Circulating tumour cells as a window into lethality in prostate cancer

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Author contributions

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Abstract

Prostate cancer is characterized by multifocality, inter- and intra-patient tumour heterogeneity, and differences in risk of progression to metastatic disease, castration resistance, and lethality, which can make prognosis challenging. Consequently, sampling methods that provide accurate insight into disease phenotype to facilitate risk-stratification of patients are crucial. The variable biology of prostate cancer seems to be recapitulated in the phenotypic heterogeneity of circulating tumour cells (CTCs). CTC sampling offers a liquid biopsy method to achieve minimally invasive longitudinal sampling for disease monitoring. CTC analysis has also offered crucial insight into aggressive phenotypes, disease metastasis and treatment response, particularly in clinical trials. The clinical use of CTC count for prognosis in advanced prostate cancer has been approved by the USA FDA, but is not routinely used clinically, as these cells are technically challenging to isolate and analyse. However, methodological advances continue to improve CTC enrichment and profiling. Understanding the clinical utility of CTCs and future innovations is crucial to incorporate CTCs into clinical management of prostate cancer.

Key points

- Circulating tumour cells (CTCs) can be detected in blood from subsets of patients with prostate cancer by using antigen-dependent or -independent methods of enrichment.
- New label-free enrichment strategies are renewing interest in CTCs after advances in next-generation sequencing had previously shifted the focus of liquid biopsy to cell-free DNA (cfDNA).
- Molecular profiling of prostate cancer CTCs might offer a stratification tool to select patients for systemic therapy.
- CTCs provide insight into the clinical and biological heterogeneity of prostate cancer, potentially offering markers of prognosis and/or treatment response.
- Further study is needed on the role of CTCs in localised prostate cancer, requiring increased sensitivity and specificity of enrichment methods.

[H1] Introduction

The clinical, morphological, molecular, and intra-tumour spatial heterogeneity of prostate cancer is well-established.[1–3] Prostate cancer can be indolent, showing a slow course of clinical progression, or can develop into aggressive disease with metastatic and lethal phenotypes.[4–7] Treatment landscape in advanced prostate cancer is rapidly changing, and current methods of diagnosis often lead to overdiagnosis of tumours that are unlikely to be clinically significant within the patient's lifetime, leading to a risk of over-treatment. In this scenario, accurate risk stratification and tumour profiling to inform therapeutic approach are particularly important.[8] Studies carried out to unravel intra-tumour spatial heterogeneity of prostate cancer led to the identification of clonal evolution of the disease both within and outside of the prostate gland.[1,2,9–12] Understanding the interplay between tumour heterogeneity, classically defined "index lesions," and the biology of disease spread is crucial to inform risk-stratification of patients and clinical decision making.

Circulating biomarkers have garnered much attention over the past decade, as cancer research and clinical practice recognise the potential of these markers for non-invasive risk stratification and personalised treatment strategies. Circulating tumour cells (CTCs) are tumour cells shed either from primary tumour or metastatic sites that travel through the bloodstream, with some having the potential to seed disease in distant sites. CTC liquid biopsy offers a minimally invasive approach, to sample molecular tumour phenotypes and genotypes with potentially lower healthcare costs than tissue biopsy. CTCs were considered as a promising circulating biomarker to dominate future cancer liquid biopsy development, with the FDA approval of CellSearch-based CTC analysis for advanced breast, colorectal and prostate cancer prognosis.[13,14] However, with the advance of DNA sequencing technology, circulating tumour DNA (ctDNA) as a biomarker for cancer has been developed through the

investigation of ctDNA fraction[15,16], genomic alterations[17] and fragmentomics[18], overtaking CTCs as the mainstream circulating biomarker for clinical application owing to the technology readiness and improved standardisation, as well as ease of sample storage and transportation. Androgen receptor (AR) status detected in plasma DNA has also been used as a biomarker of treatment selection in prostate cancer[19], with sequencing of ctDNA used to identify AR alterations that associate with androgen receptor signalling inhibitors (ARSI) resistance[20] , and patterns of methylation[21] and fragmentation[22] that can identify transformation to neuroendocrine prostate cancer (NEPC). Plasma cell-free or ctDNA assays are commercially available and can also provide insight into tumour burden, progression, copy number variants, and methylation patterns [23,24]. However, definitively identifying the origin of plasma DNA poses a challenge. Fragmentomics, which identifies size distributions of cell-free (cf) DNA fragments unique to cancer patients and subtypes, offers the possibility to distinguish tumour-derived ctDNA.[18] However, the relevance of ctDNA to tumour evolution and metastasis is limited, as whether ctDNA arises from the primary tumour, metastatic sites, or CTCs is not clear. Moreover, fragments could be representative of apoptotic tumour cells lacking potential to seed metastatic disease, and ctDNA could fail to capture the homogenisation of shedding sites.[25]

CTCs shed from the primary tumour or metastatic sites and enter the circulation, with some having the potential to seed distant metastases, propagating the haematogenous spread of cancer.[26–28] Steven Paget's "seed and soil" hypothesis introduced in 1889 suggested a reciprocal determinism in which both the metastatic potential and microenvironments of cells shed from primary tumour and metastatic niches (such as bone marrow) influence 'successful' propagation of tumours to distant sites.[29,30] This theory is exemplified by the biological phenomenon of "dormancy" observed in prostate and other cancers, as both tumour microenvironmental drivers and autogenous cell programs are thought to regulate

cellular dormancy.[31] Subsequent research across cancer types has corroborated this hypothesis and begun to unravel the molecular details of these tumour and metastatic niche profiles, suggesting the successful propagation of metastasis and treatment resistance are mediated by a mesenchymal or cancer stem-cell (CSC) phenotype.[32–34] Importantly, CTCs offer the ability to analyse phenotypic and genotypic characteristics cells (or "seeds") that have survived in circulation as they are travelling to or from metastatic niches (or "soil"), whereas other liquid biopsy modalities such as ctDNA are limited to genotypic analysis and could represent apoptotic cells or "shrapnel" that are not biologically relevant to cancer spread.

Access to CTC molecular profiles offers great promise to further delineate the metastatic potential of these cells and could enable to gain insight into metastatic sites such as the skeleton, which are particularly challenging to sample directly and often deliver minimal usable material for analyses.[35,36] Furthermore, the non-invasive nature of CTC profiling enables to achieve longitudinal real-time monitoring of disease burden and dynamic biology, including response to therapy.[37,38] However, this promise to understand the biology behind prostate cancer metastasis has been limited by the rarity of CTCs in blood and the current technologies available to enrich for these cells. These technologies are often limited by trade-offs between sensitivity (which increases with negative selection or antigen-independent enrichment) and specificity (which increases with positive selection or immune-affinity), and by the ability to retain live cells and carry out downstream analyses beyond enumeration.[39] However, technological developments in isolation and the advent of advanced molecular profiling, including single-cell technologies and cell culture, could reinvigorate the field of CTC research.

Importantly, the presence of CTCs is associated with poor overall survival (OS) in patients with metastatic castrate resistant prostate cancer (mCRPC).[40] CTCs have been

identified in the blood of patients with localised prostate cancer, but the prognostic significance of CTCs remains unclear outside the realm of metastatic disease.[8,41–44] Nonetheless, the analysis of prostate cancer CTCs holds great promise for insight into heterogeneous tumour biology[36,45], prediction of treatment response, and elucidation of the metastatic pathway.[46,47] Previous Reviews discussing the role of CTCs in prostate cancer have focussed on biomarkers used in prostate cancer detection and management[8,48], urological malignancies as a whole[25,49], and metastatic prostate cancer.[50–52] In this Review, we summarise the state-of-the-art for CTCs in all stages of prostate cancer development and treatment, describing advances in CTC isolation and enrichment, subsequent detection through enumeration, and molecular analysis of these cells, with a focus on unpacking the biological heterogeneity of prostate cancer to provide a window into lethality. The literature search methods are detailed in the Supplementary Information and Supplementary Figure 1. We also highlight research on the prognostic and clinical utility of prostate cancer CTCs, including the role of AR and its variants. Future innovations incorporating CTCs into clinical management of prostate cancer are also discussed.

[H1] Isolation and enrichment of prostate cancer CTCs

Methods for CTC enrichment can be broadly categorised as antigen-dependent, such as immune-affinity based methods, or antigen-independent, based on biological and physical properties, holding different capture efficiencies (% prostate cancer cells captured) and purity level (background level of white blood cells; Supplementary Table 1). The isolation of CTCs from blood underwent substantial advances over the past decade, with platforms such

as CellSearch™ obtaining FDA-approval in metastatic breast, prostate, or colorectal cancer, and Parsortix® obtaining FDA-approval in metastatic breast cancer.[53,54]

Immune-affinity methods target cell surface antigens, selecting for or against CTCs (positive versus negative enrichment). Immune capture is often achieved by using antibody-conjugated magnetic nanoparticles [55–60] or microfluidic chips with antibody-coated walls or solid support structures [Figure 1].[61–66] Negative enrichment targeting CD45 is used to deplete white blood cells (WBCs),[67,68] whereas positive enrichment of prostate cancer CTCs typically occurs by targeting the epithelial cell adhesion molecule (EpCAM). The most widely used platform for isolation of prostate cancer CTCs for prognosis is CellSearch™, which uses the CellTracks ™ AutoPrep ™ System to immunomagnetically enrich an EpCAM-positive cell population. However, isolation targeting EpCAM alone fails to capture the phenotypic heterogeneity of CTCs owing to the presence of epithelial-to-mesenchymal transition (EMT), a process in which expression of epithelial markers such as EpCAM is lost as CTCs extravasate into circulation; additionally, non-specific EpCAM-positive cells are also potentially captured with this strategy.[69–72] Thus, alternative recognition ligands have been used[51,59,73–76], including E-selectin[77], PSMA[62,78–80], PSA[62,78], vimentin[34,44,58,76], EGFR[59,74], c-Met[81], OB-cadherin[56], HER-2[74], TROP-2 [82], and PSCA[74] (Table 1 and Supplementary Table 2[83–93]). Multiplexed antibody-based approaches showed higher capture efficiency than single-antibody (typically EpCAM)-based capture.[58,79,94–96] In one of these studies, capture efficiencies of 74% and 85% were achieved by targeting EpCAM alone and by using an anti-EpCAM and anti-PSMA functionalised microfluidic platform, respectively.[94 82]Further research is needed to understand the implications of different capture ligands on which CTCs are captured from a heterogeneous population, specifically in regard to if the biological importance and metastatic potential of selected CTCs modulates with different capture targets.

Most antigen-independent techniques leverage the unique physical properties of CTCs— specifically size, density, and deformability—to separate CTCs from other blood components by using microfluidic platforms. This separation is commonly achieved through filter-based microfluidic devices with fixed pore size that capture CTCs and enable smaller blood cells to pass or squeeze through.[97] Prostate cancer CTCs typically have a diameter of 9.1–15.1 μm.[98,99] Thus, in studies using filtration to enrich for prostate cancer CTCs, pore sizes of 7.5–8 μm have been used[98,100], with pore sizes generally slightly smaller than the targeted cell size to facilitate deformability. In a proof-of-concept study including nine patients with prostate cancer, enrichment with the ScreenCell filtration technique enabled the use of fluorescence *in situ* hybridisation (FISH) to quantify telomere numbers and sizes.[101] In another study, ScreenCell filtration enabled the isolation and detection of CTCs from the blood of 41 patients with low-, intermediate-, and high- risk prostate cancer (GG1-5 on biopsy, no imaging details included).[98] The Parsortix® system is another size- and deformability- based CTC enrichment method. In one study including seven patients with prostate cancer, Parsortix® enabled the harvest of a significantly greater number of CK^+ CTCs from patient blood samples than CellSearch™ (mean of 32.1 and 10.1, respectively; $p=0.04$).[102] Furthermore, in this study, CK^+/$Vimentin^+$/$CD45^-$ and CK^-/$Vimentin^+$/$CD45^-$ CTCs were found through Parsortix® enrichment (mean 34.4 per patient, range 4-115) in blood samples from 4 of 5 patients with prostate cancer, and rarely in 3 healthy controls (zero CK^+/ $CD45^-$ cells and 0, 2, and 3 CK^-/$Vimentin^+$/$CD45^-$ CTCs detected), indicating that this method facilitates antigen-independent enrichment of CTCs with both epithelial and mesenchymal features. This evidence is important because CTCs might lose expression of canonical markers upon migration into the systemic circulation.

Other methods of CTC enrichment that use microfluidic techniques include dielectrophoresis, which describes the movement of cells in a non-uniform electric field, and

acoustophoresis, in which sound waves are used to exploit the variance in size and density of CTCs from healthy blood cells.[102–105] In one study, acoustophoresis was used to isolate CTCs from five patients with mCRPC to investigate PSMA expression, showing that 64.5–81.5% of CTCs (defined as CK8/18^{+}, DAPI^{+}, and CD45^{-} through immunofluorescence) were PSMA^{+}, demonstrating the ability to detect a potential therapeutic target through CTC liquid biopsy.[105] Dielectropohoresis is the core concept of DEPArray^{TM}, a platform that was shown to work well following isolation with CellSearch^{TM} to generate up to 30,000 "DEP cages" into which single cells can be sorted based on immunofluorescence imaging.[104] Importantly, this allows for downstream next generation sequencing (NGS) analysis of single CTCs, which is critical to study the heterogeneity of tumour cells.

Antigen-independent enrichment enables to capture a phenotypically heterogeneous CTC population, but isolation is limited by the relative rarity of CTCs in blood (1 CTC/billion blood cells).[106,107] To address this issue, Diagnostic LeukApheresis (DLA) uses continuous centrifugation for collection of peripheral blood mononuclear cells (PBMCs), to separate the mononuclear cell population from larger volumes of blood, and followed by CTCs isolation from this enriched fraction.[108] In a study including 22 patients with metastatic prostate cancer that aimed to collect a minimum DLA volume of 40 mL, processing a DLA aliquot of $2x10^{8}$ white blood cells (WBCs) by using CellSearch^{TM} increased CTC yield by up to 32-fold compared with that retrieved in a 7.5 ml blood samples with a standard CellSearch^{TM} protocol.[108] Another CTC enrichment tool, the GILUPI CellCollector®, uses an antibody-coated (e.g., EpCAM, PSA, or PSCA) medical wire inserted within the lumen of a blood vessel to collect CTCs from circulating blood.[96,109,110] This method enabled isolation of $\geq$1 CTC (identified through PanCK^{+}, Hoescht^{+}, CD45^{-}immunofluorescence staining) from the blood of 78.9% (56 of 71) and 46.3% (24 of 53) patients with metastatic and localised prostate cancer, respectively.[109] In another study using the GILUPI CellCollector®,

enrichment through prostate-specific markers (PSMA, PSA, PSCA, and EpCAM vs EpCAM alone) resulted in more sensitive detection rates (86.7% vs 73.3% of patients with ≥1 CTC detected) and higher CTC counts (median 9 vs. 3 CTCs) than those achieved by using EpCAM targeting alone in 25 patients with metastatic prostate cancer treated with ADT and/or chemotherapy.[96]

Overall, CTC isolation methodology determines the cell population that will be studied and, therefore, is crucial to any downstream analyses. Antigen-independent methods yield 'label-free' live cells, which facilitate the integration with a wide range of downstream biological and functional analyses, whereas methods relying on antigen targeting result in cells affixed to an antibody complex. Staining cells for protein expression typically requires fixation on a slide, and, therefore, can exhaust the sample available for further biological profiling. Thus, further development of CTC enrichment platforms that enable sensitive and specific detection at a reasonable cost and elution of live cells for functional analyses is crucial.

[H1] Enumeration and detection of prostate cancer CTCs

After capturing, potential CTCs need to be validated and quantified. Techniques to detect and enumerate (quantify) isolated CTCs are typically based on cancer cell associated antigens (for example, immunofluorescence), or nucleic acid-targeting (such as RT-PCR or FISH) (Supplementary Table 2 and Figure 1). Immunofluorescence-based enumeration methods, including the CellSearch™ system, traditionally detect CTCs based on a CK^+/$EpCAM^+$/DAPI+/$CD45^-$ signature.[111,112] To improve inter-reader variability in counting CTC from the CellSearch™ output, automated algorithms have been evaluated in patients

with metastatic prostate cancer, and showed comparable accuracy and ability to automatically extract morphological features.[113]

However, concerns exist about the over-dependence of these enumeration strategies on EpCAM and CK, owing to the phenotypic heterogeneity of CTCs that arises from EMT, which leads to loss of expression of typical epithelial markers.[114,115] A variety of additional markers have been identified, including CD133 (a marker of prostate stem cells)[116,117], E-Cadherin (CDH1)[116], PSMA (*FOLH1*)[118], PSA (*KLK3*)[119], AMACR[119], CXCR4, CD34, CD117[120], insulin-like growth factor-IR (IGF-IR)[121], and syntaptophysin (*SYP*)[122] (Table 1 and Supplementary Table 2).[123–130] [116]In study of 303 patients undergoing prostate biopsy, the enumeration of $PSA^+/P540S^+$ CTCs (positive test for CTC defined as $\geq$ 1 cell $PSA^+/P540S^+$) before diagnostic biopsy had a sensitivity and specificity of 88.5% and 88.4%, respectively, for detecting patients who required treatment (as compared to active observation). [118] In a another study, the detection of $EpCAM^+/PSMA^+/AMACR^+/PanCK^+/CD45^-$ CTCs showed a sensitivity and specificity of 91.2% and 100%, respectively, for diagnosing prostate cancer.[119] Interestingly, in a prostatectomy cohort in which blood immunostaining was carried out pre- and post-operatively to detect $CD133^+$, $CXCR4^+$, $CD34^+$ or $CD117^+$ cells, only $CD117^+$ cell percentages decreased after radical prostatectomy while the others remained near preoperative levels. [120] Furthermore, the percentage of $CD117^+$ cells did not decline in patients with BCR, and positively correlated (Spearman r=0.4851, p=0.03) with high PSA values, suggesting CD117 as a potential biomarker of therapy response.[120] As such, these enumerative studies of molecular markers for diagnosis and prognosis of prostate cancer could have a future role in identifying patients that need additional treatment, as well as in guiding treatment selection based on detection of therapeutically actionable markers.

Nucleic acid-targeting approaches for CTC detection in prostate cancer have historically relied largely on reverse transcription polymerase chain reaction (RT-PCR), in

which detection of prostate- or epithelial-specific mRNA transcripts was used as a surrogate for CTC presence in blood.[131,132] RT-PCR for *KLK2* and *KLK3* mRNAs in peripheral blood from patients with localised and metastatic prostate cancer has shown strong concordance (80–85%) with CellSearch™ enumeration results (positive result defined as ≥ 5 CTCs per 7.5ml blood sample).[133] In other studies, platforms such as the commercial Adnatest™ were used to immunomagnetically enrich for cells expressing markers such as EpCAM and HER-2 before detecting CTCs through prostate-specific transcripts (such as *PSMA, PSA,* and *EGFR*), with one study showing a 100% specificity and 100% sensitivity for detection of cancer in patients with benign prostatic hyperplasia (BPH) and high risk prostate cancer.[134–136] The Versatile Exclusion-based Rare Sample Analysis (VERSA) system is also based on the enrichment for $EpCAM^+$ cell populations in combination with quantitative RT-PCR targeting AR, AR splice variants and AR-regulated genes. In a prospective trial including 99 patients with mCRPC, the VERSA system was used to identify patterns of gene expression in CTCs, finding patients who had high expression of transcriptional AR targets were significantly more likely to have CRPC versus HSPC and had higher levels of serum PSA (median 216 ng/mL) compared to patients with low to absence of detected AR-regulated genes (median 11.22 ng/mL, $p < 0.0001$).[137] However, RT-PCR is limited by poor specificity[138], mainly ascribed to low sample purity, which results in lack of discrimination of transcripts derived from CTCs as opposed to contaminating leukocytes[139].

Studies in the past decade focused on CTC detection through FISH[34], targeting of mRNA transcripts[140], and identification of live cells actively secreting prostate cancer markers.[141] In one study, FISH analysis was used downstream of enrichment with the Parsortix platform and immunostaining to probe for nine genes and genomic regions commonly altered in prostate cancer, including 6q16, *NKX3.1, C-MYC, PTEN, CCND1*, *RB1*, 16q22.1, *ERG*, and *AR* in 81 patients with mCRPC or localised prostate cancer; changes were

observed in >30% of the genomic regions in the majority of mesenchymal ($VIM^+/CD45^-$) CTCs.[34] In a small group of patients with localised and metastatic prostate cancer, a novel system using a triple targeting nano-vector to isolate epithelial ($EpCAM^+$), mesenchymal ($EGFR^+$) and stem cell-like ($CD44^+$) CTCs was evaluated. By using this approach, intracellular 'molecular beacons' were created to target and visualise mRNA (such as RPL15 and E-cad) in living CTCs, and the number of $RPL15^+$ and $E\text{-}cad^+$ CTCs positively correlated with the presence of bone as opposed to nodal metastases, showing the ability to successfully detect live CTCs via mRNA visualisation and suggesting the potential for this strategy to provide accurate insight into tumour metastasis.[140] The visualisation of a heterogeneous CTC population, which reflects the heterogeneous tumour biology of the primary tumour, is crucial, but is limited by EpCAM-based targeting. EPithelial ImmunoSPOT (EPISPOT^{TM}) is an EpCAM-independent technology to enumerate CTCs by using a functional approach to detect viable cells that are actively secreting proteins such as PSA and FGF2 following CD45-cell depletion. In patients with localised prostate cancer before and after radical prostatectomy[141] or radiotherapy,[142,143] a higher CTC yield was observed with CellCollector^{TM} and dual fluoro-EPISPOT^{TM} than with CellSearch^{TM}, indicating the potential of EPISPOT to identify molecular heterogeneity of prostate cancer in CTCs and highlighting the importance of non-epithelial CTCs.

[H1] Molecular analyses of CTCs

CTCs offer a wealth of information beyond enumeration, as molecular profiling (Table 2) of these cells can provide a sample of tumour biology at the cellular level with minimally invasive methods. Most of the work undertaken to date has focussed on the profiling of CTCs within the field of metastatic prostate cancer.[38,144–149]

[H2] Transcriptome analysis of CTCs in metastatic prostate cancer

Transcriptomic profiling enables to identify genes and pathways implicated in CTC survival and dissemination.[150] Targeted PCR of CTCs has been widely used to predict prostate cancer survival.[151–153] Results from these studies have shown that prognostic gene signatures, including expression of genes such as *AR, ARv7, FOLH1 (PSMA), KLK2, KLK3,* and *TMPRSS2* in samples enriched for CTCs**,** can outperform PSA decline in predicting OS[153] and PFS[152] in patients with mCRPC. Furthermore, PSMA expression in CTCs has been shown to be an independent biomarker of poor prognosis, with significantly shorter PSA-PFS (12 weeks versus 30 weeks, p=0.008), and OS (13 months versus 27 months, p=0.010) in patients with vs. without $PSMA^+$ CTCs.[151] In a cohort of 43 patients with mHSPC, the expression of *ZEB1, SNAI1 and ADAMTS1* detectable in CTCs by RT-qPCR was shown to be significantly predictive of progression to mCRPC within 24 months (AUC= 0.77, p=0.0092; AUC= 0.71, p=0.039; AUC= 0.71, p=0.043, respectively).[47]

Transcriptomic analyses of CTCs might also serve to identify biomarkers of disease state and tumour molecular profile.[154–156] Results from some studies have shown that selected EMT-related genes (such as *PTPRN2, ALDH1, ESR2, WNT5A)* are more highly expressed in CTCs enriched from the blood of patients with mCRPC than mHSPC[154]; *AR* and *AR*-regulated gene expression is increased in matched CTCs and metastatic biopsy samples[155]; and *PSA* and *PSMA* expression is higher in CTCs enriched from the patients with mHSPC or mCRPC than in CTCs from patients with localised prostate cancer[156]. In another study, a multigene score was created to identify metastatic prostate cancer, including mHSPC and mCRPC. Results from this study showed that *KRT19* and *EpCAM* expression were 336.3-fold and 8.7-fold higher, respectively, in the blood enriched for CTCs from patients with metastatic versus localised disease, with *EpCAM* being detected in 100% of mCRPC CTCs.

Metastatic prostate cancer is widely treated with second-line therapies, yet no definitive method to predict therapy response, or to detect if early signs of treatment resistance in these patients exists. To address this gap, CTCs can be studied to identify biomarkers of therapeutic effectiveness and treatment response.[46,157–159] However, results from available studies are not consistent. In one study, the non-canonical (nc)-WNT signalling pathway was shown to be significantly enriched in 36 intact CTCs isolated from the blood of 5 patients (mean 6 CTCs/patient) progressing under enzalutamide AR inhibition vs. 41 CTCs from 8 untreated patients (p=0.0064), with the most significantly enriched downstream components of nc-WNT signalling being *RAC1, RHOA,* and *CDC42*.[157] Another study of pools of up to 10 CTCs isolated from patients who were resistant to abiraterone/enzalutamide therapies did not show significant enrichment of the these nc-WNT pathway targets (*RAC1, RHOA,* and *CDC42)* nor was the nc-WNT identified as enriched in pathway analysis.[158] However, both studies found increased expression of canonical WNT signalling-associated genes including *FZD4* & *LEF1*, and increased expression of *WNT5A* (a nc-Wnt pathway activator) and in CTCs from patients who developed AR-inhibitor resistance.[157,158] Increased *WNT5A* expression in CTCs has been shown to be independently predictive of OS ((HR 3.62, 95% CI 1.63 – 8.05, p=0.002) in 41 patients with mCRPC.[160] CTCs enriched from the blood of patients with resistance to enzalutamide and/or abiraterone have also been shown to have increased expression of *AR*-regulated genes, *AR* splice variants, neuroendocrine markers[159], and TGFβ and cyclin D1 (*CCND1*) signalling pathways[158]. These findings are from cohorts with small sample sizes, and further analysis of larger cohorts could be carried out to investigate CTC-based gene expression signatures as potential markers of treatment response and resistance, and to identify candidate pathways for therapeutic targeting.

Lineage plasticity has been identified in both preclinical models and tissue biopsy samples from patient cohorts with prostate cancer, but the mechanisms of how AR-directed therapies shape clonal selection and biological states that underlie lineage plasticity and treatment resistance is not well established.[161] Transcriptomic profiling of prostate cancer CTCs with qRT-PCR panels targeting AR splice variants (*AR-V7*[162,163] and *AR-V9*[137]) and AR-regulated genes (*AR-V7, AR-V9, TMPRSS2, KLK2, KLK3, and FOLH1)*[137] has shown that the expression of these targets is prognostic for response to ARSIs and capable of detecting transition to NEPC.[164] However, the role of *AR-V7* in prostate cancer prognosis is contentious as studies investigating *AR-V7* CTC positivity for prostate cancer prognosis have shown conflicting results

[H2] Genomic analyses of CTCs in metastatic prostate cancer

Genomic analyses of CTCs can offer crucial insight into tumour biology and clonal evolution of disease.[38,147,148] In this regard, alterations such as *PTEN* gene loss[38], *AR*-amplification[148], and *ERG*-rearrangements[148] in prostate cancer CTCs have been shown to match those on tumour tissue samples in mCRPC. Furthermore, results from some studies showed that 70% of somatic single-nucleotide variants (SSNVs) detected in prostate cancer CTCs were also present in matched primary tumour and lymph node metastases[149]; additionally, 84% agreement in *PTEN* loss was reported between CTCs and primary tumour[38], and 88% concordance in *ERG*-rearrangement was shown between metastatic biopsies and CTCs.[148] Non-100% concordance indicates genomic differences between primary tumour, CTCs, and metastatic sites, suggesting that CTCs could reliably reflect the biology of disease spread.

Genomic deletions present in primary prostate cancer and metastatic sites have also been detected in CTCs.[147] Microarray analysis identified 6q16 and *FXBL4* loss in six samples of prostate cancer bone metastases , and qRT-PCR found a significant reduction in *FXBL4* expression in five bone metastases and four primary prostate cancer samples as compared to BPH samples (p=0.001). [147] This study then used FISH detect *FXBL4* deletion in CTCs enriched from blood samples in 6 of 7 patients with bone metastatic prostate cancer, and 1 patient with lymph node metastases, showing that genomic deletions present in primary tumour.[147] Subsequent *in vitro* work in prostate cancer cells lines indicated that *FBXL4* is a putative prostate cancer tumour suppressor gene involved in migration and invasion regulation. [147] Taken together, these findings indicate that CTCs might be considered as proxies for tumour tissue in the detection of genomic alterations, with a potential role in selecting treatment for patients stratified by molecular subtype and distinguishing patients who carry indolent disease versus those with a malignancy that is likely to metastasise.

Genetic mutations in CTCs could be used to monitor evolutionary mechanisms of disease metastasis and genomic alterations over the course of therapy, independent of concordance with tumour tissue samples.[38,144–146,149] In a cohort of 16 samples from 12 patients with mCRPC, reproducible copy number alteration (CNA) gains and losses in CTCs associated with abiraterone- or enzalutamide-resistance have been identified, with gains noted in AR signalling pathway genes in all 16 samples, led by *AR* (50%), *FOXA1* (31.25%), *CYP11B1* (31.25%), and *UGT2B17* (31.25%).[146] While inter-patient molecular profiles were heterogeneous, such reproducible common CNAs in AR signalling genes might be useful as predictive biomarkers of therapeutic effectiveness (e.g., *AR*-directed therapies) or therapy selection, such as CNAs in loci containing known prostate cancer drug targets (e.g., *ERG* and *BRD4*).[146] In another study, sequential analyses of CTCs were carried out in a single patient over the course of therapy and progression from mHSPC to mCRPC. Results showed that

targeted ADT but not standard chemotherapy was associated with the drastic depletion of an initial clone 'A' (as assessed through phenotypic AR status, and genome-wide CNA profile) and the emergence of two molecularly distinct CTC populations, named clones 'B' and 'C'.[145] These analyses could provide insights into the clonal dynamics that underlie treatment resistance, but further studies with increased cohorts are needed. Comparing primary tumour cells to CTCs has shown that although single nucleotide variations (SNVs) in primary cells and CTC occurred sporadically, CNAs occurred cumulatively in primary cells and converged towards the CTC CNA profile, suggesting a convergent evolutionary pattern toward tumour metastasis.[144] In a proof-of-concept study including two patients with mCRPC, comparative analysis of SSNVs in CTCs led to the identification of trunk mutations also present in matched tissue samples (primary or lymph node metastasis).[165] Further investigation into this topic is needed, and offers promise, as founder mutations have served as successful targets in other cancers (for example, *BRAF* in malignant melanoma).[166] Thus, understanding clonal dynamics in CTCs is crucial in targeting evolutionary processes implicated in metastasis.

If matched primary or metastatic tumour tissue is not available, cell-free nucleic acid profiling can be combined with CTC genetic analysis to obtain comprehensive molecular insight.[167,168] However, this multiparametric liquid biopsy analyses have yielded differing results, perhaps owing to the uncertainty of origin of cfDNA and, consequently, its genomic alterations. In one study in which matched CTC and cfDNA samples were compared in 18 patients with mCRPC, SSNVs unique to CTCs (20.7%), unique to cfDNA (65.5%), and shared (13.8%) were identified, suggesting that CTC analysis alone might miss genomic alterations.[168] Conversely, in another study, somatic variants in prostate cancer-related genes were detected in CTCs in 92% of patients and in cfDNA in 45% of patients, with more SSNVs detected across all patients' CTCs (n=38) than across all patients' cfDNA (n=16). Overall, further study is needed to confirm whether SSNVs in prostate cancer-related genes

are more often detected in CTCs or cfDNA.[167] Matched cfDNA and CTC samples from 57 patients with mCRPC showed that CTCs were more frequently detectable and evaluable for CNA analysis (73.7% vs. 42.1%, respectively), indicating that CTCs may offer greater insight into clonal evolution. [169] CNA analysis has also showed high consistency of AR copy number gains between patient-matched cfDNA and CTC DNA data; however, most SSNVs were unique to either CTCs or cfDNA, further suggesting that CTCs and cfDNA may offer differing insights into the biology of prostate cancer[167,168,170]

Single-cell analyses of CTCs offer great promise to study phenotypic heterogeneity and investigate the EMT.[157,169] Analysiss of 257 prostate cancer CTCs at the single-cell level in 47 patients with mCRPC (range 1-22 CTCs/patient) showed combined (≥2 genes) TSG loss in *TP53*, *PTEN*, and/or *RB1* on the same CTC was associated with shorter median PFS (median 3.6 vs. 7.2 mo,, p=0.042) and greater genomic instability as defined by large-scale transitions scores (p=0.0015), demonstrating the ability of genomic molecular CTCs features (beyond enumeration) to offer clinically-relevant information.[169] RNA-seq of 77 single, intact CTCs isolated from 13 patients (mean 6 CTCs per patient) with metastatic and localised prostate cancer showed higher intercellular heterogeneity in individual CTC transcriptional profiles than single cells from prostate cancer cell lines (mean correlation coefficient 0.10 vs. 0.44, $p<1\times10-20$). [157]. This study also found that putative stem cell markers including *ALDH7A1*, *CD44*, and *KLF4* were present in 60% of CTCs and epithelial markers were expressed in 92% of CTCs analysed, while mesenchymal genes were not upregulated as compared to prostate cancer cell lines, suggesting an epithelial or stem-cell like phenotype of prostate CTCs. [157] However, this study isolated CTCs using a microfluidic chip coated with anti-EpCAM antibody, biasing captured CTCs towards and epithelial phenotype. However, single-cell analyses of prostate CTCs are currently technically limited by the low purity of

CTC-enriched samples, especially in those that elute live whole cells free of immune-affinity labels, and high single-cell technology costs.

[H2] Current limitations of molecular analyses of CTCs

Molecular analyses of CTCs are limited by low quantity and quality of enriched cells, alongside the costs of sequencing enriched cells, which still include many peripheral blood mononuclear cells. Attempts were made to overcome this limitation by performing low-pass whole genome sequencing (WGS) to predict the quality of single CTC DNA sequencing libraries before moving to costly whole exome sequencing (WES).[149] High concordance (96.3% of genes covered in both methods) was observed between targeted NGS panels and WES of CTCs (observed in 2 patients with mCRPC; 4 CTCs per patient), suggesting that these targeted panels could provide a cost-effective substitute for detecting clinically significant alterations in prostate cancer-related genes.[171]

Current studies focusing on molecular analysis of CTCs in patients with mCRPC rely on small sample-size cohorts, with often less than a few hundred cells examined in total.[149,157,171] Studies in which molecular analyses of CTCs are carried out in patients with localised prostate cancer are particularly limited in number, with RT-PCR of immunomagnetically enriched cells often used to differentiate between metastatic and localised disease states based on gene expression.[156,172] Notably, in one study, transcriptomic sequencing of CTCs— enriched from the blood of 98 patients pre-prostate biopsy and 155 patients with localised prostate cancer— led to the identification of a 12-gene panel that improved the prediction of biopsy outcomes of clinically significant prostate cancer (AUC 0.764 vs. 0.826, p=0.03) for PSA alone vs. combined with CTC score.[44] Future studies with larger sample sizes or integrative analysis of existing datasets could increase statistical

power, facilitating the identification of small effects or subtle associations of genomic or transcriptomic alterations with disease phenotypes and disease state.

CTC detection is limited by technological development and low CTC numbers in many patients. An integrated panel including other liquid biopsy techniques such as cfDNA, microRNAs (miRNA), or epithelial cells isolated from urine could combine the best of these several liquid biopsy options.[170,173 178,179180] Enrichment methods are also crucial to carry out downstream analyses, with selective upstream isolation potentially affecting prostate cancer CTCs that undergo molecular profiling, thus influencing phenotypic findings (e.g., epithelial vs. mesenchymal phenotype).[35,174]

[H1] Functional analyses and culture of CTCs

Functional analysis of CTCs offer the possibility to isolate specific pathways and manipulate metabolic function in prostate cancer CTCs.[175–177] In one study of metabolic markers, a glucose-metabolic (GM^+) CTC subtype was identified, characterised by expression of both of *PGK1* and *G6PD*; in this study, an increased number of hypermetabolic GM^+CTCs were associated with metastasis and advanced tumour stages ($p<0.05$), and outperformed EMT-based CTC subtypes in discerning metastatic from non-metastatic prostate cancer (AUC 0.780 for GM^+CTCs vs. EMT CTCs subtypes epithelial AUC 0.729 and mesenchymal AUC 0.648).[177] Investigations of proteins expressed on the surface of prostate cancer CTCs have focused on markers different from those typically used for enumeration (such as Pan-CK, EpCAM, CD45) and including PSMA, CD133, Ki67, and AR.[175,176] CTC PSMA expression in patients with metastatic prostate cancer shows substantial intra-patient heterogeneity, and the presence of PSMA-negative CTCs in patients with PSMA-positive primary tumours could explain disease progression despite treatment with PSMA-targeted therapies.[176] CD133-positive CTCs have been shown to have elevated

Ki67 expression and no change in AR expression nor AR cellular co-localisation with nuclear markers, suggesting that CD133 expression is a marker of CTC proliferation and is independent from AR pathway activity.[175] This study demonstrates potential use of CTC profiling to identify markers of proliferative potential. Further studies to identify other markers of proliferation and therapeutic-actionability could be used to achieve patient risk stratification and monitor response to therapy.

Perhaps, the most appealing prospect for functional CTC analysis would be *ex vivo* expansion of CTCs for biological analyses and drug-responsivity testing. In one study, cultivation and successful growth of candidate prostate cancer CTCs (isolated through CellCollector and identified by CK 8, 9, and/or 10 expression) was achieved in 1 out of 3 samples from a patient with treatment-naïve metastatic prostate cancer.[178] A PSA level of 0.48 ng/ml was detected in the culture medium after 10 days, which declined to 0.02 ng/ml after 3 weeks, indicating that PSA-secreting cells were successfully isolated and initially cultured but could not be sustained long-term.[178] In other studies,[179] organoid models have been used to attempt CTC culture in 3D. In one study, organoid culture of CTCs enriched for diagnostic leukapheresis (DLA) was achieved from 14 out of 40 patients with metastatic prostate cancer, with the majority of organoids maintained for 6–8 weeks, and two culture maintained for >6 months [180]. Validation through qRT-PCR showed that *AR*-V7 was expressed in one of the CTC organoids models, the *TMPRSS2-ERG* fusion transcript in three, and a majority of the isolated samples were positive for *AR* and/or *KLK3*, suggesting that patient-derived CTC organoids could potentially be used to infer information about the primary tumour, but further studies with matched samples are needed.[180] Furthermore, patient-specific somatic SNVs in *TP53* and *PTEN* identified in metastatic biopsy samples were found in matched organoid CTC cultures in three samples, further indicating that organoid CTC cultures could be representative of the tumour molecular profile.[179] Successful

ex vivo CTC expansion would offer great promise to unpack the functional biology of CTCs. However, attempts to expand prostate cancer CTCs often report low rates of successful culture and failure of long-term maintainence.[178–180] These studies are also limited by the challenge of definitively identifying cultured cells as CTCs.

[H1] CTCs and clinical prognosis in prostate cancer

To date, research on enumeration of CTCs to predict treatment response has focused on metastatic disease (Figure 2, Supplementary Table 3). In this studies, CellSearch® has been predominantly used to enumerate CTCs (typically identified as $CD45^-$, $EpCAM^+$, and CK 8, 18, and/or 19^+) and predict response to anti-androgen hormonal agents, radiotherapy, and chemotherapy in patients with mCRPC.[181–188] In several studies, detection of $\geq$5 CTCs per 7.5 ml of peripheral blood in patients with mCRPC (vs. patients with <5 CTCs/7.5 ml blood) was associated with shortened progression-free survival (PFS) and/or overall survival (OS) before treatment with cabazitaxel (chemotherapy) (OS 8.8 vs. 28 months, p=0.002, PFS not significant[181]); before treatment with abiraterone or enzalutamide (second-line hormonal therapies) (abiraterone: PFS p=0.043, OS p=0.027 [183];enzalutamide: PFS p=0.021, OS p=0.003[185]); or after first treatment cycle with cabazitaxel and docetaxel (chemotherapies) (docetaxel: OS p=0.004, PFS not evaluated [182]).[181–183,185] Detection of $\geq$5 CTCs/7.5 ml in blood was also associated with shortened PFS and OS (vs. <5 CTCs/7.5 ml) in patients with mCRPC before and after 12 weeks of radium-223 radiotherapy (PFS 17.1 vs. 25 weeks, p=0.061, OS 29.4 vs. 57.9 weeks, p=0.032).[184] In another study, high radium-223 therapy completion rate (6-month injection course) was reported in patients with a $\geq$5 baseline CTC count.[187] Importantly, in one of these studies, a decrease in CTC count between 3- and 6-

month measurements was associated with improved radiological PFS (rPFS) and OS, indicating the utility of accessible longitudinal sampling.[185]

In one study, CTC-positive status, irrespective of detection threshold (≥1 CTC), was associated with shorter rPFS (CTC-positive vs. CTC-negative, p=0.043) in patients with mCRPC receiving second-line hormonal therapy with abiraterone[183], and with shorter OS after first treatment cycle in patients receiving cabazitaxel chemotherapy (CTC-positive vs. CTC-negative, p=0.047)[181]. Importantly, in another study, the predictive accuracy of ≥5 CTCs/7.5 ml blood for OS after first treatment cycle with docetaxel (75%, p=0.02) was shown to be similar to that of biochemical and radiological progression after the fourth cycle of therapy (73%, p=0.03), suggesting that CTC enumeration could be an earlier predictor of survival and treatment response than current objective response approaches[182].

Few studies have been carried out to investigate CTC quantification in mHSPC.[47,189,190] Baseline CTC presence before docetaxel treatment has been shown to be independently predictive of shortened OS in patients with mHSPC.[47] In another study, baseline CTC presence before docetaxel treatment in patients with mHSPC was predictive of progression to mCRPC.[189] Importantly, patients with mHSPC with baseline undetectable CTCs were shown to have ~9-fold higher odds of achieving 7-month PSA <0.2 (versus PSA >4, p< 0.001) and 4-fold higher odds of achieving PFS at 2 years (p< 0.001) than patients with baseline ≥5 CTCs.[190] In another study in which prostate cancer CTCs from patients with oligometastatic HSPC were classified as epithelial, mesenchymal, and bi-phenotypic, mesenchymal CTCs had the highest accuracy in predicting progression to mCRPC (AUC: 0.64 versus 0.60 versus 0.61, respectively) and cancer-specific survival (CSS) (AUC: 0.86 versus 0.58 versus 0.67, respectively) after a 3-year follow-up time .[191] The potential utility of CTCs in predicting treatment response and identifying patients who are likely to progress to

mCRPC is an attractive prospect, but further studies with larger cohorts are needed to prove that CTC detection is a reliable tool to reach this aim.

The potential of CTCs in screening, diagnosis, or treatment of localised prostate cancer has been only assessed in a small number of studies (Supplementary Table 4).[41,192–195] Importantly, the presence of CTCs in pre-biopsy blood samples was superior to the Montreal nomogram (AUC 0.84 versus 0.78, p=0.018) in predicting high-risk prostate cancer (defined as not complying with Epstein criteria for active surveillance).[195] In other studies, CTC count and enumeration of AR-positive CTCs in 49 patients with localised prostate cancer were shown to be associated with the occurrence of BCR (AR-positive CTC count p=0.0.03) and the presence of metastases (total CTC count p=0.03, AR-positive CTC count p=0.0.03) following radical prostatectomy.[41] The incorporation of $PSMA^{+}/CD45^{-}$ CTC presence 90-120 days after surgery into a clinicopathological risk score (CAPRA) was shown to improve prediction of 5-year BCR in a cohort of 321 patients who underwent radical prostatectomy as monotherapy for prostate cancer (Harrell's C index of 0.73 for CAPRA score alone vs. 0.86 combined with CTC presence).[193] Thus, CTC detection shortly after surgery might provide insight into the benefits of early adjuvant treatment.

Studies on the use of prostate cancer CTCs for clinical prognostication suggest that the baseline presence of CTCs pre-treatment, and sustained CTC presence during treatment, are prognostic of treatment failure. [181–188] Identification of quantitative CTC cut-off values predictive of disease metastasis and progression have a clear translational utility, and large prospective studies are needed to reach this objective.

[H1] Detection of the AR and AR variants in prostate cancer CTCs

The *AR* gene is implicated in treatment failure and disease progression in mCRPC through mechanisms including *AR* overexpression, *AR* gene amplification, and expression of *AR* variants (AR-Vs).[196,197] Consequently, transcriptomic and proteomic expression of AR and AR variants in prostate cancer CTCs isolated from the blood of patients with mCRPC has been investigated to predict response to second-line hormonal agents, radiotherapy, or chemotherapy. Mutations within *AR* can be detected in CTCs isolated from patients with mCRPC[198] with particular attention to AR-V7, the most well-studied AR-V.[163,199–210] Importantly, AR-V7 CTC positivity has been associated with indicators of advanced and high-volume disease at the baseline —including high PSA levels and number of bone metastases —in enzalutamide-, abiraterone-, and chemotherapy-naïve patients with mCRPC.[213] The role of CTCs as a measure of AR activity in advanced prostate cancer is, perhaps, the most established area for CTC use in clinical prostate cancer practice.

In a study aimed at predicting treatment resistance, the presence of AR-V7-positive CTCs in 62 patients with mCRPC receiving enzalutamide or abiraterone was associated with lower PSA response rates (0% vs. 61%, $p<0.001$), shorter PSA-PFS (median, 1.4 vs. 6.0 months, $p<0.001$), shorter clinical or rPFS (median, 2.1 vs. 6.4 months, $p<0.001$), and reduced OS (9.2 vs >11.9 months, $p<0.001$). [163] Furthermore, results from a study including 36 patients with mCRPC receiving taxane chemotherapy or anti-androgens showed that patients with AR-V7-positive CTCs (vs. AR-V7-CTC-negative patients) had superior PSA response rates (41% vs 0%, $p<0$ .001) and longer PSA-PFS (HR, 0.19 [95% CI, 0.07-0.52], $p<0.001$) and PFS (HR, 0.21 [95% CI, 0.07-0.59], $p<0.003$) under taxane treatment compared to treatment with abiraterone or enzalutamide, whereas outcomes did not differ by

treatment type in men with AR-V7–negative CTCs, suggesting AR-V7 CTC positivity as a potential biomarker for treatment selection in mCRPC.[211] In a follow-up study, CTC presence and AR-V7 status (present or absent) were assessed through a CTC-based mRNA assay in 202 patients with mCRPC before starting first- and second-line anti-androgen therapy.[212] PFS and OS at a median follow-up time of 15–22 months were best in the CTC-absent population, intermediate in the CTC present/AR-V7 absent group, and worst in CTC present/AR-V7-present group.[212]

These findings indicate that CTC-AR-V7 status is promising for monitoring disease progression and burden but are not consistent with results from other studies showing mixed reliability of CTC-AR-V7 status in predicting response to next generation ADT.[214,215] In these studies, no association was reported between CTC AR-V7 positivity and post-therapy PSA changes, time on therapy, or rPFS.[216,217] In another study, AR-V7 protein expression in CTCs assessed through immunohistochemistry was not predictive of taxane or anti-androgen response in patients with mCRPC.[219] Importantly, in another work, absence, rather than and compared to presence, of AR-V7 transcript expression in CTCs —assessed through mRNA assay— was associated with shorter biochemical and rPFS and OS in patients receiving anti-androgens.[220] Results from further studies have shown that CTC AR-V7 was associated with reduced rPFS, PSA-PFS[226], OS[226,227], and time to treatment failure[227] in patients with mCRPC receiving abiraterone or enzalutamide, and reduced rPFS and OS in patients who progressed after first-line ADT and received radium-223 radiotherapy.[228] The largest prospective trial to evaluate AR-V7-positive CTCs was PROPHECY, in which both a modified AdnaTest mRNA assay and the Epic Sciences nuclear-specific protein assay were used to evaluate AR-V7 CTC positivity in 118 patients with mCRPC beginning treatment with enzalutamide or abiraterone.[162] In this study, shorter PFS (modified AdnaTest hazard ratio, 1.9 [95% CI, 1.1 to 3.3; p=0.032] and Epic Sciences 2.4 [95% CI, 1.1 to 5.1; p=0.020]) and OS (modified

AdnaTest hazard ratio, 4.2 [95% CI, 2.1 to 8.5] and Epic Sciences 3.5 [95% CI, 1.6 to 8.1), were reported in patients with AR-V7-positive CTCs (vs. AR-V7-negative CTCs). However, only 10–24% of patients had AR-V7 positive CTCs, and many patients with AR-V7–negative CTCs still did not respond to ARSIs, suggesting that AR-V7 status alone is not a sensitive predictor of resistance.[162] Additionally, the Epic Sciences AR-V7 assay is limited by dependence on nuclear localization for positivity and is no longer available for purchase.[229] Thus, the role of AR-V7 detection in prostate cancer CTCs in treatment selection and prediction of response has elicited considerable debate in the literature.[221–225] Small sample sizes, different isolation and detection methods, and study design or failure to reach endpoint could contribute to discordant results.

Most studies on CTCs have involved patients with mCRPC, but CTCs could also help identify which patients are likely to progress from mHSPC to castrate resistant disease. In 42 patients with oligometastatic HSPC, AR-positive CTC status assessed by immunofluorescence (Epic Sciences assay) at the baseline pre-radiotherapy was associated with shorter PFS compared to AR-negative CTC status (p=0.011, median PFS: AR+ = 9.3 vs. AR+ = 27.1 months).[230] In another study, AR-positive CTC status (vs. AR-negative CTC status) at the baseline was associated with shorter time from mHSPC to CRPC (4.9 versus 8.9 months, p=0.02) and significantly shorter CSS (14.3 versus 33.0 months, p=0.002), with all patients with AR-V7-positive CTCs dying of prostate cancer within the study follow-up monitoring.[231] Thus, AR-V7 presence in prostate cancer CTCs could serve as an important marker of disease progression to castrate resistance.

Taxanes affect cytoplasmic-to-nuclear AR-trafficking, which makes AR a potential target for taxane therapy. Patients with nuclear AR-V7 positivity in CTCs showed improved OS with taxane therapy compared with ARSIs, indicating that assessing nuclear AR-V7

positivity could inform treatment decisions relative to standard-of-care measures in mCRPC.[229,232] This association was no longer significant (p=0.55) if nuclear-agnostic criteria were used[224], suggesting that molecular detail beyond the presence or absence of AR or AR splice variants might be important for prognostication and to guide treatment decisions. In a study in which a multigene signature of disease progression was established to identify proteomic and transcriptomic changes in AR signalling and genomic alterations in 115 patients with mCRPC starting chemotherapy or AR pathway inhibitor therapy, the number of positive transcripts of AR-V7 or androgen-regulated genes *GRHL2, HOXB13,* and *FOXA1*, could predict OS (median OS: not reached vs 24.8 mo vs 16.2 mo for 0, 1, and ≥2 transcripts, respectively; p=0.0052).[233] In another study, microfluidic capture and single-cell immunofluorescence analysis of "AR-on (PSA+/PSMA-), AR-off (PSA-/PSMA+), and AR-mixed (PSA+/PSMA+)" CTC populations showed predominantly "AR-on" CTC signatures in patients with mHSPC, and heterogeneous "AR-on, AR-off, and AR-mixed" populations in patients with mCRPC.[234] Initiation of first-line ADT induced a switch from "AR-on" to "AR-off" CTCs, whereas secondary hormonal therapy in CRPC resulted in variable responses. These results suggest that CTC-based AR analysis could potentially be useful to guide treatment decisions, but validation in larger cohorts is needed.[234]

Single-cell analysis of CTCs enriched from patients with NEPC showed that ARv567es was the predominant transcript in the CTCs of these patients, with full length-AR-detected in 2 of 17 CTCs (12%), AR-V7 in 0 of 17 (0%) and AR-v567es in 10 of 17 (59%).[174] The presence of the AR v567es splice variant was also shown to predict shortened survival on taxane treatment in patients with mCRPC (PFS 12.71 vs. 7.29 for patients with ARv567es-negative vs. ARv567es-positive CTCs), although AR-V7 had the strongest influence in predicting outcomes (PFS 12.02 vs. 8.48 months for patients with AR-V7-negative vs. AR-V7-positive CTCs, HR = 0.38, p = 0.01).[236] Thus, the differential detection

of AR splice variants in prostate cancer CTCs could aid diagnosing tumour type and predicting treatment outcomes.

Overall, detection of AR and AR variants in CTCs offers potential insight into both prostate cancer biology and prognostics. However, trials to date have been limited by small sample sizes, incomplete follow-up, and conflicting results, and thus such methods are not currently used in clinical practice. The methodology of isolation is crucial to downstream biological analyses, as, for example, AR-V7 expression was shown to be lower in EpCAM-positive than matched EpCAM-negative CTCs, suggesting that AR-V7 prevalence is underestimated with EpCAM-based enrichment tools.[174] CellSearch® has been shown to capture CTCs with AR-amplification, whereas with ISET, only CTCs with AR gain of copies are captured.[35] Furthermore, in one study, poor concordance was shown between AdnaTest transcriptomic analyses of CTCs and matched mCRPC biopsy samples, with AR-V7 detected in matched tumour tissue samples of 63% of patients with AR-V7-negative CTCs, suggesting that CTC analysis alone might miss patients with treatment-resistant disease.[237] Results from further studies of genomic paired analyses of cfDNA and CTC DNA have shown discordant AR gene rearrangements in patients with mCRPC.[238] These findings suggest the potential need of parallel liquid biopsy analyses to capture the heterogeneity of genetic and transcriptomic alterations.

[H1] Conclusions

Understanding metastatic dissemination of prostate cancer is crucial to explain the biological mechanisms underlying potentially lethal phenotypes. CTCs are believed to be an important step in disease spread. Thus, analysis of these cells offers great promise for risk-stratification and disease monitoring. Inter- and intra-patient genetic and phenotypic heterogeneity of CTCs reflect the spatial and clinical heterogeneity of prostate cancer to some

extent, with genomic (i.e., CNAs and SSNVs) and transcriptomic (i.e., heterogenous expression of EMT markers) sequencing demonstrating intra-patient CTC heterogeneity on the single-cell level.

The prognostic significance of ≥5 CTCs/7.5 ml blood in patients with mCRPC at the baseline before treatment with chemotherapy, second-line hormonal therapy[185], and radiotherapy [187]for PFS and OS shows the potential utility of CTC enumeration for risk-stratification. Additionally, results from studies including patients with mHSPC highlight a potential role for CTC in predicting treatment response and progression to mCRPC. Further studies on the enumeration of CTCs in patients with localised prostate cancer are needed to confirm these findings, and also to potentially predict BCR following prostatectomy and identify patients who would benefit from early adjuvant treatments.

Analysing prostate cancer CTCs offers insight on multiple levels: through enumeration for prognostics; and through detection of AR alterations and splice variants (specifically AR-V7) to guide treatment selection. Studies on detection of AR and AR variants have produced conflicting results; however, increasing assay sensitivity might improve the detection of AR-V7 in CTCs to predict resistance to hormonal therapies, including abiraterone and enzalutamide. In the future, detection of AR splice variants in CTCs could also be used to gain insight into primary tumour biology and treatment resistance.

CTCs, as whole cells, offer great potential to gain insight into functional biology, and provide certainty that the cells analysed have metastatic potential. However, this promise has been undermined in past years by challenges in achieving sensitive and specific enrichment. Enumerative studies of CTCs that garnered popularity in the past decade have lost traction, probably owing to advancements in WGS that propelled research on ctDNA, and to the proven limitations of enumerative studies of CTCs for treatment selection and disease

progression. With ongoing advancements in isolation techniques, such as label-free microfluidics and the advent of single-cell RNA sequencing, molecular analyses of CTCs could now aim to improve the evaluation of biologically relevant pathways of prostate cancer metastasis. In this regard, molecular analysis of prostate cancer CTCs has led to identification of prognostic gene signatures predictive of survival in metastatic disease; disease state; and tumour molecular profile. Molecular profiling of CTCs could be used to identify prognostic biomarkers with the ability to enable risk-stratification of patients upon diagnosis and predict therapy response throughout the course of treatment. Furthermore, CTCs offer an attractive evolutionary link between the prostate and metastatic sites through analysis of copy number alterations and tumour mutational profile. In the future, this information could be leveraged to gain an improved understanding of the biological mechanisms underlying prostate cancer lineage plasticity and treatment resistance. In summary, CTCs are an essential link in the prostate cancer metastatic cascade of events and, therefore, can provide important insight into risk stratification at diagnosis, progression, response to treatment and lethality of this common and ubiquitous disease.

Table 1: Cell-surface and intracellular markers to target prostate cancer CTCs for isolation and detection.

Marker abbreviation	**Marker name**	**Alternative names**	**Details**	**Marker use**	**Refs**
EpCAM	Epithelial cell adhesion molecule*	CD326	Transmembrane protein involved in cell-to-cell adhesion, signalling, migration and growth; strong expression in various carcinomas including PCa compared with normal epithelia	Isolation	68-71, 83
N/A	E-selectin	Endothelial-leukocyte adhesion molecule 1 (ELAM-	Glycoprotein that promotes endothelial		76, 84

		1), CD62E or leukocyte-endothelial cell adhesion molecule 2 (LECAM-2)	leukocyte interactions; has an important role in the adhesion of tumour cells to the vascular endothelium		
PSMA	Prostate-specific membrane antigen*	Glutamate carboxypeptidase II	Transmembrane protein expressed by the prostate tissue		62, 77-79, 85
PSA	Prostate-specific antigen*	Kallikrein III or gamma-seminoprotein	Androgen-regulated serine protease produced by prostate epithelial cells and prostate cancer cells		62, 77, 86
N/A	Vimentin*	Fibroblast intermediate filament	Intermediate filament protein typically upregulated in cells undergoing EMT		57, 75, 87
EGFR	Epidermal growth factor receptor	HER-1, ErbB-1	Transmembrane glycoprotein and type I tyrosine kinase receptor involved in cell survival and growth		59, 73, 80, 88, 91
N/A	c-MET	Hepatocyte growth factor receptor (HGFR), Met proto-oncogene, or Tyrosine-protein kinase Met	Receptor tyrosine kinase expressed on the surface of mesenchymal cells, fibroblasts, and smooth muscle cells, acts through a paracrine mechanism to activate HGF—c-Met signalling		80, 89
N/A	OB-cadherin	Cadherin-11	Ca^{2+}-dependent homophilic cell adhesion molecule, expressed in osteoblasts and prostate cancer cells		56, 90

HER-2	Human epidermal growth factor receptor-2	HER2, ErbB-2 or Neu	Receptor tyrosine kinase expressed in normal and malignant epithelial cells, regulates cell proliferation, survival, and differentiation		73, 91
TROP-2	Tumour-associated calcium signal transducer 2	Epidermal glycoprotein 1 (EGP-1) or membrane component chromosome 1 surface marker 1 (M1S1)	Cell surface glycoprotein that regulates tumour growth and metastatic ability of prostate cancer cells		81, 92
PSCA	Prostate stem cell antigen	N/A	Glycosylphosphatidylinositol (GPI)-anchored cell surface protein up-regulated in several major cancers including prostate, bladder and pancreatic cancers		73, 93
CD133	Cluster of differentiation 133	Prominin-1	Transmembrane protein expressed in cancer stem cells, regulates metastasis, drug resistance, and stemness properties in various cancer cells	Detection	113, 114, 120
E-cad	E-cadherin	Cadherin-1 or CD324	Ca^{2+}-dependent cell-cell adhesion molecule involved in epithelial cell behaviour, tissue formation, and suppression of cancer		113, 121
AMACR	Alpha-methylacyl-CoA racemase	P504S, 2-Methylacyl-CoA Racemase or RACE 2	Mitochondrial and peroxisomal enzyme involved in branched-chain		116, 122

			fatty acid and bile acid metabolism; useful diagnostic biomarker for prostate cancer and other malignancies		
CXCR4	C-X-C chemokine receptor type 4	CD184 or fusin	G protein coupled receptor for ligand SDF1-α, involved in cancer cell adhesion, invasion, and proliferation		117, 123
CD34	N/A	Gp105-120 and My10	Transmembrane phosphoglycoprotein, mediates attachment of stem cells to extracellular matrix in bone marrow or tissue		117, 124
CD117	N/A	C-KIT, tyrosine-protein kinase KIT or mast or stem cell growth factor receptor (SCFR)	Prostate cancer stem cell marker, type III tyrosine kinase receptor involved in cell signalling, survival, metabolism, proliferation, apoptosis, migration, and differentiation		117, 125
IGF-IR	Insulin-like growth factor-IR	Insulin-like growth factor 1 receptor (IGF-1R)	Essential for development, survival, and proliferation of many cell types, and upregulated in prostate cancer tissue		118, 126
SYP	Synaptophysin	P38	Integral synaptic vesicle protein, marker of neuroendocrine prostate cancer		119, 127

*Also used for detection of prostate cancer CTCs
N/A: not available

Table 2 – Molecular analyses on CTCs from patients with localised and metastatic prostate cancer.

Analyses	**Genes or targets studied**	**Genomics or transcriptomics (single cell or bulk)**	**Disease state** (n=# patients)	**Comparator**	**Findings**	**Refs**
FISH Isolation: Enrichment free Epic platform (Epic Sciences)	PTEN, ERG, AR	Genomics (single cell)	mCRPC	Matched archival and fresh cancer tissue	PTEN loss associated with worse survival in univariate analysis (median survival 7.0 vs 12.1 month), but not in a multivariate analysis including additional parameters. PTEN status concordant in CTCs and fresh tissue in 32 of 38 (84%) patients. AR expression in CTCs did not correlate with PTEN or ERG status.	38
qPCR of total RNA from whole blood	KLK2 and TMPRSS2	Transcriptomics (bulk)	mCRPC	Healthy volunteers	A 2-gene panel of KLK2 and TMPRSS2 was created. Unfavourable results (defined as ≥1 marker positive) independently predicted OS in multivariate analysis. Conversion to a favourable result during treatment was associated with improved OS, suggesting the score can predict treatment benefit.	150
RT-PCR of mRNA	EZH2	Transcriptomics (bulk)	Metastatic PCa (n=20)	Healthy volunteers (n=10)	EZH2 expression was greater in patients with	168

Isolated through anti-Ber-EP4 immunomagnetic beads			and localised PCa (n=20)		metastatic PCa (mean expression density 2040.5 ± 1881.3 intensity/mm^2)than in localised PCa (349.4 ± 156.7 intensity/mm^2, p=0.019) and healthy controls (345.7 ± 131.8 intensity/mm^2, p=0.023).	
WGA with low-pass WGS for CNV analysis NGS for SSNV analysis	WGA: 58-gene panel NGS: 27-gene panel (Cynvenio)	Genomic (WGA: single cells; NGS – bulk)	mCRPC (n=20)	Matched tumours where available	For each patient, a profile of cfDNA, cfRNA, CTC DNA, and germline (WBC) DNA was developed. Alterations unique to CTC DNA (20.7%), unique to cfDNA (65.5%), and shared (13.8%) were detected	165
Single-cell RNA profiling through qPCR	RT^2 Profiler Human Prostate Cancer PCR Array	Transcriptomic (single cell)	Prostate cancer (no further detail)	Individual CTCs	Profiling showed subgroups of CTCs with differing phenotypes within patient samples, suggesting that this method can be used to study intra-patient heterogeneity	147
IF (CTCs) IHC (primary tumours)	PSMA	N/A	Clinically-proven mPCa	Primary tumours	CTC PSMA expression was heterogeneous. In patients with CTCs, 12 of 20 (67%) had $PSMA^+$ CTCs. All patients had heterogenous CTC PSMA phenotypes. All "favourable" (<5 CTCs/ml blood vs. unfavourable,	173

					≥5 CTCs/ml) patients in this study all PSMA-negative CTCs. Several patients with $PSMA^+$ primary tumours had $PSMA^-$ CTCs.	
WGA	Whole exome	Genomic (single cell)	mCRPC (n=2)	Matched primary and metastatic tissue samples	A new census-based WES protocol to call SSNVs from prostate CTCs was introduced.	146
aCGH	CNVs (gains and losses)	Genomics (bulk)	Abiraterone or enzalutamide-resistant mCRPC (n=16)	Matched leukocytes	Genomic gains occurring in >25% of patients: *AR, FOXA1, ABL1, MET, ERG, CDK12, BRD4, ZFHX3* Genomic losses in: *PTEN, ZFHX3, PDE4DIP, RAF1, GATA2.* The molecular profile is complex, but reproducible common CNVs might be useful as predictive biomarkers of therapeutic effectiveness.	36
RNA	HyCEAD assay (with tailored PCa gene panel, 64 genes).	Transcriptomics (bulk)	mCRPC	CTCs from other patients with mCRPC	A custom panel consisting of *AR, ARv7, FOLH1 (PSMA), KLK2, KLK3,* and *TMPRSS2* was developed.	149
WGA	CNA, SNV, and SV analyses.	Genomics (single cell)	Colon, breast, gastric or prostate (n=5) cancer	Matched single primary tumour cells and CTCs	SNVs in primary cells and CTC occurred sporadically, whereas CNAs occurred	141

					accumulatively in primary cells and converged towards the CTC CNA profile. This homogeneity might be valuable in understanding and targeting evolutionary processes implicated in metastasis.	
Epic Sciences single-cell CNA analysis pipeline	Genome-wide CNAs Gene-based copy number analysis: 578 cancer genes from Roche Comprehensive Cancer Design Panel	Genomics (single cell)	mCRPC clinically-defined as AVPC (n=21) and 'conventional' (non-AVPC) mCRPC (n=26)	WBCs	Loss of ≥2 of these genes was more frequently resolved in CTC DNA (35% of patients) than ct DNA (21% of patients). Higher genomic instability (assessed through CTC LST) was observed in patients with clinically defined AVPC.	166
RT-PCR	84 EMT-related and reference genes	Transcriptomics (single cell)	PCa (n=8; mCRPC=5, metastatic castration sensitive=2, non-metastatic castration sensitive=1)	Housekeeping gene (UBB)	Individual CTCs were highly heterogeneous, but select EMT-related genes (*PTPRN2*, *ALDH1*, *ESR2*, *WNT5A*) were more highly expressed in patients with castration-resistant cancer than castration-sensitive cancer.	151
RNA-seq of single cells(amplification + next	N/A	Transcriptomics (single cell level)	77 CTCs from 12 patients with metastatic PCa and 1 patient with	Bulk transcriptomes of primary PCa from a separate	60% of the 77 CTCs analysed robustly expressed putative stem-cell markers,	Miyamoto

generation RNA-seq)			localised PCa	cohort (n=12 patients).	92% expressed epithelial markers, but mesenchymal genes were not upregulated compared with primary tumours. Non-canonical Wnt signalling was activated in mPCa patients progressing under enzalutamide AR inhibition relative to enzalutamide untreated patients in a retrospective analysis (p= 0.0064).	
WGA, then NGS	Genome-wide CNV profiles	Genomics (single cell)	mCRPC (n=1)	CTCs compared at 4 sequential timepoints	Targeted ADT but not standard chemotherapy was associated with the drastic depletion of an initial clone 'A' and the emergence of 2 molecularly distinct clones 'B' and 'C' (assessed through phenotypic AR status and genome-wide CNV profile).	142
RT-PCR	*PSMA, AR-V7, AR, EGFR*	Transcriptomics (bulk)	mCRPC (n=79)	N/A	In mCRPC, PSMA expression in CTCs (vs. $PSMA^-$ CTCs) is an independent biomarker of poor prognosis (shorter PSA-PFS (12 weeks vs 30 weeks, p=0.008) and OS (13 months vs 27	148

					months, p=0.010)).	
CTCs: FA-FISH Tissue: FISH, IHC	*ERG* (FISH and IHC), *AR* (FISH)	Genomics (single cell)	mCRPC (n=54, n=28 for CTC sampling)	Matched tissue biopsy samples	CTCs and metastatic biopsy samples showed 88% concordance in ERG-rearrangement, with further ERG-rearrangement patterns detected in CTCs, indicating greater heterogeneity in CTCs than biopsy samples.	145
RT-PCR	*AR1/2, AR 4/5, AR-V7, AR-V9, KLK2, KLK3, TMPRSS2, FOLH1, NKX3.1, SYP, CHGA, CHGB, MYCN, NKX2.2*	Transcriptomics	mCRPC (11 patients with paired baseline and progression CTC samples)	Baseline vs at progression after ENZ	At progression, increased expression of AR-regulated genes, AR splice variants, and neuroendocrine markers were shown in CTCs. In patients with metastatic biopsies harbouring AR alterations, increased AR and AR-regulated gene expression was found in matched CTCs as well.	152
NGS	Targeted amplicon cancer hotspot panel, 52 cancer-related genes	Genomics (single cell)	mCRPC (n=22)	Paired cfDNA, leukocyte, bcDNA.	SSNVs in PCa-related genes were detected in CTCs in 92% of patients and cfDNA in 45% of patients. CNA analysis showed high consistency of AR copy number gains between patient-matched cfDNA and CTC DNA; however, most	164

					SSNVs were unique to either CTCs or ctDNA.	
RT-ddPCR	*AR, AR-V7, PSA, PSMA, EpCAM, KRT19*	Transcriptomics (bulk)	Localised PCa (n=26), mHSPC (n=10), mCRPC (n=28)	Localised vs mHSPC vs mCRPC vs healthy donor	A multigene score was defined as the sum of the individual gene expression scores for the six genes. Logistic regression analysis showed that the multigene model was better at identifying metastatic prostate cancer than individual gene models.	153
Flow cytometry (ImageStreamX)	*PROM1* (CD133)	Proteomics	Patients with mCRPC (n=20)	CD133+ vs CD133- CTCs	CD133 was detected in all patients. CD133+CTCs had elevated Ki67 expression vs CD133-CTCs, suggesting a greater proliferative potential. AR expression and AR nuclear colocalization have similar levels in CD133+ and CD133- CTCs, suggesting CD133 expression is a marker of CTC proliferation and is independent from AR pathway activity.	172
Multi-RNA-ISH	*HK2, PDP2, G6PD, PGK1, PHKA1, PYGL,*	Metabolomics	mPCa (n=29)	Non-metastatic PCa (n=25)	$GM^{+}CTC$ (determined through the expression of 8 metastasis-related metabolic genes) were	174

	PDK1, PKM2				detected in 64.8% of patients. Increased hypermetabolic GM^+CTCs were associated with metastasis and advanced tumour stages ($p<0.05$). The triple tPSA-Gleason-GM^+CTC marker outperformed tPSA-Gleason-H-CTC marker (AUC=0.904 vs 0.874) in discriminating mPCa vs. localised PCa.	
NGS	62 PCa-associated genes and recurring gene fusions with ETS family members (via tiling)	Genomics (single cell)	mCRPC (n=2)	WES of the same samples	High concordance was found between WES and targeted NGS, with almost all alterations detected in each dataset being present in the other one.	167
RNA-Seq	N/R	Transcriptomics (bulk)	mCRPC (n=36; after RNA-seq data QC=12, DGEA of drug-resistant and drug-sensitive CTCs =5 samples each (unpaired))	Sequential samples, treatment responders vs patients showing resistance	CTCs in patients with treatment-resistant tumours showed upregulation of TGFβ and CCND1 signalling pathways. Resistant CTCs had significantly increased WNT transcripts (*WNT7B* and *WNT5A*) and increased canonical WNT signalling associated genes (including *FZD4* and *LEF1*).	155

FISH	FBXL4	Genomics	Bone metastatic PCa (n=7)	2 patients with lower Gleason score (3+3; 4+3)	*FBXL4* deletion was detected in CTCs of 6 out of 7 patients with bone metastatic PCa, and in CTCs from a patient with lymph node only metastasis; no loss was detected in the two patients with lower Gleason scores. Subsequent in vitro work suggested FBXL4 as a putative PCa TSG involved in migration and invasion regulation, suggesting a potential role for CTC FBXL4 loss as a potential prognostic marker in liquid biopsy.	144
WES	N/A	Genomics (single cell)	mCRPC (n=2)	2 patients with CTCs, 1 patient with paired lymph node metastasis and 9 cores of primary tumour	70% of SSNVs detected in prostate cancer CTCs were also present in matched primary tumour and lymph node metastases	146
RNA-seq	78 prostate cancer-related target genes	Transcriptomics (bulk)	mCRPC (n=41)	N/A	*TMPRSS2:ERG* fusion was expressed in 41% of CTC samples, and the aggressive prostate cancer associated long non-coding RNA *SChLAP1* was upregulated in	157

		70% of CTC samples. CTC expression *WNT5a*, *AURKA*, and *BMP7* were independently predictive of overall survival.

Abbreviations: Prosate cancer (PCa). Castration-resistant prostate cancer (CRPC). Metastatic castration-resistant prostate cancer (mCRPC). Androgen receptor (AR). Homozygous (HO). Heterozygous (HE). Diethylstilbestrol (DE). Quantitative polymerase chain reaction (qPCR). Immunofluorescence (IF). Whole Exome Sequencing (WES). Somatic SNVs (SSNVs). Array-based comparative genomic hybridisation (aCGH). Copy Number Variant (CNV). Copy Number Alteration (CNA). Progression-free survival (PFS). Single Nucleotide Variant (SNV). Structural variant (SV). Aggressive variant prostate cancer (AVPC). Large-scale transitions (LST). Epithelial-mesenchymal transition (EMT). Differential gene expression (DGE). Androgen deprivation therapy (ADT). Prostate-specific antigen progression-free survival (PSA-PFS). Overall survival (OS). Fluorescence in situ hybridization (FISH). Filter-adapted FISH (FA-FISH). Immunohistochemistry (IHC). Enrichment-immunofluorescence in situ hybridization (SE-iFISH). Cell-free DNA (cfDNA). Reference Single Nucleotide Polymorphism (rs). metastatic hormone-sensitive prostate cancer (mHSPC). Buffy coat DNA (bcDNA). Next Generation Sequencing (NGS). Droplet digital PCR (ddPCR). Real-time droplet digital PCR (RT-ddPCR). Multi-RNA in situ hybridisation (multi-RNA-ISH). Receiver operating characteristic (ROC). Area Under the Curve (AUC). Total PSA (tPSA). Hybrid CTCs (H-CTCs) [regarding epithelial and mesenchymal phenotypes]. Enzalutamide (ENZ). Abiraterone acetate (ABI). Tumour suppressor gene (TSG). Benign prostatic hyperplasia (BPH)

Figures

Figure 1 – A. CTCs are captured from blood using antigen-dependent and/or antigen-independent isolation and enrichment methods. B. Captured cells are identified as CTCs (detected) based on immunofluorescence staining of cell-surface markers or RT-PCR of prostate-specific or -related transcripts. C. Captured cells are profiled using genomic, transcriptomic, and proteomic techniques.

PSMA, prostate-specific membrane antigen; EpCAM, epithelial cell adhesion molecule; PSCA, prostate stem cell antigen; FGF2, fibroblast growth factor 2.

Fig 1

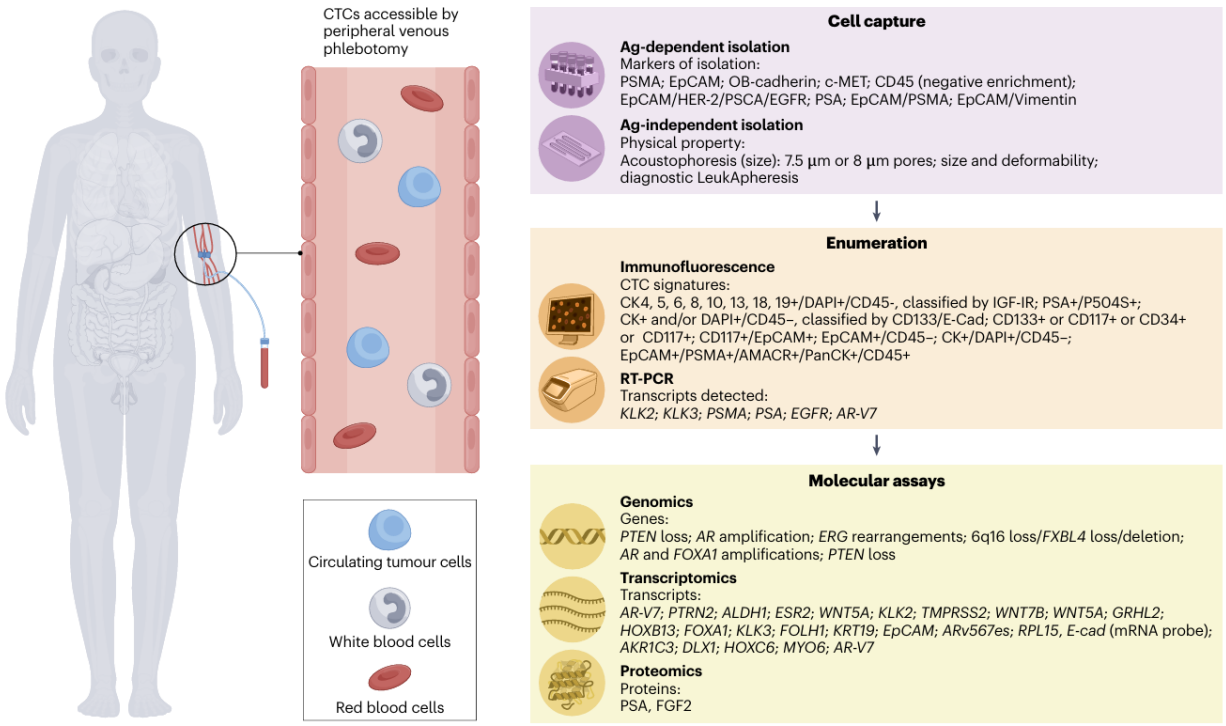


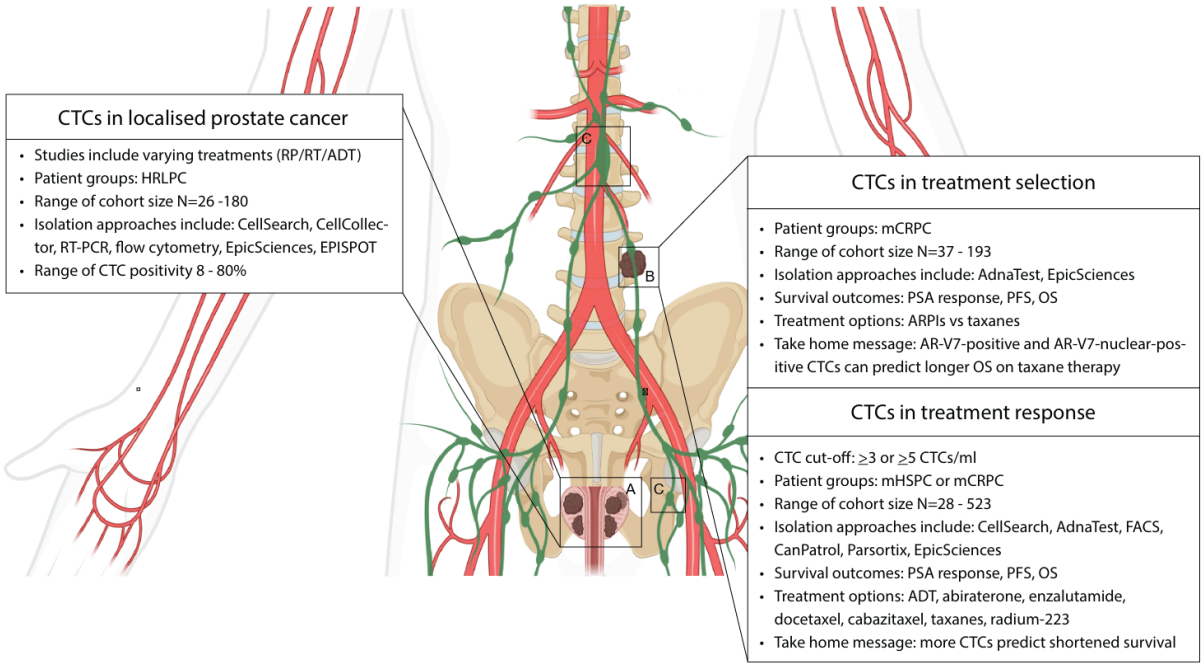
Figure 2 – Current clinical utility for circulating tumour cell (CTC) detection in localised prostate cancer and metastatic prostate cancer

A. CTC positivity (%) from blood of patients with localised PCa. Most studies stratified as "high-risk". See supplementary table 4.

B. CTC analysis from blood of patients with metastatic PCa to predict treatment selection/response. See supplementary table 3.

C. Potential for future studies on dissemination of tumour cells via lymphatic fluid, possible routes including pelvic lymphatic aspiration or cisterna chyli drainage.

CTCs, circulating tumour cells; RP, radical prostatectomy; RT, radiotherapy; ADT, androgen deprivation therapy; HRLPC, high-risk localised prostate cancer; PSA, prostate-specific antigen; PFS, progression-free survival; OS, overall survival.



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Supplementary Materials

Supplemental Table 1 - Summary of methods used to isolate or enrich for prostate cancer CTCs. Capture efficiency (% cancer cells captured from sample/cancer cells in sample), purity (measurement of background contaminant white blood cells) and comments on platform advantages and notable characteristics are detailed.

Isolation Method	Capture Efficiency	Purity	Comment	Reference
Antigen-Dependent (Immuno-affinity) Isolation Methods				
Positive enrichment with anti-EpCAM MNPs; negative Enrichment with anti-CD45 MNPs	84-98%	95 WBCs per mL	Automated platform which facilitates negative and positive selection and on-chip immunostaining. Integrated RNA and DNA extraction processes. mRNA was extracted from cells captured from patients with PCa following positive selection via EpCAM, transcripts detected included *AR* variants and downstream *AR* transcripts.	[1]
Antibody (anti-EpCAM) functionalised polymer on an in-vivo device (CellCollector)	10-35%	50-94%	Suitable for temporary venous implantation to isolate CTCs from a larger sample volume. Mean CTC counts 4.6 CTCs in patients with localized PCa, 16.8 CTCs in patients with locally advanced PCa, and 26.8 CTCs in patients with metastatic PCa. Detected tumor-associated transcripts of EGFR and PSMA in patients with metastatic PCa in 42.8% and 14.3% of the analyzed samples, respectively. Demonstrated CTC capture *in-vivo*, optimal for isolation from large volumes of blood. 78.9% (56/71) of patients with metastatic disease (PCa-m) and 46.3% (24/53) of patients with localized disease (PCa-l) had ≥1 captured CTC. CTCs detected in 65.7% of samples with CellCollector in comparison to 44.4% with CellSearch.	[2]
Positive enrichment by multi-antibody (anti-EpCAM only vs. anti-PSMA, anti-PSA, anti-PSCA and anti-	Single antibody (30%), multi-	-	Comparison of CellCollector (anti-EpCAM (CC-EpCAM) vs. anti- PSMA, PSA, PSCA, and EpCAM versions (CC-EpCAM)), demonstrating an	[3]

EpCAM) coated hydrogel modified steel wire	antibody (48%)		increase in capture efficiency when a multi-antibody approach was employed. ScreenCell, and Ficoll enrichment methods used for cultivation experiments.	
Immunomagnetic isolation (anti-EpCAM) on a PDMS microfluidic chip	97%	-	Use of micromagnets improved the trap density on the chip, reducing opportunity for cell and nanoparticle aggregation. Provides spatial information that could be used to phenotype cancer cell based on biomarker expression levels	[4]
Dual antibody (anti-EpCAM and anti-PSMA) functionalised microfluidic platform	74% (single antibody), 85% (dual-antibody)	-	Demonstrated that dual antibody method yielded a greater capture efficiency than single. qRT-PCR of bulk long non-coding RNA from captured CTCs demonstrated *SChLAP1* and *PSA* expression level in prostate cancer CTCs are significantly correlated with lymph node metastasis and bone metastasis.	[5]
Antibody (anti-E-Selection) modified nanotubes	50%	18-80%	Isolated 20–704 viable CTCs per 3.75-mL sample. Comparatively high flow rate (4.8 mL/hour) used for CTC isolation, enabled by rapid Selectin binding. Comparison to CellSearch demonstrated substantial discord with the selectin-functionalized device: 5 of 12 samples processed by CellSearch were negative for CTCs, whereas these 5 patients were positive for CTCs with the selectin-functionalized device.	[6]
Immunomagnetic isolation (anti-EpCAM) in bulk solution and FACS (DAPI-positive, EpCAM-positive, CD45-negative) in bulk solution	-	-	WGA of DNA from CTCs was less efficient when CTCs were isolated after 48 hours post-phlebotomy. Cell counts via CellSearch™ correlated closely with cell counts via IE/FACS. CTCs from 9 patients were observed to have multiple CNAs including those previously reported in primary prostate tumors such as gains in 8q and losses in 8p. High-level copy number gains at the AR locus observed in 7 (78%) cases	[7]

			in CTCs, but not matched archival primary tumours.	
Antibody (anti-EpCAM)/PLGA modified microfluidic chip	75%	-	Laser assisted single cell isolation with downstream WGA. Shared mutations between CTCs and WBCs were fewer in number than shared mutations among CTCs.	[8]
Microfluidic channel with self-assembled array of antibody (anti-EpCAM) functionalised MNP columns	70%	<100 cells in 7.5ml	A pre-enrichment step was completed to obtain a high purity output. Detected cancer-related PIK3CA gene mutation in 20 cells captured in the chip using RT-PCR.	[9]
Antibody (anti-EpCAM, anti-PSMA and anti-PSA) functionalised herringbone microfluidic chip	80%	11%	Multiple CTC cell surface antigens targeted on a single chip to account for heterogeneity. CTC counts ranging from as low as 6 CTCs/mL (patients with localized prostate cancer) to as high as 280 CTCs/mL (patients with metastatic prostate cancer). CTC clusters with sizes of 40–50 cells were also captured.	[10]
Antibody (anti-EpCAM and anti-Vimentin) modified liposome magnetic nanoparticles	90.85%	-	Liposome magnetic beads specific to two CTC surface antigens were employed to enhance CTC capture. Integrated FISH. Multiple antibody isolation was significantly higher than the capture efficiency of EpCAM or Vimentin based isolation alone.	[11]
Antibody (anti-EpCAM and anti-PSMA) modified rGO microfluidic chip	90.50%	-	rGO demonstrated large surface area for antibody attachment and CTC capture. Optimal capture efficiency at antibody feeding ratio of 50% anti-EpCAM/anti-PSMA. In patients with a PSA in the gray-zone of 4-10 ng/mL, combination of CTC detection based on wrinkled rGO coatings and PSA-based tests via machine-learning analysis could increase sensitivity to 91.7% from 58.3% in regular PSA tests.	[12]
Immunomagnetic (anti-EpCAM, anti-EGFR, anti-FGFR) isolation	98.30%	-	Multiplexed antibodies may enable a more heterogenous CTC capture. Isoalted CTCs	[13]

			with anti-EpCAM magnetic beads with the same efficiency as CellSearch® in 9% of the samples and with even higher efficiency in 45%. Total amount of CTCs (isolated with anti-EpCAM, EGFR and FGFR) was higher than that detected by CellSearch® in 68.18% of samples.	
Immunomagnetic (anti-EpCAM and anti-Vimentin) isolation	50-100%	200 WBCs per mL	Dual antibody immunomagnetic approach was shown to achieve high capture efficiency with low WBC contamination. Achieved an average capture of 13.29, 11.13, and 27.95 CTCs/mL isolated using EpCAM alone, vimentin alone, and both antibodies, respectively.	[14]
Antibody (anti-FAPα and anti-EpCAM) functionalised microfluidic channels	83%	93%	Enabled CTC release and downstream detection. *PSA* and *PSMA* mRNA were expressed in both CTCs isolated with FAPα and EpCAM.	[15]
Immunomagnetic (anti-c-MET) isolation	20-40%	-	Use of c-MET (oncogene involved in tumorigenesis) for isolation.	[16]
Immunomagnetic (anti-OB-cadherin) isolation	50%	-	OB-cadherin (Marker associated with mesenchymal cell behaviour) used for CTC isolation. AR amplification, TMPRSS2-ERG fusion, and homozygous PTEN deletion was present in both EpCAM and OB-cadherin captured cells	[17]
Antibody (anti-EpCAM, anti-HER-2, anti-PSCA, and anti-EGFR) functionalised substrate	<80%	9-74%	Combination of multiple antibodies with a dendrimer and E-selectin modified surface enabled high recovery yield of CTCs. Antibody mixture of anti-HER-2/anti-EGFR/anti-EpCAM outperformed anti-PSCA/anti-EGFR/anti-EpCAM from prostate cancer patient samples.	[18]
Antibody (anti-PSMA) modified microfluidic geometries	85%	62%	Size dependant collisions of CTCs with posts was optimised by tuning chip geometry. CTCs found in the blood of 90% patients with mCRPC.	[19]
Antibody (anti-EpCAM) modified	-	86%	Fluidic architecture of the disposable chip was optimised	[20]

cyclic olefin copolymer microfluidic channels			to eliminate bubbles from the device to enhance CTC capture.	
Multi-antibody (anti-EpCAM, anti-PSA and anti-PSMA) modified microfluidic channel	80%	8.70%	Enabled nanomechanical characterisation of CTCs by AFM. Demonstrated average capture of 2.2 CTCs/mL, 1.1 CTCs/mL, and 1.8 CTCs/mL using anti-EpCAM, anti-PSA, and anti-PSMA antibodies, respectively (from patients with localized prostate cancer), and 12.5 CTCs/mL using anti-EpCAM antibodies (from patients with metastatic prostate cancer).	[21]
Immunomagnetic (anti-EpCAM and anti-TROP-2) isolation	-	-	Similar gene expression patterns were found in EPCAM and TROP-2–captured CTCs. In one patient with NEPC, more CTCs were captured with EPCAM antibodies (606 CTCs) than with TROP-2 antibodies (380 CTCs).	[22]
Antigen-Independent (Physical) Isolation Methods				
Physical isolation by filtration with 7.5 um pores (ScreenCell)	74-91%	-	Rapid isolation completed in 2-3 minutes, method was shown to preserve micro-emboli of CTCs. Conducted downstream staining of telomeres in CTC nuclei.	[23]
Microfluidic size- and deformity- based isolation (Parsortix™)	54%	3.10%	Direct comparison of Parsortix™ to IsoFlux and CellSearch®. Demonstrated capture of a similar number of CK-positive CTCs using Parsortix and IsoFlux from 7.5 mL of blood (average of 33.8 and 37.6 respectively). Purity of CTCs harvested was higher with Parsortix (3.1%) than IsoFlux at (1.0%). Parsortix harvested more CK-positive CTCs than CellSearch (average of 32.1 and 10.1 respectively).	[24]
Microfluidic acoustophoresis	83.70%	93%	Label-free approach to CTC isolation, with no detectable loss of cell viability subsequent to acoustophoresis.	[25]
7.5 um pore size based filtration (ScreenCell)	-	-	Captured CTCs were immunofluorescently stained for AR expression. Isolated CTCs from patients with prostate cancer across all risk groups and stages	[26]

Dielectrophoresis of CTCs from blood samples on a microfluidic chip (DEPArray™)	99.70%	100%	Isolating single cells by DEP enabled staining and picking of IF stained CTCs, which can then be analysed with NGS methods.	[27]
Apheresis of whole blood with mononuclear cell collection (Diagnostic LeukApheresis, DLA),	-	-	Liters of blood screened to better assess CTC heterogeneity, direct comparison with CellSearch. Leukocyte depletion of 18 mL followed by CellSearch yielded a relative decrease in yield (37%) versus CellSearch DLA.	[28]
Acoustic isolation	86%	-	Enabled the separation of both single CTCs and CTC clusters from blood, with elution of proliferative cells. CTC counts ranging from 0.93 to 400 CTCs/mL from patients with mCRPC and widespread bone metastases.	[2924]
Methods using Antigen-Dependent and -Independent Isolation				
Antibody (anti-CD45) functionalised, 3D printed microfluidic channels and size-based filter (3 um pores)	90%	10^5 WBCs from 10 mL sample	Dual isolation approach employing size-based filtration following WBC depletion.	[29]
On-chip microfluidic inertial focussing and immunomagnetic (anti-CD45) WBC depletion	99.5%	445 WBCs/mL blood	Rapid and automated isolation of CTCs by multimodal separation. CTCs detected in 96.4% of patients with mCRPC. EpCAM-negative CTCs found in 20% of patients with mCRPC.	[30]
Branched aptamer (towards EpCAM and Cadherin) modified cage structure on an integrated chip	95%	-	Cage structure for trapping live CTCs following aptamer "baiting" on device, exploiting CTC filopodia. Achieved detection of living CTCs in with high sensitivity (100%) and specificity (86%) in patients with suspected prostate cancer (PSA > 4 ng/mL).	[31]

MNPS, magnetic nanoparticles; EpCAM, epithelial cell adhesion molecule; CD45, cluster of differentiation 45; WBCs, white blood cells; AR, androgen receptor; PSMA, prostate-specific membrane antigen; PSA, prostate-specific antigen; PSCA, prostate stem cell antigen; SChLAP1, second chromosome locus associated with prostate-1; FACS, fluorescence activated cell sorting; WGA, whole genome amplification; IE, immunomagnetic enrichment; CNAs, copy number aberrations; PLGA, Poly(lactic-co-glycolic acid); PIK3CA, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha; PEDOT, poly(EDOT-PBA-co-EDOT-EG3); KLK3, kallikrein-related peptidase 3; FOLH1, folate hydrolase 1; FISH, fluorescence in situ hyrbidisation; rGO, reduced graphene oxide; EGFR, epidermal

783 growth factor receptor; FGFR, fibroblast growth factor receptor; FAPα, Fibroblast activation
784 protein-alpha; c-MET, cellular-mesenchymal epithelial transition factor receptor tyrosine
785 kinase; OB-cadherin, osteoblast cadherin; mCRPC, metastatic castration-resistant prostate
786 cancer; AFM, atomic force microscopy; NEPC, neuroendocrine prostate cancer; CK,
787 cytokeratin; NGS, next-generation sequencing;
788

789 **Supplementary Table 2** – Summary of enumerative analyses conducted on CTCs from
790 patients with localised and metastatic prostate cancer

Enumeration Method	Disease State	Description	Markers Used	Reference
Immunostaining-based				
CellSearch®	Localised		EpCAM, E-cadherin, CD133	32,33
CellCollector®	Both		EpCAM, PanCK, CD45	34
AdnaTest®	Localised	Combined with PCR for (PSMA, PSA, EGFR)	EpCAM	35
	Metastatic			36
EPISPOT®	Metastatic	Detects only viable tumour cells and protein secretion	PSA, FGF2	37
EPIC-PSMA	Metastatic		PSMA, CK, CD45	38
Nanoprobe	Metastatic		EpCAM, EGFR, CD44, RPL15, E-Cad	39
Immunostaining	Both		EpCAM, CK8, CK18, CK19, CD45	40
	Localised		EpCAM, PSMA, CD45	41
			AR, CD45, CK	42
			EpCAM, PanCK, CD45	43
	Localised		PSA	44–50
	Localised		PSA, CD82	51,52
	Localised		EpCAM, PSMA, AMACR, CD45	53
	Localised	FISH	CEP8, CD45	54
	Both		PSA, P504S	55,56
	Localised		CD117/c-kit, CD133, CXCR4/CD184, CD34	57
	Metastatic		PSA, P540s	58
	Metastatic		84-1, PD-L1	59
	Metastatic		EpCAM, CK, CD45, CD66	60
	Metastatic	FACS	AR, Ki67, EpCAM, Pan-CK, CD45	61
	Metastatic		IGF-IR, EpCAM, CK, CD45	62
PCR-based				
PCR	Metastatic		AR-V7	63
	Metastatic		PSMA, ARV7, PSA	64
	Metastatic		ABCG2, PROM1, PSCA, TWIST1, vimentin	65
	Metastatic		KLK2, TMPRSS2	66
Comparison studies				

CSV based vs CellSearch®	Both	Comapred to CellSearch, the CSV-based method had greater sensitivity and specificity.	84-1 mAB against CSV	67
CellSearch® vs CellCollector®	Localised	CellCollector® resulted in a significantly higher percentage of CTC-positive samples compared to CellSearch® and yielded significantly higher CTC numbers		68
CellSearch® vs EPISPOT vs CellCollector®	Localised	Only CTC count ≥ 1, determined using the CellSearch® system, was a significant predictor of a worse OS during at least a 5-year follow-up.		69
	Localised	The highest percentage of CTC-positive patients was detected with the CellCollector® (48%) and -EPISPOT (42%) assays, while the CellSearch® system presented the lowest rate (14%). Concordance among methods was only 23%.		70
	Localised	CTCs were detected in 37%, 54.9% and 58.7% of patients using CellSearch, CellCollector and EPISPOT, respectively		71
CellSearch® vs PCR	Both	RT-PCR and CellSearch CTC results were strongly concordant (80%-85%). Among CRPC patients, KLK mRNAs and CellSearch CTCs were closely associated with clinical evidence of bone metastases and with survival.	KLK3, KLK2, PSCA	72
ISET vs CellSearch®	Metastatic	Concordant results were obtained in 60% (12 out of 20) of the patients with prostate cancer.		73

795 **Supplementary Table 3 –** Studies investigating CTC analysis to predict treatment response
796 or inform treatment selection in patients with metastatic prostate cancer are listed. Cohort size
797 (N), disease state, platform used for CTC isolation or enrichment, treatment, study aim, and
798 take-home message are detailed.

Cohort Size (N)	Disease State	Isolation Platform	Treatment	Study Aim	Take-home message	Reference
33	mHSPC	CellSearch	ADT	Treatment response	Baseline CTC enumeration with CellSearch® (>3 CTCs/ml) was predictive of progression to mCRPC in 33 patients with mHSPC receiving ADT	[74]
62	mCRPC	AdnaTest	enzalutamide or abiraterone		Detection of AR-V7-positive CTCs was predictive of shortened PFS and OS in patients receiving abi or enza	[75]
33	mCRPC	CellSearch	docetaxel		CTC counts appear to be an earlier and more sensitive predictor for survival and treatment response than current objective response criteria	[76]
202	mCRPC	AdnaTest	enzalutamide or abiraterone		Detection of AR-V7-positive CTCs was predictive of poorer PSA response and shortened PSA-PFS in patients receiving abi or enza	[63]
45	mCRPC	CellSearch	radium-223 RT		Baseline CTC enumeration (>5 CTCs/ml) was predictive of failure to complete radium-223 therapy and shortened OS	[77]
45	mHSPC	FACS	enzalutamide		Baseline CTC enumeration (≥ 5 CTCs/ml) was predictive of worse rPFS and OS	[78]

533	mHSPC	CellSearch	ADT		Baseline CTC enumeration with CellSearch® (>5 CTCs/ml) was predictive of lower 7-month PSA and achieving 2-year PFS	[79]
40	mCRPC	CellSearch	cabazitaxel		Baseline CTC enumeration (>5 CTCs/mL) was predictive of shortened PFS and OS	[80]
98	mCRPC	CellSearch	abiraterone		Baseline CTC enumeration (>5 CTCs/mL) was predictive of shortened OS	[81]
28	mCRPC	CellSearch	radium-223 RT		Baseline CTC enumeration (>5 CTCs/mL) was predictive of and shortened OS	[82]
18; 43	mHSPC; mCRPC	Parsortix	docetaxel		Baseline CTC detection was predictive of shortened OS	[83]
37	mCRPC	AdnaTest	docetaxel or cabazitaxel	Treatment selection	PSA responses were higher and PSA-PFS and PFS were longer in mCRPC patients with AR-V7-positive CTCs treated with taxanes (vs. abi or enza), but no differences in outcomes between treatment in AR-V7-CTC-negative patients	[84]
161	mCRPC	Epic	ARSI or taxane therapy		Detection of AR-V7-nuclear-positive CTCs was predictive of longer OS on taxane therapy (vs. ARSIs)	[85]
193	mCRPC	Epic	ARSI or taxane therapy		Detection of AR-V7-nuclear-positive CTCs was predictive of longer OS on taxane therapy (vs. ARSIs) with mCRPC	[86]

mHSPC, metastatic hormone-sensitive prostate cancer; ADT, androgen deprivation therapy; mCRPC, metastatic castration-resistant prostate cancer; PFS, progression-free survival; OS, overall survival; abi, abiraterone; enza, enzalutamide; RT, radiotherapy; FACS, fluorescence-activated cell sorting; rPFS, radiographic progression-free survival; AR-V7, androgen receptor splice variant 7; PSA, prostate-specific antigen; PSA-PFS, PSA-based progression-free survival; ARSI, androgen receptor signaling inhibitor.

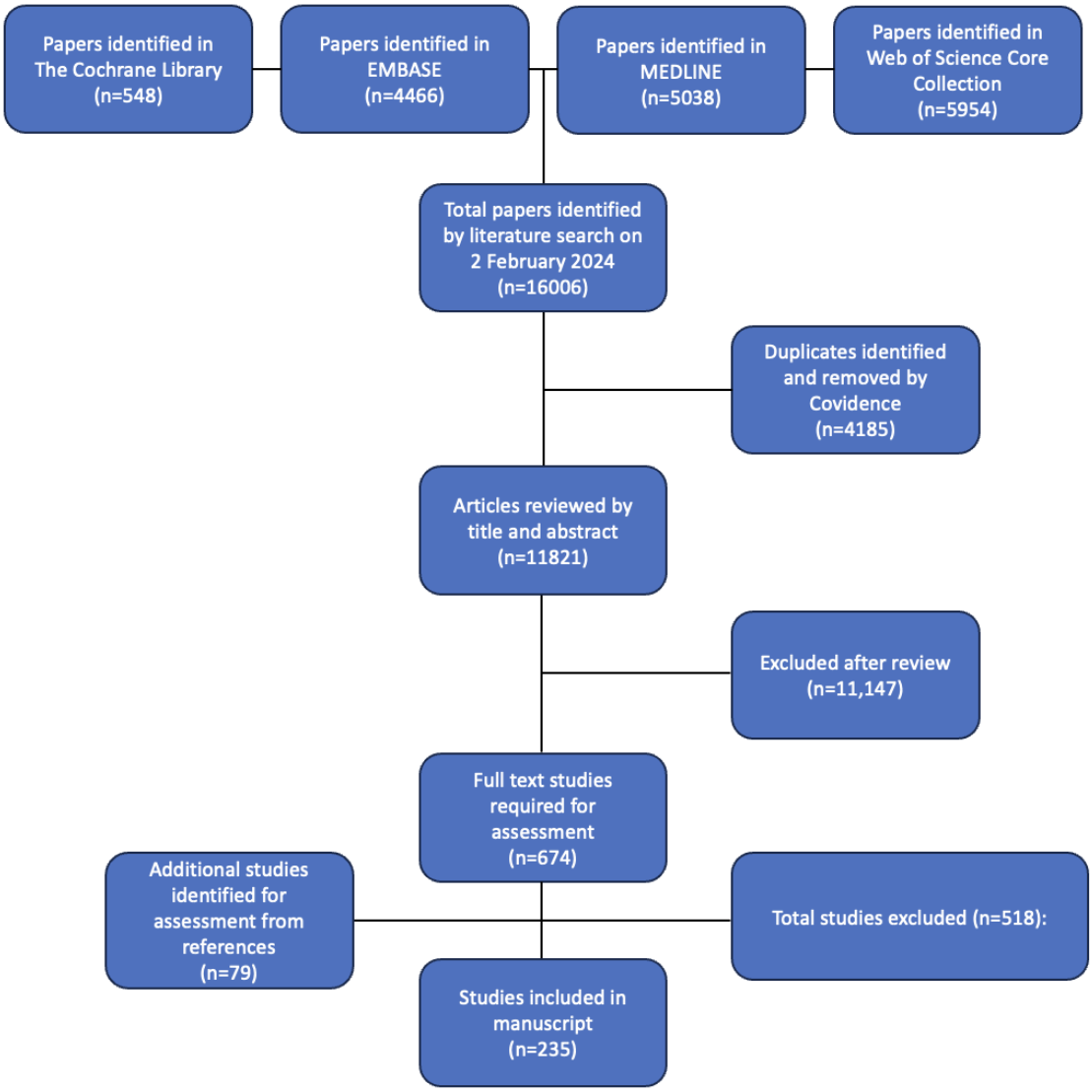
Supplementary Table 4 – Studies on CTCs in the blood of patients with localised prostate cancer are listed. Cohort size (N), detection method, treatment, and the percentage of patients with CTCs detected in their blood (% positivity) are detailed.

Cohort size (N)	Detection Method	Treatment	% Positivity	Reference
180	RT-PCR	RP or RT	8%	[72]
35	CellSearch	HRLPC RP	49%	[32]
115	Flow cytometry	HRLPC RP	3% of buffy coat cells were CD117+	[57]
107	CellSearch, CellCollector, and EPISPOT	HRLPC RP	81.3%*	[71]
51	CellCollector and CellSearch	HRLPC RT	33.7% and 18.6%, respectively	[68]
131	CellSearch, CellCollector, and EPISPOT	HRLPC RT+ADT	79%* (14%, 48%, and 42%, respectively)	[70]
45	EpicSciences	HRLPC RT+ADT or RP	73.3%	[42]
26	RT-PCR	N/R	0.4–30.5 (mean 6.5) CTCs/ml	[87]

*Cumulative positivity across multiple isolation platforms

RP, radical prostatectomy; RT, radiotherapy; HRLPC, high-risk localised prostate cancer; ADT, androgen deprivation therapy

Supplementary Figure 1 - Consort diagram for literature that was screened for inclusion in the current manuscript.



Search Methods

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were limited to exclude Medline records. No other limits or filters were applied. Results were deduplicated using Covidence.

11,821 abstracts were independently reviewed by SMA and TA for inclusion in full text review. Inclusion criteria included analysis of circulating tumour cells using clinical samples obtained from patients with prostate cancer. Exclusion criteria included only pre-clinical work (no use of patient samples), publication prior to 2005, non-English language, and no prostate-relevance. Conflicts were resolved following discussion according to author consensus opinion. 674 papers continued to full text review. 156 papers found in literature search and 42 additional papers identified during writing were included in review to total 198 as according to author consensus opinion.

Supplementary 1: EMBASE Search Strategy. Platform=OvidSP, coverage=1974-present, search completed 23 February 2024, link to search=https://ovidsp.ovid.com/ovidweb.cgi?T=JS&NEWS=N&PAGE=main&SHAREDSEARCHID=6pSX0jzOYZzIXP9w8IYxpwEGcMDpP92HRoqpZUoj0ubMuzPHT3QTG9tw19SfND2AQ.

	EMBASE Search Terms (1974-present)	Number of Results
1	((circulating adj1 (tumo?r* or cancer* or neoplas*)) or ((circulating or cell free) adj1 (dna or rna or nucleic acid or deoxyribonucleic acid or ribonecleic acid)) or ctDNA or cfDNA or cfRNA).ti,ab,kf.	43877
2	exp cell free nucleic acid/ or circulating tumor cell/	32642
3	1 or 2	52257

4	liquid biops*.ti,ab,kf.	15011
5	liquid biopsy/	12268
6	4 or 5	17257
7	((((local* or adjacent or proximal) adj3 recur*) and (cancer* or tumo?r* or neoplas* or adenocarcinoma* or carcinoma* or malignan* or oncol*)) or micro metasta* or micrometasta*).ti,ab,kf.	89693
8	(cancer recurrence/ and (local* or adjacent or proximal).ti,ab,kf.) or micrometastasis/	74908
	7 or 8	127157
9		
10	((Prostat* or urolog* or genital*) adj3 (cancer* or tumo?r* or neoplas* or adenocarcinoma* or carcinoma* or malignan* or oncol*)).ti,ab,kf.	282449
11	exp prostate cancer/ or male genital tract cancer/ or urogenital tract cancer/ or urinary tract cancer/ or prostate tumor/	312548
12	10 or 11	355651
13	(single adj3 cell).ti,ab,kf.	120626
14	exp single cell analysis/	45188
15	13 or 14	130021
16	3 and 12	4875
17	3 and 9	1443
18	6 and 12	1561
19	4	1762
20	16 or 17 or 18 or 19	8208
21	limit 20 to "remove medline records"	4466

Supplementary 2: Cochrane Central Database of Controlled Trials Search Strategy. Platform=Cochrane Library, Wiley, coverage=Issue 2 of 12, February 2024, search completed 23 February 2024.

	Cochrane Library Search Terms (Issue 2 of 12, February 2024)	Number of Results
#1	((circulating NEAR/1 (tumo?r* or cancer* or neoplas*)) or ((circulating or "cell free") NEAR/1 (dna or rna or nucleic acid or "deoxyribonucleic acid" or "ribonecleic acid")) or CTC or CTCs or ctDNA or cfDNA or cfRNA)	3703
#2	MeSH descriptor: [Cell-Free Nucleic Acids] explode all trees	210
#3	MeSH descriptor: [Neoplastic Cells, Circulating] this term only	261
#4	#1 or #2 or #3	3828
#5	(liquid NEXT biops*)	286
#6	MeSH descriptor: [Liquid Biopsy] this term only	21
#7	#5 or #6	286
#8	(((local NEAR/3 recur*) and (cancer* or tumo?r* or neoplas* or adenocarcinoma* or carcinoma* or malignan* or oncol*)) or (micro NEXT metasta*) or micrometasta*)	10888
#9	MeSH descriptor: [Neoplasm Recurrence, Local] this term only	7487
#10	MeSH descriptor: [Neoplasm Micrometastasis] this term only	30
#11	#8 or #9 or #10	10888

#12	((Prostat* or urolog* or genital*) NEAR/3 (cancer* or tumo?r* or neoplas* or adenocarcinoma* or carcinoma*))	20281
#13	MeSH descriptor: [Prostatic Neoplasms] explode all trees	8696
#14	MeSH descriptor: [Urogenital Neoplasms] this term only	69
#15	MeSH descriptor: [Genital Neoplasms, Male] this term only	36
#16	MeSH descriptor: [Urologic Neoplasms] this term only	158
#17	#12 or #13 or #14 or #15 or #16	20323
#18	(single NEAR/3 cell)	754
#19	MeSH descriptor: [Single-Cell Analysis] explode all trees	21
#20	#18 or #19	754
#21	#4 and #17	407
#22	#4 and #11	164
#23	#7 and #17	32
#24	#4 and #20	20
#25	#21 or #22 or #23 or #24	578
	Results in CENTRAL	548

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Supplementary 3: MEDLINE Search Strategy (Ovid MEDLINE® Epub Ahead of Print, In-Process & Other Non-Indexed Citations, Ovid MEDLINE® Daily and Ovid MEDLINE®). Platform=OvidSP, coverage=1946-present, search completed 23 February 2024, link to search= https://ovidsp.ovid.com/ovidweb.cgi?T=JS&NEWS=N&PAGE=main&SHAREDSEARCHID=20MGH2C2YbcCvWDcgScvcGbcc69MqWU0mIxpr3NvNdPbO5dA5PnigtUK41NU1Mn7D.

	MEDLINE Search Terms (1946-present)	Number of Results
1	((circulating adj1 (tumo?r* or cancer* or neoplas*)) or ((circulating or cell free) adj1 (dna or rna or nucleic acid or deoxyribonucleic acid or ribonecleic acid)) or ctDNA or cfDNA or cfRNA).ti,ab,kf.	24767
2	exp Cell-Free Nucleic Acids/ or Neoplastic cells, circulating/	20003
3	1 or 2	33622
4	liquid biops*.ti,ab,kf.	9620
5	Liquid biopsy/	2896
6	4 or 5	10084
7	((((local* or adjacent or proximal) adj3 recur*) and (cancer* or tumo?r* or neoplas* or adenocarcinoma* or carcinoma* or malignan* or oncol*)) or micro metasta* or micrometasta*).ti,ab,kf.	58057
8	Neoplasm recurrence, local/ or Neoplasm Micrometastasis/	148628
9	7 or 8	183418

10	((Prostat* or urolog* or genital*) adj3 (cancer* or tumo?r* or neoplas* or adenocarcinoma* or carcinoma* or malignan* or oncol*)).ti,ab,kf.	190419
11	exp Prostatic neoplasms/ or urogenital neoplasms/ or genital neoplasms, male/ or urologic neoplasms/	163562
12	10 or 11	222425
13	(single adj3 cell).ti,ab,kf.	87767
14	exp Single-Cell Analysis/	14326
15	13 or 14	90149
16	3 and 12	2130
17	3 and 9	1955
18	6 and 12	702
19	3 and 15	926
20	16 or 17 or 18 or 19	5038

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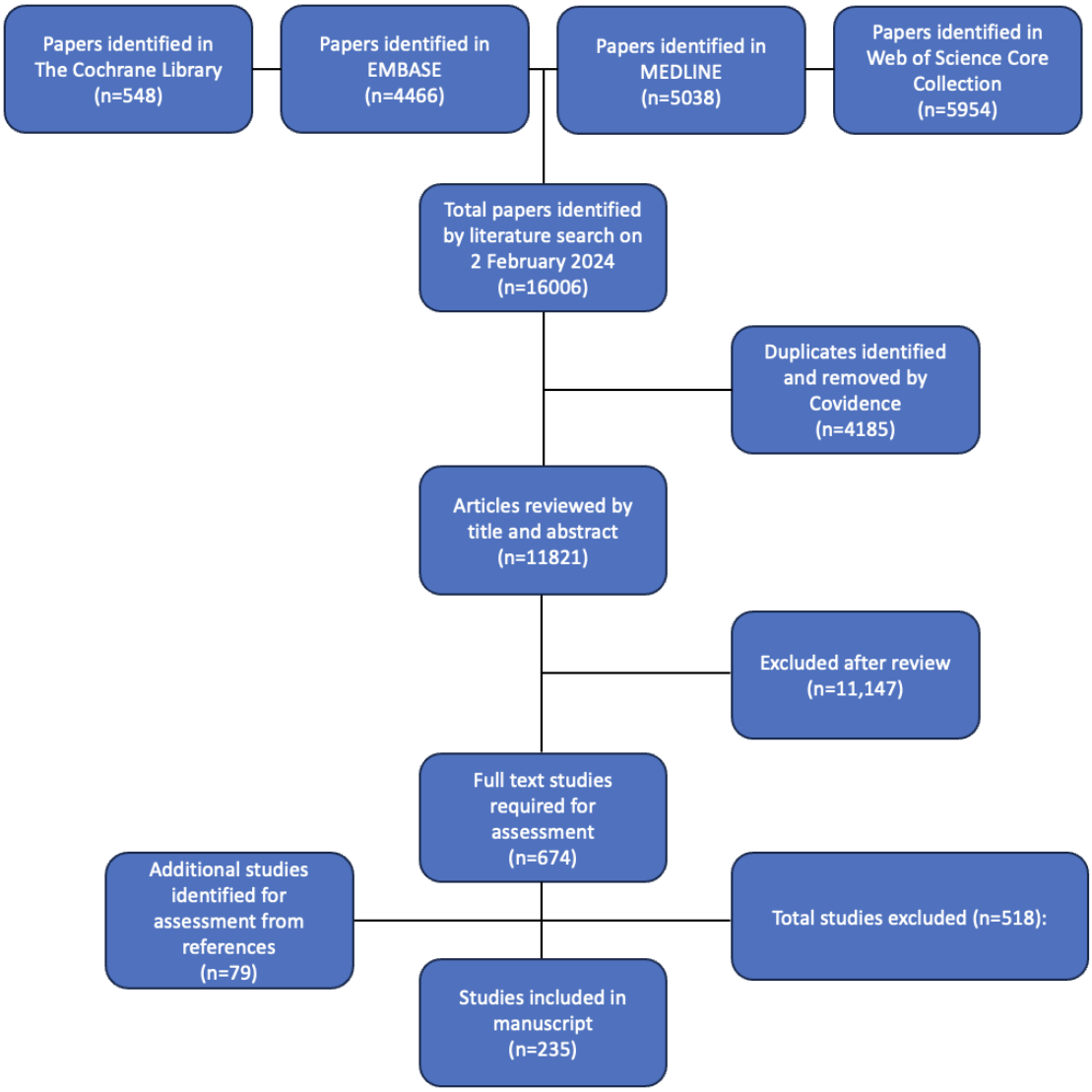
Supplementary 4: WoS Search Strategy (Science Citation Index and Conference Proceedings Citation Index – Science). Platform=Web of Science Core Collection, coverage=WOS.SCI 1900-present, WOS.SSCI 1900-present, search completed 23 February 2024.

	Web of Science Search Terms (1900-present)	Number of Results
1	TS=((circulating NEAR/1 (tumo$r* OR cancer* OR neoplas*)) OR ((circulating or "cell free") NEAR/1 (dna OR rna OR "nucleic acid" OR "deoxyribonucleic acid" OR "ribonecleic acid")) OR ctDNA OR cfDNA OR cfRNA)	41887
2	TS=((Prostat* or urolog* or genital*) NEAR/3 (cancer* or tumo$r* or neoplas* or adenocarcinoma* or carcinoma* or malignan* or oncol*))	274661
3	#1 AND #2	3462
4	TS=((((local* or adjacent or proximal) NEAR/3 recur*) and (cancer* or tumo?r* or neoplas* or adenocarcinoma* or carcinoma* malignan* or oncol*)) or "micro metasta*" or micrometasta*)	47545
5	#1 AND #4	812
6	TS=("liquid biops*")	11456
7	#6 AND #2	947
8	TS=(single NEAR/3 cell)	154836
9	#1 AND #8	1620
10	#3 OR #5	4062

Supplementary Materials

Supplementary Figure 1: Consort diagram for literature that was screened for inclusion in the current manuscript.



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Supplementary 3: MEDLINE Search Strategy (Ovid MEDLINE® Epub Ahead of Print, In-Process & Other Non-Indexed Citations, Ovid MEDLINE® Daily and Ovid MEDLINE®). Platform=OvidSP, coverage=1946-present, search completed 23 February 2024, link to search= https://ovidsp.ovid.com/ovidweb.cgi?T=JS&NEWS=N&PAGE=main&SHAREDSEARCHID=20MGH2C2YbcCvWDcgScvcGbcc69MqWU0mIxpr3NvNdPbO5dA5PnigtUK41NU1Mn7D.

	MEDLINE Search Terms (1946-present)	Number of Results
1	((circulating adj1 (tumo?r* or cancer* or neoplas*)) or ((circulating or cell free) adj1 (dna or rna or nucleic acid or deoxyribonucleic acid or ribonecleic acid)) or ctDNA or cfDNA or cfRNA).ti,ab,kf.	24767
2	exp Cell-Free Nucleic Acids/ or Neoplastic cells, circulating/	20003
3	1 or 2	33622
4	liquid biops*.ti,ab,kf.	9620
5	Liquid biopsy/	2896
6	4 or 5	10084
7	((((local* or adjacent or proximal) adj3 recur*) and (cancer* or tumo?r* or neoplas* or adenocarcinoma* or carcinoma* or malignan* or oncol*)) or micro metasta* or micrometasta*).ti,ab,kf.	58057
8	Neoplasm recurrence, local/ or Neoplasm Micrometastasis/	148628
9	7 or 8	183418

10	((Prostat* or urolog* or genital*) adj3 (cancer* or tumo?r* or neoplas* or adenocarcinoma* or carcinoma* or malignan* or oncol*)).ti,ab,kf.	190419
11	exp Prostatic neoplasms/ or urogenital neoplasms/ or genital neoplasms, male/ or urologic neoplasms/	163562
12	10 or 11	222425
13	(single adj3 cell).ti,ab,kf.	87767
14	exp Single-Cell Analysis/	14326
15	13 or 14	90149
16	3 and 12	2130
17	3 and 9	1955
18	6 and 12	702
19	3 and 15	926
20	16 or 17 or 18 or 19	5038

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911 Supplementary 4: WoS Search Strategy (Science Citation Index and Conference Proceedings
912 Citation Index – Science). Platform=Web of Science Core Collection, coverage=WOS.SCI
913 1900-present, WOS.SSCI 1900-present, search completed 23 February 2024.
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	Web of Science Search Terms (1900-present)	Number of Results
1	TS=((circulating NEAR/1 (tumo$r* OR cancer* OR neoplas*)) OR ((circulating or "cell free") NEAR/1 (dna OR rna OR "nucleic acid" OR "deoxyribonucleic acid" OR "ribonecleic acid")) OR ctDNA OR cfDNA OR cfRNA)	41887
2	TS=((Prostat* or urolog* or genital*) NEAR/3 (cancer* or tumo$r* or neoplas* or adenocarcinoma* or carcinoma* or malignan* or oncol*))	274661
3	#1 AND #2	3462
4	TS=((((local* or adjacent or proximal) NEAR/3 recur*) and (cancer* or tumo?r* or neoplas* or adenocarcinoma* or carcinoma* malignan* or oncol*)) or "micro metasta*" or micrometasta*)	47545
5	#1 AND #4	812
6	TS=("liquid biops*")	11456
7	#6 AND #2	947
8	TS=(single NEAR/3 cell)	154836
9	#1 AND #8	1620
10	#3 OR #5	4135

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