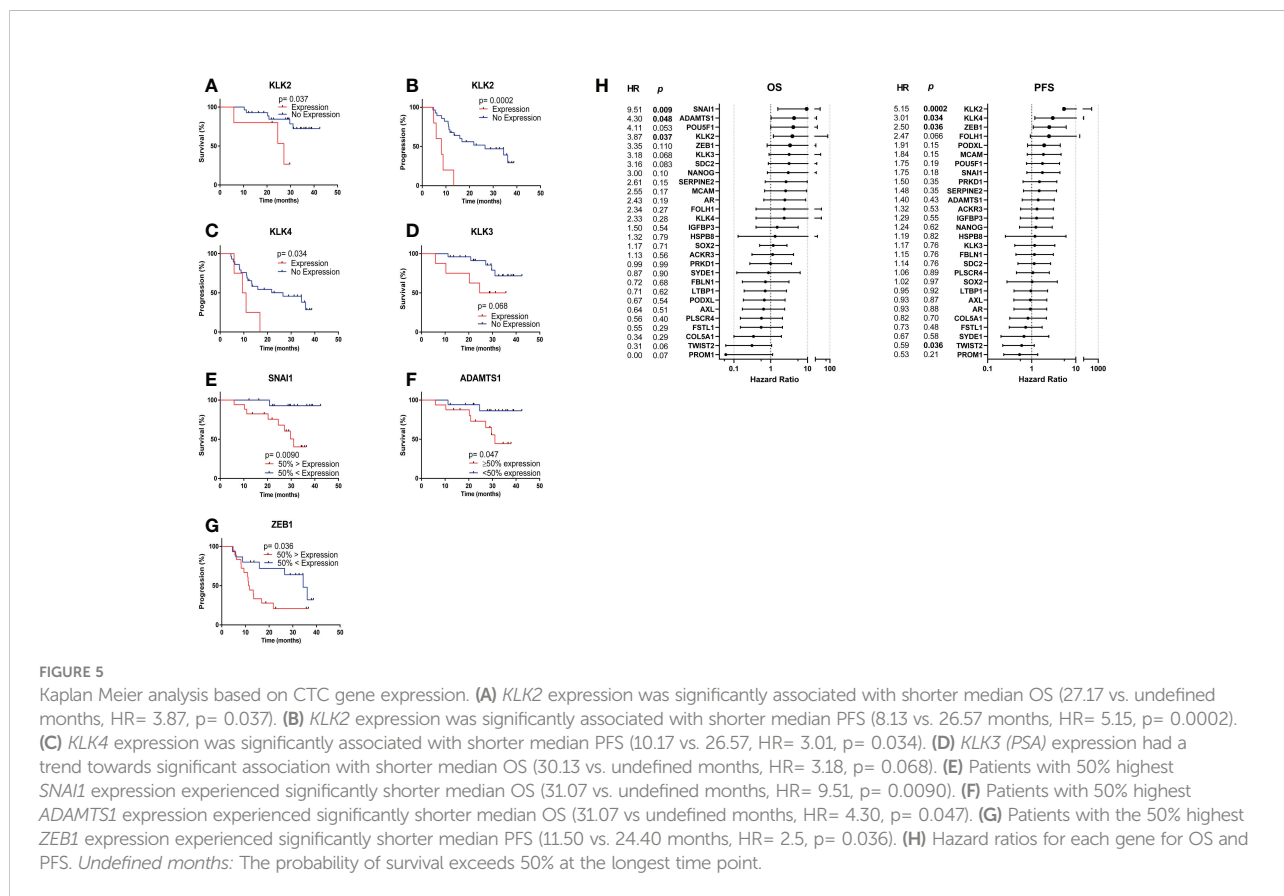
Using receiver operating characteristic curve analysis, high expression of *ADAMTS1* was significantly predictive of shorter OS with an AUC of 0.73, p= 0.043 (Figure 7A). Neither serum PSA or ALP levels significantly differentiated patients based on

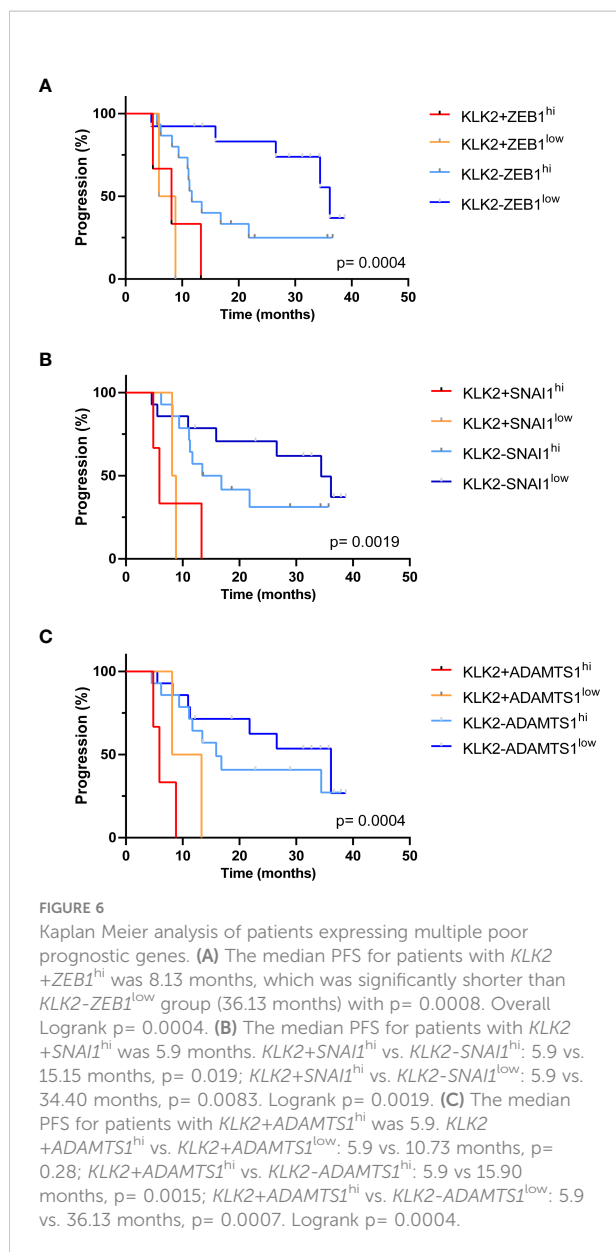
OS, although ALP showed a trend towards significance (AUC= 0.55, p= 0.66; AUC= 0.73, p= 0.072, respectively) (Figure 7A). Combining *ADAMTS1*, ALP and ≥5 total CTCs to form a CRS as CRS-AA5o= 0.06386 * *ADAMTS1* + 0.001465 * ALP + 2.169 * ≥5 total CTCs, which increased the AUC to 0.83, p= 0.0070 (65% sensitivity and 87.5% specificity at a cut off of <0.48) vs AUC of 0.73 for ALP alone, (Figure 7A) however without a significant difference (p= 0.38).

We then evaluated the candidate genes for the prediction of PFS and found that high expression of *ZEB1*, *SNAI1* and *ADAMTS1* was significantly predictive of progression within 24 months (AUC= 0.77, p= 0.0092; AUC= 0.71, p= 0.039; AUC= 0.71, p= 0.043, respectively) (Figure 7B). Expression of *KLK2* had a trend of correlated with <24 months PFS, but the AUC was not significant (AUC= 0.64, p= 0.18) (Figure 7B). ALP but not serum PSA levels significantly discriminated patients with <24 months PFS from those with ≥24 months PFS (AUC= 0.86, p= 0.0015; AUC= 0.52, p= 0.86, respectively) (Figure 7B).

Discussion

Improvements in our understanding of the genetic landscape of PCa have advanced treatments for metastatic



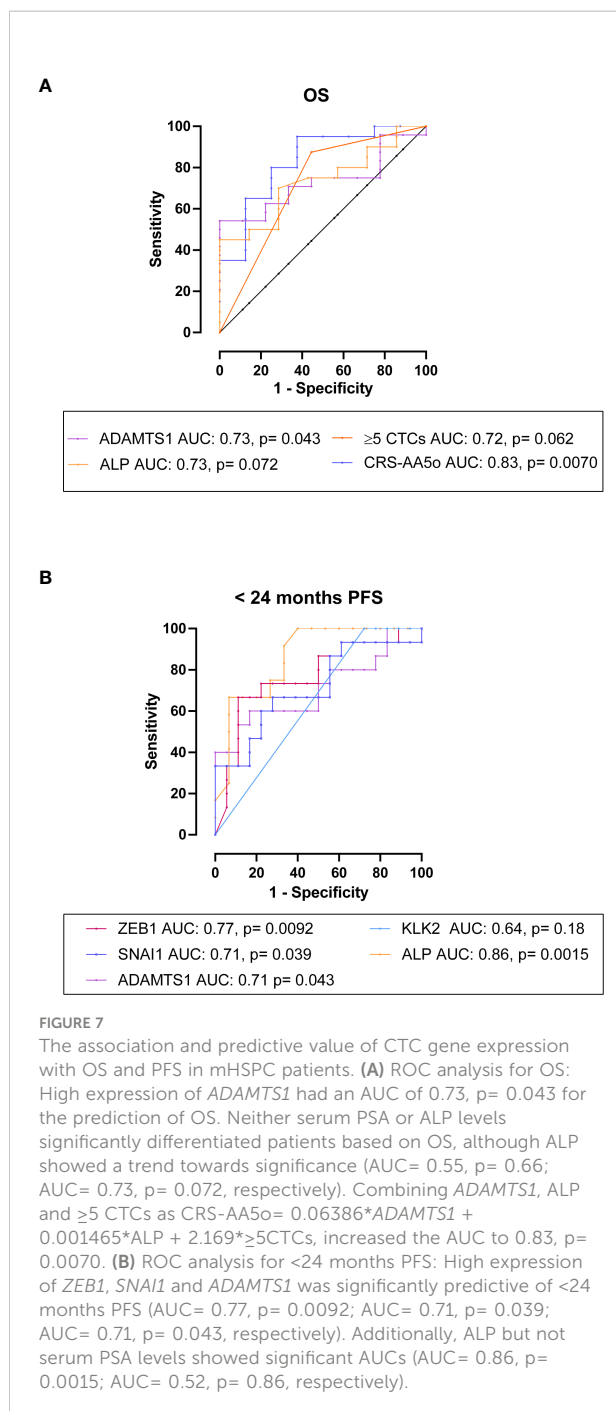


disease. These treatments comprise androgen-receptor targeting therapies (abiratorine, enzalutminde) bone-targeting radiotherapies (Radium-223), immunotherapies, and cytotoxic chemotherapies (docetaxel, cabazataxel). Docetaxel is now a first-line therapy in both mCRPC and mHSPC, however its efficacy is limited by the vast inter- and intra-tumour heterogeneity of PCa, resulting in clonal populations with inherent and/or acquired resistance in a proportion of patients. Although multiple docetaxel resistance mechanisms have been revealed through extensive research, such as upregulation of drug efflux pumps (e.g. ABCB1) (40), alterations to β -tubulin and expression of tubulin isoforms (41), deregulation of apoptosis and survival signalling pathways (42), induction of EMT and cancer stem cells

phenotypes (36, 43, 44), and deregulation of AR signalling (45), the development of clinically useful tools to predict response is currently still required (46–48). Thus, patients with resistance may undergo systemic chemotherapy with little survival benefit (49, 50). Our ability to elucidate biomarkers of resistance is limited by tissue biopsy, which samples only a small fraction of the entire heterogeneous tumour, is practically difficult in metastatic disease and as a repeated measure. However, liquid biopsy analysis of CTCs offers a minimally invasive, easily repeatable tool for cancer specific interrogation. Using the Parsortix[®] CTC isolation system, we investigated the potential of using CTCs, including subtype and CTC gene expression analysis, to predict docetaxel response and survival benefit in both mCRPC and mHSPC patients.

We found that individual CTC measurements alone showed some, but limited associations with RECIST response in mCRPC and mHSPC patients. However, when combined with serum biomarkers in mCRPC patients, initial progressive disease could be predicted with high accuracy. Furthermore, combining CTCs with serum biomarkers efficiently detected mCRPC patients at risk of shorter OS, supporting the potential use of CTCs to triage patients for docetaxel treatment. Importantly, we showed that ≥ 5 pre-docetaxel CTCs/7.5mL were also significantly associated with poor PFS in mHSPC patients treated with first-line chemo-hormonal therapy. Additionally, molecular analysis of CTC samples revealed that the expression of the candidate docetaxel-resistance gene *ADAMTS1*, and EMT transcription factors *ZEB1* and *SNAI1* along with the PCa specific kallikreins *KLK2* and *KLK4* significantly correlated with poor mHSPC patient outcome.

While the detection of CTCs is now a well-established marker of aggressive cancer with poor survival outcome, it is yet to be determined if CTCs have an association with initial docetaxel response. Immediate radiological or RECIST response criteria are commonly used to determine a treatment response upon the completion of a therapeutic regimen. In our study, although potentially due to the limited cohort sizes, individual CTC subtypes did not significantly discriminate between patients with a partial response and those with stable disease and/or progressive disease. This may suggest that in the pre-docetaxel setting the CTC subtype has limited relation to inherent docetaxel sensitivity and RECIST response. Nevertheless, we found that total CTC numbers showed potential for predicting initial radiological treatment response when used in combination with serum biomarkers (PSA and ALP) in mCRPC patients, with a good AUC of 0.90. Although we observed a higher number of mesenchymal CTC in mHSPC patients lacking a partial response to treatment, they did not add significant predictive value. Newly diagnosed mHSPC patients are treated with first-line ADT for a short period prior to starting and throughout docetaxel therapy. Unlike mCRPC patients, all mHSPC patients should be responsive to ADT at this time. Therefore, our results indicate that responsiveness to first-line



ADT might affect the value of CTCs for the prediction of near-term clinical response to docetaxel. This is also reflected in the lack of significant correlations between serum PSA level and CTC numbers before docetaxel treatment in mHSPC patients. In summary, CTCs might have a potential value in predicting docetaxel response, but data here is insufficient to make a conclusion. Further investigations in larger cohorts are required.

While pre-docetaxel CTC measurements showed limitations in the prediction of initial radiological response, we showed that

CTCs were associated with OS and PFS subsequent to docetaxel treatment. The significant correlation of epithelial CTCs with shorter OS and/or PFS confirmed previous research findings in mCRPC patients to be treated with docetaxel (19, 23, 24, 26, 27, 51). However, by using the epitope independent Partsortix[®] isolation system, we were able to capture and analyse three different CTC subtypes. Interestingly, in the mCRPC patient cohort, the detection of ≥ 1 EMTing-CTCs ($p= 0.0001$) and a positive CTC-score ($p= 0.0005$) were the most significant predictors of shorter OS among the different CTC measurements. Our previous studies have demonstrated both to be valuable PCa biomarkers, and associated with increased metastatic burden (32, 38). Patients with a positive-CTC score and/or ≥ 1 EMTing-CTC present in 7.5mL of pre-docetaxel blood experienced an approximate three-fold reduction in median OS time compared to those with a negative CTC-score or no EMTing-CTCs. This demonstrates the value of analysing different subtypes of CTCs and the good prognostic value of the CTC score, in which mesenchymal CTCs were a component. A positive CTC-score combined with serum PSA (AUC= 0.96 for OS <24 months) may flag high-risk mCRPC patients with high sensitivity (88.89%) and specificity (100%) and facilitate timely therapeutic intervention post-docetaxel, such as Cabazitaxel administration, which has been shown to retain activity in patients after docetaxel treatment (52).

To date, reports on the use of CTCs as a prognostic biomarker in mHSPC patients treated with ADT plus docetaxel are limited. The prediction of significantly shorter OS by ≥ 5 total-CTCs in mHSPC patients may be useful for treatment stratification in this cohort. In this study, CTCs expressing cytokeratin alone did not significantly differentiate mHSPC patients with shorter OS from those with a good response and prolonged OS after docetaxel treatment. Again, these findings highlight the advantage of epitope independent CTC isolation, which allows for the capture and analysis of multiple CTC subtypes.

We showed that the RNA expression of certain genes in CTCs correlated with shorter OS and/or PFS in mHSPC patients. These poor prognostic genes included the EMT transcription factors *ZEB1* and *SNAI1*. EMT is responsible for tumour cell migration and metastasis, and is associated with drug resistance in multiple solid tumour types (53). In docetaxel resistance, upregulation of EMT genes has been shown to mediate resistance emergence in PCa cell line models (36, 54, 55) and increased expression in primary tumours prior to therapy has been correlated with radiological relapse (36). Several EMT genes have been assessed in CTCs, the most common being Vimentin, for detection of a mesenchymal subtypes which have been linked to higher metastatic burden, a more aggressive phenotype and disease progression in PCa (32, 56, 57). The shorter OS and/or PFS associated with the RNA expression of *ZEB1* and *SNAI1* by CTCs may indicate a more aggressive and potentially docetaxel resistant phenotype being present in the primary or metastatic tumour sites, which may promote disease progression in mHSPC patients.

Additionally, these findings highlight the importance of considering multiple different markers corresponding to the same cellular phenotype, as genes may be exhibiting different patterns of spatial-temporal expression under pathological conditions, yet could be controlled by different upstream signals.

Patients with mHSPC who had detectable *KLK2* RNA expression in CTCs were more likely to suffer shorter PFS compared to patients with *KLK3* (PSA) expression. The protein encoded by *KLK2*, hK2, has been utilised in the 4Kscore[®] Test to predict risk of aggressive PCa (58). Our findings suggest that *KLK2*/hK2 may also be used as biomarker to identify mHSPC patients who are likely to progress under chemo-hormonal treatment.

The metalloprotease, *ADAMTS1*, was upregulated in docetaxel-resistant cells in microarray analysis and was associated with both PFS and OS. Combining *ADAMTS1* expression with ALP levels and ≥ 5 CTCs predicted shorter median OS with high sensitivity and specificity. For PFS, ALP was highly predictive, with an AUC of 0.86, leaving little margin for improvement, so combined biomarker analysis was not performed.

The variety of CTC derived genes that were identified as biomarkers of poor prognosis indicates the heterogeneity of CTCs between patients. CTC heterogeneity may be the result of a spatio-temporally different microenvironment surrounding the tumour lesions, in the circulation, as well as differences in therapy response (14, 59). In the case of the mHSPC patient cohort, differing levels of response to initial ADT may alter tumour biology, influence CTC gene expression and fitness, and subsequent response to docetaxel therapy, such as induction of EMT machinery (60–62). To explore this, further analysis of CTC gene expression changes over multiple time points during therapy is warranted.

The limitations of this study include: 1. Small patient cohort, particularly for the mCRPC patients, although we observed several significant correlations. The small sample size was due to the several effective therapies been developed in recently years for PCa, leading to competing treatment options. 2. The candidate CTC genes were selected based on the microarray dataset gene expression profile from 2D-cultured docetaxel-resistance cell line models, as currently, datasets from better models for docetaxel resistant versus sensitive samples are not available. It is well-known that 2D-induced resistance creates artificial resistance mechanisms. Therefore, validation of these candidate genes in clinically relevant samples is critical. 3. As the harvested CTC samples that we used for gene expression analysis were not pure CTCs (with predominantly leucocyte contamination), we had to exclude a large number of candidate docetaxel resistance genes which were expressed in leucocyte. This led to only 23 selected from the initial 237 candidate genes, thus potentially missing genes with good docetaxel therapeutic response prediction value. Further pure CTC selection (although a challenging task) or single cell RNA sequencing may be explored in the future to address this issue.

In summary, our study demonstrated that in mCRPC, elevated numbers of CTCs were indicators of poor initial

response when combined with serum biomarkers, and CTC measurements could be used to predict short OS and/or PFS in mCRPC and mHSPC patients. Additionally, we showed that measuring RNA expression of candidate docetaxel-resistance and PCa related genes from CTC samples increased our ability to predict patient outcome in the mHSPC patient cohort. Importantly, we found that combining CTC data with clinical serum biomarkers has the potential to predict poor docetaxel treatment response, although this should be confirmed in a large series of samples.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/[Supplementary Material](#).

Ethics statement

The studies involving human participants were reviewed and approved by UK Research Ethics Committee: London - City & East Research Ethics Committee. The patients/participants provided their written informed consent to participate in this study.

Author contributions

Conceptualization: CD, PR, AG, GSh, JS, and Y-JL. Validation: CD and LX. Formal analysis: CD and Y-JL. Investigation: CD, TG, EB, ES, LX, and XM. Data Curation: CD, EB, ES, XM, and GSc. Writing – Original Draft: CD. Visualization: CD. Writing – Review and Editing: TG, ES, XM, PR, AG, TO, JS, and Y-JL. Methodology: XM, Y-JL, LX. Resources: GSh, PR, KT, CA, AW, MG, SC, TP, AG, SK, GSc, DB, and JS. Supervision: JS and Y-JL. Project administration: Y-JL. Funding: Y-JL. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fonc.2022.1060864/full#supplementary-material>

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