

# Circulating tumour cells as a window into lethality in prostate cancer

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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 a 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 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 incorporating CTCs into the clinical management of prostate cancer.

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## 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 for selecting 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 localized prostate cancer, requiring increased sensitivity and specificity of enrichment methods.

## Introduction

The clinical, morphological, molecular and intra-tumour spatial heterogeneity of prostate cancer is well established<sup>1–3</sup>. Prostate cancer can be indolent, showing a slow course of clinical progression, or can develop into aggressive disease with metastatic and lethal phenotypes<sup>4–7</sup>. The 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<sup>8</sup>. Studies carried out to unravel intra-tumour spatial heterogeneity of prostate cancer led to the identification of the clonal evolution of the disease both within and outside the prostate gland<sup>1,2,9–12</sup>. 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 recognize the potential of these markers for non-invasive risk stratification and personalized 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 sampling molecular tumour phenotypes and genotypes with potentially lower health care costs than tissue biopsy. CTCs were considered 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<sup>13,14</sup>. 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<sup>15,16</sup>, genomic alterations<sup>17</sup> and fragmentomics<sup>18</sup>, overtaking CTCs as the mainstream circulating biomarker for clinical application owing to the technology readiness and improved standardization, 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<sup>19</sup>, with sequencing of ctDNA used to identify AR alterations associated with androgen receptor signalling inhibitor (ARSI) resistance<sup>20</sup> and patterns of methylation<sup>21</sup> and fragmentation<sup>22</sup> that can identify transformation to neuroendocrine prostate cancer (NEPC). Plasma cell-free or ctDNA assays are commercially available and can also provide an insight into tumour burden, progression, copy number variants and methylation patterns<sup>23,24</sup>. However, definitively identifying the origin of plasma DNA poses a challenge. Fragmentomics, which enables identification of size distributions of cell-free DNA fragments unique to patients and cancer subtypes, offers the possibility of distinguishing tumour-derived ctDNA<sup>18</sup>. However, the relevance of ctDNA to tumour evolution and metastasis is limited, as it is not clear whether ctDNA arises from the primary tumour, metastatic sites or CTCs. Moreover, fragments could be representative of apoptotic tumour cells lacking the potential to seed metastatic disease, and ctDNA could fail to capture the homogenization of shedding sites<sup>24</sup>.

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<sup>25–27</sup>. 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<sup>28,29</sup>. This theory is exemplified by the biological phenomenon of 'dormancy' observed in prostate and other cancers, as both tumour microenvironmental drivers and autogenous cell programmes are thought to regulate cellular dormancy<sup>30</sup>. Subsequent research across cancer types has corroborated this hypothesis and begun to unravel the molecular details of these tumour and metastatic niche profiles, suggesting that the successful propagation of metastasis and treatment resistance are mediated by a mesenchymal or cancer stem cell (CSC) phenotype<sup>31–33</sup>. Importantly, CTCs enable analysis of phenotypic and genotypic characteristics cells (or 'seeds') that have survived in circulation, as these cells are travelling to or from metastatic niches (or 'soil'), whereas other liquid biopsy modalities such as ctDNA are limited to genotypic analysis, and could identify apoptotic cells or 'shrapnel' that are not biologically relevant to cancer spread.

Access to CTC molecular profiles offers great promise in further delineating the metastatic potential of these cells and could enable insight to be gained into metastatic sites such as the skeleton, which are particularly challenging to sample directly and often deliver minimal usable material for analyses<sup>34,35</sup>. Furthermore, the non-invasive nature of CTC profiling enables longitudinal real-time monitoring of disease burden and dynamic biology to be achieved, including response to therapy<sup>36,37</sup>. Yet, 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<sup>38</sup>. 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)<sup>39</sup>. CTCs have been identified in the blood of patients with localized prostate cancer, but the prognostic significance of CTCs

remains unclear outside the realm of metastatic disease<sup>8,40–43</sup>. Nonetheless, the analysis of prostate cancer CTCs holds great promise for an insight into heterogeneous tumour biology<sup>35,44</sup>, prediction of treatment response and elucidation of the metastatic pathway<sup>45,46</sup>. Previous Reviews discussing the role of CTCs in prostate cancer have focussed on biomarkers used in prostate cancer detection and management<sup>8,47</sup>, urological malignancies as a whole<sup>24,48</sup> and metastatic prostate cancer<sup>49–51</sup>.

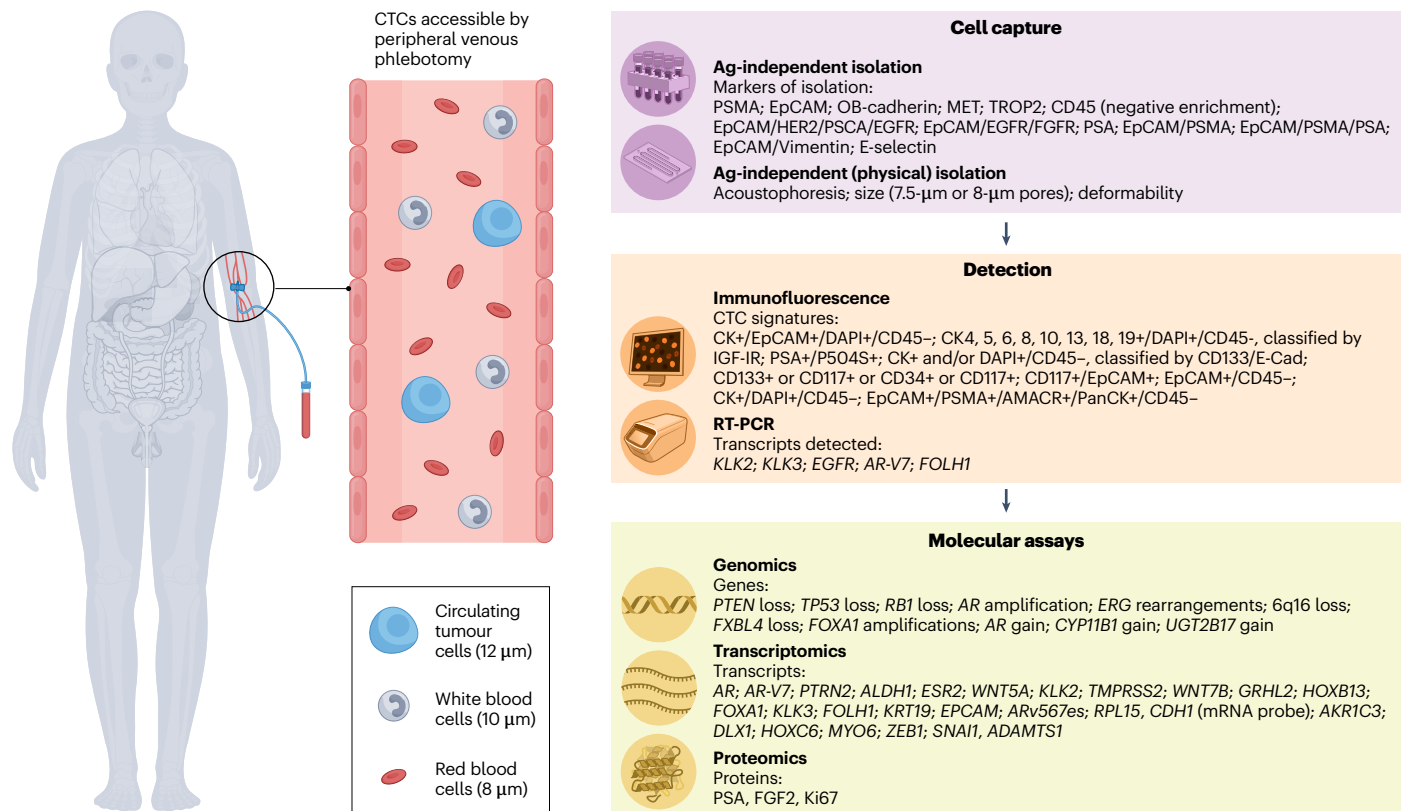
In this Review, we summarize the state-of-the-art for CTCs at all stages of prostate cancer development and treatment, describing advances in CTC isolation and enrichment, subsequent detection through enumeration and molecular analysis of these cells, with a focus on unpacking the biological heterogeneity of prostate cancer to provide a window into lethality. The literature search methods are detailed in the Supplementary Information and Supplementary Fig. 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 the clinical management of prostate cancer are also discussed.

## Isolation and enrichment of prostate cancer circulating tumour cells

Methods of CTC enrichment can be broadly categorized as antigen dependent, such as immune-affinity-based methods, or antigen

independent, based on biological and physical properties, holding different capture efficiencies (percentage of circulating prostate cancer cells captured) and purity levels (background level of white blood cells (WBCs); Supplementary Table 1). The isolation of CTCs from blood has undergone 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<sup>52,53</sup>.

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<sup>54–59</sup> or microfluidic chips with antibody-coated walls or solid support structures<sup>60–65</sup> (Fig. 1). Negative enrichment targeting CD45 is used to deplete WBCs<sup>38,66</sup>, 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 the circulation; additionally, non-specific



**Fig. 1 | Isolation, detection and profiling workflow of circulating tumour cells.** Circulating tumour cells (CTCs) are captured from blood by using antigen (Ag)-dependent and/or Ag-independent isolation and enrichment methods. Captured cells are identified as CTCs (detected) based on immunofluorescence staining of cell-surface markers or real-time PCR of prostate-specific or prostate-related transcripts. Captured cells are then profiled by using genomic,

transcriptomic and proteomic techniques. AMACR,  $\alpha$ -methylacyl-CoA racemase; CK, cytokeratin; E-cad, E-cadherin; EGFR, epidermal growth factor receptor; EpCAM, epithelial cell adhesion molecule; FGF2, fibroblast growth factor 2; FGFR, fibroblast growth factor receptor; IGF-IR, insulin-like growth factor-IR; PSCA, prostate stem cell antigen; PSMA, prostate-specific membrane antigen.

EpCAM-positive cells are also potentially captured with this strategy<sup>67–70</sup>. Thus, alternative recognition ligands have been used<sup>50,58,71–74</sup>, including E-selectin<sup>75</sup>, prostate-specific membrane antigen (PSMA)<sup>61,76–78</sup>, PSA<sup>61,76</sup>, vimentin<sup>33,43,57,74</sup>, epidermal growth factor receptor (EGFR)<sup>58,72</sup>, MET<sup>79</sup>, OB-cadherin<sup>55</sup>, HER2 (ref. 72), TROP2 (ref. 80), and prostate stem cell antigen (PSCA)<sup>72</sup> (Table 1, Supplementary Table 2 and refs. 81–91). Multiplexed antibody-based approaches showed higher capture efficiency than single-antibody (typically EpCAM)-based capture<sup>57,62,74,77,92</sup>. 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 functionalized microfluidic platform, respectively<sup>62</sup>. 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 whether 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<sup>93</sup>. Prostate cancer CTCs typically have a diameter of 9.1–15.1  $\mu\text{m}$ <sup>94,95</sup>, while WBCs have diameters of approximately 10  $\mu\text{m}$  (ref. 96) and RBCs are 7.5–8.7  $\mu\text{m}$  (ref. 97). Thus, in studies in which filtration was used to enrich for prostate cancer CTCs, pore sizes of 7.5–8  $\mu\text{m}$  have been used<sup>93,94</sup>, with pore sizes generally slightly smaller than the targeted cell size to facilitate deformability-based capture. 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 hybridization (FISH) to quantify telomere numbers and sizes<sup>98</sup>. 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)<sup>94</sup>. 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 cytokeratin (CK)<sup>+</sup> CTCs from patient blood samples than CellSearch (mean of 32.1 and 10.1, respectively;  $P = 0.04$ )<sup>99</sup>. Furthermore, in this study, CK<sup>+</sup>/Vimentin<sup>+</sup>/CD45<sup>-</sup> and CK<sup>-</sup>/Vimentin<sup>+</sup>/CD45<sup>-</sup> 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 individuals (0 CK<sup>-</sup>/CD45<sup>-</sup> cells and 0, 2 and 3 CK<sup>-</sup>/Vimentin<sup>+</sup>/CD45<sup>-</sup> 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<sup>99–102</sup>. One study using acoustophoresis to isolate CTCs from five patients with mCRPC to investigate PSMA expression found that 64.5–81.5% of CTCs (defined as CK8/18<sup>+</sup>, DAPI<sup>+</sup> and CD45<sup>-</sup> through immunofluorescence) were PSMA<sup>+</sup>, showing the ability to detect a potential therapeutic target through CTC liquid biopsy<sup>102</sup>. Dielectrophoresis is the core concept of DEPArray, a platform that was shown to work well following isolation with CellSearch to generate up to 30,000 'DEP cages' into which single cells can be sorted based on immunofluorescence imaging<sup>101</sup>. Importantly, this approach

enables downstream next-generation sequencing analysis of single CTCs to be obtained, which is crucial to studying the heterogeneity of tumour cells.

Antigen-independent enrichment enables a phenotypically heterogeneous CTC population to be captured, but isolation is limited by the relative rarity of CTCs in blood (1 CTC/billion blood cells)<sup>25,103</sup>. To address this issue, diagnostic leukapheresis (DLA) – based on continuous centrifugation for collection of peripheral blood mononuclear cells – has been used to separate the mononuclear cell population from larger volumes of blood, followed by CTC isolation from this enriched fraction<sup>104</sup>. In a study including 22 patients with metastatic prostate cancer aimed at collecting a minimum DLA volume of 40 ml, processing a DLA aliquot of  $2 \times 10^8$  WBCs by using CellSearch increased CTC yield by up to 32-fold compared with that retrieved in 7.5-ml blood samples with a standard CellSearch protocol<sup>104</sup>. Another CTC enrichment tool, the GILUPI CellCollector, uses an antibody-coated (such as EpCAM, PSA or PSCA) medical wire inserted within the lumen of a blood vessel to collect CTCs from circulating blood<sup>92,105,106</sup>. This method enabled isolation of  $\geq 1$  CTC (identified through PanCK<sup>+</sup>, Hoescht<sup>+</sup>, CD45 immunofluorescence staining) from the blood of 78.9% (56 of 71) and 46.3% of patients (24 of 53) with metastatic and localized prostate cancer, respectively<sup>105</sup>. In another study in which the GILUPI CellCollector was used, enrichment through prostate-specific markers (PSMA, PSA, PSCA and EpCAM versus EpCAM alone) resulted in more sensitive detection rates (86.7% versus 73.3% of patients with  $\geq 1$  CTC detected) and higher CTC counts (median: 9 versus 3 CTCs) than those achieved by using EpCAM targeting alone in 25 patients with metastatic prostate cancer receiving androgen deprivation therapy (ADT) and/or chemotherapy<sup>92</sup>.

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.

## Enumeration and detection of prostate cancer circulating tumour cells

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 reverse-transcription PCR (RT-PCR) or FISH) (Supplementary Table 2 and Fig. 1). Immunofluorescence-based enumeration methods, including the CellSearch system, traditionally detect CTCs based on a CK<sup>+</sup>/EpCAM<sup>+</sup>/DAPI<sup>+</sup>/CD45<sup>-</sup> signature<sup>14,107</sup>. To improve inter-reader variability in counting CTCs 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<sup>108</sup>.

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<sup>42,109</sup>. A variety of additional markers have been identified, including CD133 (a marker of prostate stem cells)<sup>110,111</sup>,

**Table 1 | Cell-surface and intracellular markers to target prostate cancer circulating tumour cells for isolation and detection**

Marker name	Marker abbreviation	Alternative names	Details	Marker use	Refs.
Epithelial cell adhesion molecule <sup>a</sup>	EpCAM	CD326	Transmembrane protein involved in cell-to-cell adhesion, signalling, migration and growth; strong expression in various carcinomas including prostate cancer compared with normal epithelia	Isolation	67–70,81
E-selectin	NA	Endothelial-leukocyte adhesion molecule, CD62E or leukocyte-endothelial cell adhesion molecule 2	Glycoprotein that promotes endothelial leukocyte interactions; has an important role in the adhesion of tumour cells to the vascular endothelium		75,82
Prostate-specific membrane antigen <sup>a</sup>	PSMA	Glutamate carboxypeptidase II	Transmembrane protein expressed by the prostate tissue		61,76–78,83
Prostate-specific antigen <sup>a</sup>	PSA	Kallikrein III or gamma-semioprostatic protein	Androgen-regulated serine protease produced by prostate epithelial cells and prostate cancer cells		61,76,84
Vimentin <sup>a</sup>	NA	Fibroblast intermediate filament	Intermediate filament protein typically upregulated in cells undergoing EMT		56,74,85
Epidermal growth factor receptor	EGFR	HER1, ErbB-1	Transmembrane glycoprotein and type I tyrosine kinase receptor involved in cell survival and growth		58,72,79, 86,89
MET	NA	Hepatocyte growth factor receptor, 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–MET signalling		50,87
OB-cadherin	NA	Cadherin 11	Ca <sup>2+</sup> -dependent homophilic cell adhesion molecule, expressed in osteoblasts and prostate cancer cells		55,88
Human epidermal growth factor receptor-2	HER2	HER2, ErbB2 or Neu	Receptor tyrosine kinase expressed in normal and malignant epithelial cells, regulates cell proliferation, survival and differentiation		72,89
Tumour-associated calcium signal transducer 2	TROP2	Epidermal glycoprotein 1 or membrane component chromosome 1 surface marker 1	Cell-surface glycoprotein that regulates tumour growth and metastatic ability of prostate cancer cells		80,90
Prostate stem cell antigen	PSCA	NA	Glycosylphosphatidylinositol-anchored cell-surface protein up-regulated in several major cancers including prostate, bladder and pancreatic cancers		72,91
Cluster of differentiation 133	CD133	Prominin 1	Transmembrane protein expressed in cancer stem cells, regulates metastasis, drug resistance and stemness properties in various cancer cells	Detection	110,111,116
E-cadherin	E-cad	Cadherin 1 or CD324	Ca <sup>2+</sup> -dependent cell–cell adhesion molecule involved in epithelial cell behaviour, tissue formation and suppression of cancer		110,117
α-methylacyl-CoA racemase	AMACR	P504S, 2-methylacyl-CoA racemase or RACE 2	Mitochondrial and peroxisomal enzyme involved in branched-chain fatty-acid and bile-acid metabolism; useful diagnostic biomarker for prostate cancer and other malignancies		113,118
C-X-C chemokine receptor type 4	CXCR4	CD184 or fusin	G protein coupled receptor for ligand SDF1α, involved in cancer cell adhesion, invasion and proliferation		111,119
Cluster of differentiation 34	CD34	Gp105-120 and My10	Transmembrane phosphoglycoprotein, mediates attachment of stem cells to extracellular matrix in bone marrow or tissue		111,120
Cluster of differentiation 117	CD117	C-KIT, tyrosine-protein kinase KIT or mast or stem cell growth factor receptor	Prostate cancer stem-cell marker, type III tyrosine kinase receptor involved in cell signalling, survival, metabolism, proliferation, apoptosis, migration and differentiation		111,121
Insulin-like growth factor-IR	IGF-IR	Insulin-like growth factor 1 receptor	Essential for development, survival and proliferation of many cell types, and upregulated in prostate cancer tissue		114,122
Synaptophysin	SYP	P38	Integral synaptic vesicle protein, marker of neuroendocrine prostate cancer		115,123

NA, not available; SDF1α, stromal cell-derived factor 1α. <sup>a</sup>Also used for detection of prostate cancer CTCs.

E-cadherin (encoded by *CDH1*)<sup>110</sup>, PSMA (encoded by *FOLH1*)<sup>112</sup>, PSA (encoded by *KLK3*)<sup>113</sup>,  $\alpha$ -methylacyl-CoA racemase (AMACR)<sup>113</sup>, C-X-C chemokine receptor type 4 (CXCR4), CD34, CD117 (ref. 111), insulin-like growth factor-IR (IGF-IR)<sup>114</sup>, and synaptophysin (encoded by *SYP*)<sup>115</sup> (Table 1 and Supplementary Table 2 (refs. 110, 116–123)). In a study including 303 patients undergoing prostate biopsy, the enumeration of PSA<sup>+</sup>/P540S<sup>+</sup> CTCs (positive test for CTC defined as  $\geq 1$  cell PSA<sup>+</sup>/P540S<sup>+</sup>) before diagnostic biopsy had a sensitivity and specificity of 88.5% and 88.4%, respectively, for detecting patients who required treatment (compared with active observation<sup>112</sup>). In another study, the detection of EpCAM<sup>+</sup>/PSMA<sup>+</sup>/AMACR<sup>+</sup>/PanCK<sup>+</sup>/CD45<sup>+</sup> CTCs showed a sensitivity and specificity of 91.2% and 100%, respectively, for diagnosing prostate cancer<sup>113</sup>. Interestingly, in a prostatectomy cohort in which blood immunostaining was carried out pre- and post-operatively to detect CD133<sup>+</sup>, CXCR4<sup>+</sup>, CD34<sup>+</sup> or CD117<sup>+</sup> cells, only CD117<sup>+</sup> cell percentages decreased after radical prostatectomy, whereas the others remained near preoperative levels<sup>111</sup>. Furthermore, the percentage of CD117<sup>+</sup> cells did not decline in patients with biochemical resistance (BCR), and correlated positively (Spearman  $r = 0.4851$ ,  $P = 0.03$ ) with high PSA values, suggesting CD117 as a potential biomarker of therapy response<sup>111</sup>. Thus, these enumerative studies of molecular markers for diagnosis and prognosis of prostate cancer could have a future role in identifying patients who 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 RT-PCR, in which detection of prostate- or epithelial-specific mRNA transcripts was used as a surrogate for CTC presence in blood<sup>124,125</sup>. RT-PCR for *KLK2* and *KLK3* mRNAs in peripheral blood from patients with localized and metastatic prostate cancer has shown strong concordance (80–85%) with CellSearch enumeration results (positive result defined as  $\geq 5$  CTCs per 7.5-ml blood sample)<sup>126</sup>. In other studies, platforms such as the commercial Adnatest were used to immunomagnetically enrich for cells expressing markers such as EpCAM and HER2 before detecting CTCs through prostate-specific transcripts (such as *FOLH1*, *KLK3* and *EGFR*), with results from one study showing a 100% specificity and 100% sensitivity for detection of cancer in patients with benign prostatic hyperplasia and high-risk prostate cancer<sup>127–129</sup>. The Versatile Exclusion-based Rare Sample Analysis system is also based on the enrichment for EpCAM<sup>+</sup>-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 Versatile Exclusion-based Rare Sample Analysis system was used to identify patterns of gene expression in CTCs; patients who had high expression of transcriptional AR targets were significantly more likely to have CRPC versus hormone-sensitive prostate cancer (HSPC), and had higher levels of serum PSA (median 216 ng/ml) than patients with a low level or absence of detected AR-regulated genes (median 11.22 ng/ml,  $P < 0.0001$ )<sup>130</sup>. However, RT-PCR is limited by poor specificity<sup>131</sup>, mainly ascribed to low sample purity, which results in a lack of discrimination of transcripts derived from CTCs as opposed to contaminating leukocytes<sup>132</sup>.

Studies in the past decade focused on CTC detection through FISH<sup>33</sup>, targeting of mRNA transcripts<sup>133</sup> and identification of live cells actively secreting prostate cancer markers<sup>134</sup>. 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*, *MYC*, *PTEN*, *CCND1*, *RBI1*, 16q22.1, *ERG* and *AR* in 81 patients with mCRPC or localized prostate cancer; changes were observed in  $>30\%$  of the genomic regions in

the majority of mesenchymal (VIM<sup>+</sup>/CD45<sup>-</sup>) CTCs<sup>33</sup>. In a small group of patients with localized and metastatic prostate cancer, a novel system using a triple-targeting nano-vector to isolate epithelial (EpCAM<sup>+</sup>), mesenchymal (EGFR<sup>+</sup>) and stem-cell-like (CD44<sup>+</sup>) CTCs was evaluated. By using this approach, intracellular ‘molecular beacons’ were created to target and visualize mRNA (such as RPL15 and E-cad) in living CTCs, and the number of RPL15<sup>+</sup> and E-cad<sup>+</sup> CTCs correlated positively with the presence of bone as opposed to nodal metastases, showing the ability to successfully detect live CTCs through mRNA visualization, and suggesting the potential for this strategy to provide accurate insight into tumour metastasis<sup>133</sup>. The visualization 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) is an EpCAM-independent technology for enumerating 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 localized prostate cancer before and after radical prostatectomy<sup>134</sup> or radiotherapy<sup>135,136</sup>, a higher CTC yield was observed with CellCollector and dual fluoro-EPISPOT than with CellSearch, indicating the potential of EPISPOT to identify molecular heterogeneity of prostate cancer in CTCs and highlighting the importance of non-epithelial CTCs.

## Molecular analyses of circulating tumour cells

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<sup>34,35,37,137–140</sup>.

## Transcriptome analysis of circulating tumour cells in metastatic prostate cancer

Transcriptomic profiling enables identification of genes and pathways implicated in CTC survival and dissemination<sup>141</sup>. Targeted PCR of CTCs has been widely used to predict prostate cancer survival<sup>142–144</sup>. Results from these studies have shown that prognostic gene signatures, including expression of genes such as *AR*, *ARv7*, *FOLH1* (which encodes PSMA), *KLK2*, *KLK3* and *TMPRSS2* in samples enriched for CTCs, can outperform PSA decline in predicting OS<sup>144</sup> and PFS<sup>143</sup> 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 versus without PSMA<sup>+</sup> CTCs<sup>142</sup>. In a cohort of 43 patients with mHSPC, the expression of *ZEB1*, *SNAI1* and *ADAMTS1* detectable in CTCs through RT-qPCR was shown to be significantly predictive of progression to mCRPC within 24 months (area under the curve (AUC) = 0.77,  $P = 0.0092$ ; AUC = 0.71,  $P = 0.039$ ; AUC = 0.71,  $P = 0.043$ , respectively)<sup>46</sup>.

Transcriptomic analyses of CTCs might also serve to identify biomarkers of disease state and tumour molecular profile<sup>145–147</sup>. 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 rather than mHSPC<sup>145</sup>; *AR* and *AR*-regulated gene expression is increased in matched CTCs and metastatic biopsy samples<sup>146</sup>; and *KLK3* and *FOLH1* expression is higher in CTCs enriched from patients with mHSPC or mCRPC than in CTCs from patients with localized prostate cancer<sup>147</sup>. In another study, a multigene score was created to identify metastatic prostate cancer, including mHSPC and mCRPC. Results from this study showed that *KRT19* expression and *EPCAM* expression were 336.3-fold and 8.7-fold

**Table 2 | Molecular analyses on circulating tumour cells from patients with localized and metastatic prostate cancer**

Analyses	Genes or targets studied	Genomics or transcriptomics (single-cell or bulk)	Disease state (n)	Comparator	Findings	Ref.
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 months), 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	37
qPCR of total RNA from whole blood	KLK2 and TMPRSS2	Transcriptomics (bulk)	mCRPC	Healthy volunteers	A two-gene panel of KLK2 and TMPRSS2 was created; unfavourable results (defined as $\geq 1$ marker positive) independently predicted OS in multivariate analysis; conversion to a favourable result during treatment was associated with improved OS, suggesting that the score can predict treatment benefit	144
RT-PCR of mRNA Isolated through anti-Ber-EP4 immunomagnetic beads	EZH2	Transcriptomics (bulk)	Metastatic PCa (n=20) and localized PCa (n=20)	Healthy volunteers (n=10)	EZH2 expression was greater in patients with metastatic PCa (mean expression density $2040.5 \pm 1881.3$ intensity/mm <sup>2</sup> ) than in localized PCa ( $349.4 \pm 156.7$ intensity/mm <sup>2</sup> , $P=0.019$ ) and healthy individuals ( $345.7 \pm 131.8$ intensity/mm <sup>2</sup> , $P=0.023$ )	160
WGA with low-pass WGS for CNV analysis NGS for SSVN 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	157
Single-cell RNA profiling through qPCR	RT <sup>2</sup> 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	141
IF (CTCs) IHC (primary tumours)	PSMA	NA	Clinically proven mPCa	Primary tumours	CTC PSMA expression was heterogeneous; in patients with CTCs, 12 of 20 (67%) had PSMA <sup>+</sup> CTCs; all patients had heterogeneous CTC PSMA phenotypes; all 'favourable' (<5 CTCs/ml blood vs unfavourable, $\geq 5$ CTCs/ml) patients in this study had PSMA-negative CTCs; several patients with PSMA <sup>+</sup> primary tumours had PSMA <sup>-</sup> CTCs	163
WGA	Whole exome	Genomic (single cell)	mCRPC (n=2)	Matched primary and metastatic tissue samples	A new census-based WES protocol to call SSVNs from prostate CTCs was introduced	140
aCGH	CNVs (gains and losses)	Genomics (bulk)	Abiraterone or enzalutamide-resistant mCRPC (n=16)	Matched leukocytes	Genomic gains occurring in >25% of patients: <i>AR</i> , <i>FOXA1</i> , <i>ABL1</i> , <i>MET</i> , <i>ERG</i> , <i>CDK12</i> , <i>BRD4</i> , <i>ZFH3</i> Genomic losses in: <i>PTEN</i> , <i>ZFH3</i> , <i>PDE4DIP</i> , <i>RAF1</i> , <i>GATA2</i> The molecular profile is complex, but reproducible common CNVs might be useful as predictive biomarkers of therapeutic effectiveness	35
RNA	HyCEAD assay (with tailored PCa gene panel, 64 genes)	Transcriptomics (bulk)	mCRPC	CTCs from other patients with mCRPC	A custom panel consisting of <i>AR</i> , <i>ARV7</i> , <i>FOLH1</i> (PSMA), <i>KLK2</i> , <i>KLK3</i> , and <i>TMPRSS2</i> was developed	143
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 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	137
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 $\geq 2$ of these genes was more frequently resolved in CTC DNA (35% of patients) than in ctDNA (21% of patients); higher genomic instability (assessed through CTC LST) was observed in patients with clinically defined AVPC	158

**Table 2 (continued) | Molecular analyses on circulating tumour cells from patients with localized and metastatic prostate cancer**

Analyses	Genes or targets studied	Genomics or transcriptomics (single-cell or bulk)	Disease state (n)	Comparator	Findings	Ref.
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 ( <i>UBB</i> )	Individual CTCs were highly heterogeneous, but select EMT-related genes ( <i>PTPRN2</i> , <i>ALDH1</i> , <i>ESR2</i> , <i>WNT5A</i> ) were more highly expressed in patients with castration-resistant cancer than in those with castration-sensitive cancer	145
RNA-seq of single cells (amplification + next-generation RNA-seq)	NA	Transcriptomics (single-cell level)	77 CTCs from 12 patients with metastatic PCa and 1 patient with localized PCa	Bulk transcriptomes of primary PCa from a separate cohort (n=12 patients)	60% of the 77 CTCs analysed robustly expressed putative stem-cell markers, 92% expressed epithelial markers, but mesenchymal genes were not upregulated compared with primary tumours Non-canonical Wnt signalling was activated in patients with mPCa progressing under enzalutamide AR inhibition relative to enzalutamide-untreated patients in a retrospective analysis ( $P=0.0064$ )	148
WGA, then NGS	Genome-wide CNV profiles	Genomics (single cell)	mCRPC (n=1)	CTCs compared at four sequential timepoints	Targeted ADT but not standard chemotherapy was associated with the drastic depletion of an initial clone 'A' and the emergence of two molecularly distinct clones 'B' and 'C' (assessed through phenotypic AR status and genome-wide CNV profile)	138
RT-PCR	<i>FOLH1</i> , <i>AR-V7</i> , <i>AR</i> , <i>EGFR</i>	Transcriptomics (bulk)	mCRPC (n=79)	NA	In mCRPC, PSMA expression in CTCs (vs PSMA <sup>+</sup> 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 months, $P=0.010$ )	142
CTCs: FA-FISH Tissue: FISH, IHC	<i>ERG</i> (FISH and IHC), <i>AR</i> (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 <i>ERG</i> rearrangement, with further <i>ERG</i> rearrangement patterns detected in CTCs, indicating greater heterogeneity in CTCs than in biopsy samples	34
RT-PCR	<i>AR1/2</i> , <i>AR 4/5</i> , <i>AR-V7</i> , <i>AR-V9</i> , <i>KLK2</i> , <i>KLK3</i> , <i>TMPRSS2</i> , <i>FOLH1</i> , <i>NKX3.1</i> , <i>SYP</i> , <i>CHGA</i> , <i>CHGB</i> , <i>MYCN</i> , <i>NKX2.2</i>	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	146
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 SSNVs were unique to either CTCs or ctDNA	156
RT-ddPCR	<i>AR</i> , <i>AR-V7</i> , <i>KLK3</i> , <i>FOLH1</i> , <i>EPCAM</i> , <i>KRT19</i>	Transcriptomics (bulk)	Localized PCa (n=26), mHSPC (n=10), mCRPC (n=28)	Localized 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	147
Flow cytometry (ImageStreamX)	<i>PROM1</i> (CD133)	Proteomics	Patients with mCRPC (n=20)	CD133 <sup>+</sup> vs CD133 <sup>-</sup> CTCs	CD133 was detected in all patients. CD133 <sup>+</sup> CTCs had elevated Ki67 expression vs CD133 <sup>-</sup> CTCs, suggesting a greater proliferative potential; AR expression and AR nuclear colocalization have similar levels in CD133 <sup>+</sup> and CD133 <sup>-</sup> CTCs, suggesting that CD133 expression is a marker of CTC proliferation and is independent of AR pathway activity	162
Multi-RNA-ISH	<i>HK2</i> , <i>PDP2</i> , <i>G6PD</i> , <i>PGK1</i> , <i>PHKA1</i> , <i>PYGL</i> , <i>PDK1</i> , <i>PKM2</i>	Metabolomics	mPCa (n=29)	Non-metastatic PCa (n=25)	GM <sup>+</sup> CTC (determined through the expression of eight metastasis-related metabolic genes) were detected in 64.8% of patients; increased hypermetabolic GM <sup>+</sup> CTCs were associated with metastasis and advanced tumour stages ( $P<0.05$ ). The triple tPSA-Gleason-GM <sup>+</sup> CTC marker outperformed tPSA-Gleason-H-CTC marker (AUC=0.904 vs 0.874) in discriminating mPCa vs localized PCa	164

**Table 2 (continued) | Molecular analyses on circulating tumour cells from patients with localized and metastatic prostate cancer**

Analyses	Genes or targets studied	Genomics or transcriptomics (single-cell or bulk)	Disease state (n)	Comparator	Findings	Ref.
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	159
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 ( <i>WNT7B</i> and <i>WNT5A</i> ) and increased canonical WNT signalling associated genes (including <i>FZD4</i> and <i>LEF1</i> )	149
FISH	FBXL4	Genomics	Bone metastatic PCa (n=7)	2 patients with lower Gleason score (3+3; 4+3)	<i>FBXL4</i> 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 <i>FBXL4</i> as a putative PCa TSG involved in migration and invasion regulation, suggesting a potential role for CTC <i>FBXL4</i> loss as a potential prognostic marker in liquid biopsy	139
WES	NA	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	140
RNA-seq	78 prostate cancer-related target genes	Transcriptomics (bulk)	mCRPC (n=41)	NA	<i>TMPRSS2:ERG</i> fusion was expressed in 41% of CTC samples, and the aggressive prostate cancer-associated long non-coding RNA <i>SChLAP1</i> was upregulated in 70% of CTC samples; CTC expression of <i>WNT5a</i> , <i>AURKA</i> and <i>BMP7</i> were independently predictive of overall survival	150

ABI, abiraterone acetate; aCGH, array-based comparative genomic hybridization; ADT, androgen deprivation therapy; AR, androgen receptor; AUC, area under the curve; AVPC, aggressive variant prostate cancer; bcDNA, buffy coat DNA; BPH, benign prostatic hyperplasia; cfDNA, cell-free DNA; CNA, copy number alteration; CNV, copy number variant; CRPC, castration-resistant prostate cancer; CTC, circulating tumour cell; ddPCR, droplet digital polymerase chain reaction; DE, diethylstilbestrol; DGE, differential gene expression; EMT, epithelial-mesenchymal transition; ENZ, enzalutamide; FA-FISH, filter-adapted fluorescence in situ hybridization; FISH, fluorescence in situ hybridization; H-CTCs, hybrid circulating tumour cells (regarding epithelial and mesenchymal phenotypes); HE, heterozygous; HO, homozygous; IHC, immunohistochemistry; IR, immunofluorescence; LST, large-scale transitions; mCRPC, metastatic castration-resistant prostate cancer; mHSPC, metastatic hormone-sensitive prostate cancer; multi-RNA-ISH, multi-RNA in situ hybridization; NA, not available; NGS, next-generation sequencing; OS, overall survival; PCa, prostate cancer; PFS, progression-free survival; PSA-PFS, prostate-specific antigen progression-free survival; PSMA, prostate-specific membrane antigen; qPCR, quantitative polymerase chain reaction; ROC, receiver operating characteristic; rs, reference single-nucleotide polymorphism; RT-ddPCR, real-time droplet digital polymerase chain reaction; RT-PCR, reverse transcription polymerase chain reaction; SE-iFISH, subtraction enrichment-immunofluorescence in situ hybridization; SNV, single-nucleotide variant; SSNVs somatic single-nucleotide variants; SV, structural variant; TGFβ, transforming growth factor-β; tPSA, total PSA; TSG, tumour suppressor gene; WES, whole-exome sequencing, WGA, whole genome amplification; WGS, whole-genome sequencing.

higher, respectively, in the blood enriched from CTCs from patients with metastatic versus localized disease, with *EPCAM* being detected in 100% of mCRPC CTCs.

Metastatic prostate cancer is widely treated with second-line therapies, yet no definitive method for predicting therapy response, or for detecting if early signs of treatment resistance in these patients exist. To address this gap, CTCs can be studied to identify biomarkers of therapeutic effectiveness and treatment response<sup>45,146,148,149</sup>. However, results from available studies are not consistent. In one study, the non-canonical 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 versus 41 CTCs from 8 patients who did not receive treatment ( $P = 0.0064$ ), with the most significantly enriched downstream components of

non-canonical-WNT signalling being *RAC1*, *RHOA* and *CDC42* (ref. 148). Another study including pools of up to 10 CTCs isolated from patients who were resistant to abiraterone or enzalutamide therapies did not show significant enrichment of these non-canonical-WNT pathway targets (*RAC1*, *RHOA* and *CDC42*), nor was the non-canonical-WNT pathway identified as enriched in pathway analysis<sup>149</sup>. However, results from both studies showed increased expression of canonical WNT signalling-associated genes including *FZD4* and *LEF1*, and increased expression of *WNT5A* (a non-canonical-WNT pathway activator) in CTCs from patients who developed AR-inhibitor resistance<sup>148,149</sup>. 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<sup>150</sup>. 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<sup>146</sup>, and transforming growth factor- $\beta$  (TGF $\beta$ ) and cyclin D1 (encoded by *CCND1*) signalling pathways<sup>149</sup>. 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<sup>151</sup>. Transcriptomic profiling of prostate cancer CTCs with qRT-PCR panels targeting *AR* splice variants (*AR-V7* (refs. 152,153) and *AR-V9* (ref. 130)) and *AR*-regulated genes (*AR-V7*, *AR-V9*, *TMPRSS2*, *KLK2*, *KLK3* and *FOLH1*)<sup>130</sup> has shown that the expression of these targets is prognostic for response to ARSIs and capable of detecting transition to NEPC<sup>154</sup>. 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.

## Genomic analyses of circulating tumour cells in metastatic prostate cancer

Genomic analyses of CTCs can offer a crucial insight into tumour biology and clonal evolution of disease<sup>34,37,139</sup>. In this regard, alterations such as *PTEN* gene loss<sup>37</sup>, *AR* amplification<sup>34</sup>, and *ERG* rearrangements<sup>34</sup> 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<sup>140</sup>; additionally, 84% agreement in *PTEN* loss was reported between CTCs and primary tumour<sup>37</sup>, and 88% concordance in *ERG* rearrangement was shown between metastatic biopsies and CTCs<sup>34</sup>. 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<sup>139</sup>. Microarray analysis identified 6q16 and *FXBL4* loss in six samples of prostate cancer bone metastases, and a significant reduction in *FXBL4* expression was found through qRT-PCR in five bone metastases and four primary prostate cancer samples compared with benign prostatic hyperplasia samples ( $P = 0.001$ )<sup>139</sup>. In this study, FISH was then used to 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 were also present in the primary tumour<sup>139</sup>. Subsequent results from *in vitro* work in prostate cancer cell lines indicated that *FXBL4* is a putative prostate cancer tumour suppressor gene involved in migration and invasion regulation<sup>139</sup>. These findings indicate that CTCs might be considered 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 metastasize.

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<sup>35,37,137,138,140</sup>. 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%), *FOXAI* (31.25%), *CYP11B1* (31.25%) and *UGT2B17* (31.25%)<sup>35</sup>. Although inter-patient molecular profiles were heterogeneous, such reproducible common CNAs in *AR* signalling genes might be useful as predictive biomarkers of therapeutic effectiveness (for example, *AR*-directed therapies) or therapy selection, such as CNAs in loci containing known prostate cancer drug targets (*ERG* and *BRD4*)<sup>35</sup>. 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'<sup>138</sup>. 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 with 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 towards tumour metastasis<sup>137</sup>. 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)<sup>140</sup>. 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)<sup>155</sup>. 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<sup>156,157</sup>. However, these 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<sup>157</sup>. 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 studies are needed to confirm whether SSNVs in prostate cancer-related genes are more often detected in CTCs or cfDNA<sup>156</sup>. Analysis of matched cfDNA and CTC samples from 57 patients with mCRPC showed that CTCs were more frequently detectable and evaluable for CNA analysis (73.7% versus 42.1%, respectively), indicating that CTCs might offer greater insight into clonal evolution<sup>158</sup>. 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 might offer differing insights into the biology of prostate cancer<sup>156,157</sup>.

Single-cell analyses of CTCs offer great promise for studying phenotypic heterogeneity and investigating EMT<sup>148,158</sup>. Analysis of 257 prostate cancer CTCs at the single-cell level in 47 patients with mCRPC (range 1–22 CTCs/patient) showed that combined ( $\geq 2$  genes) TSG loss in *TP53*, *PTEN* and/or *RBI* on the same CTC was associated

with shorter median PFS (median 3.6 versus 7.2 months,  $P = 0.042$ ) and greater genomic instability as defined by large-scale transitions scores ( $P = 0.0015$ ), showing the ability of genomic molecular CTC features (beyond enumeration) to offer clinically relevant information<sup>158</sup>. RNA-seq of 77 single, intact CTCs isolated from 13 patients (mean 6 CTCs per patient) with metastatic and localized prostate cancer showed higher intercellular heterogeneity in individual CTC transcriptional profiles than single cells from prostate cancer cell lines (mean correlation coefficient: 0.10 versus 0.44,  $P < 1 \times 10^{-20}$ )<sup>148</sup>. Results from this study also showed 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, whereas mesenchymal genes were not upregulated compared with prostate cancer cell lines, suggesting an epithelial- or stem-cell-like phenotype of prostate CTCs<sup>148</sup>. However, in this study, CTCs were isolated using a microfluidic chip coated with anti-EpCAM antibody, biasing captured CTCs towards an epithelial phenotype. However, single-cell analyses of prostate CTCs are currently technically limited by the low purity of CTC-enriched samples – especially when live whole cells free of immune-affinity labels are eluted – and high single-cell technology costs.

## Current limitations of molecular analyses of circulating tumour cells

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 to predict the quality of single CTC DNA sequencing libraries before moving to costly whole-exome sequencing<sup>140</sup>. High concordance (96.3% of genes covered in both methods) was observed between targeted next-generation sequencing panels and whole-exome sequencing of CTCs (observed in two patients with mCRPC; four CTCs per patient), suggesting that these targeted panels could provide a cost-effective substitute for detecting clinically significant alterations in prostate cancer-related genes<sup>159</sup>.

Current studies focusing on molecular analysis of CTCs in patients with mCRPC rely on cohorts with small sample sizes, often with less than a few hundred cells examined in total<sup>140,148,159</sup>. Studies in which molecular analyses of CTCs are carried out in patients with localized prostate cancer are particularly limited in number, with RT-PCR of immunomagnetically enriched cells often used to differentiate between metastatic and localized disease states based on gene expression<sup>147,160</sup>. Notably, in one study, transcriptomic sequencing of CTCs – enriched from the blood of 98 patients pre-prostate biopsy and 155 patients with localized 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 versus 0.826,  $P = 0.03$ ) for PSA alone versus combined with CTC score<sup>43</sup>. 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.

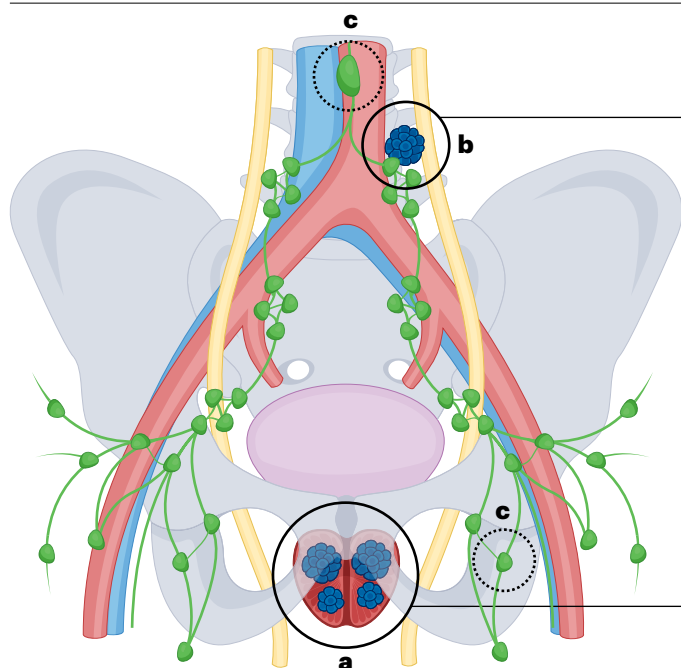
CTC detection is limited by technological development and low CTC numbers in many patients. An integrated panel including other liquid biopsy techniques such as cfDNA, microRNAs (miRNA), or epithelial cells isolated from urine could combine the best of these several liquid biopsy options<sup>36,156</sup>. Enrichment methods are also crucial to carrying out downstream analyses, with selective upstream isolation potentially affecting prostate cancer CTCs that undergo molecular profiling,

in turn influencing phenotypic findings (for example, epithelial versus mesenchymal phenotype)<sup>34,161</sup>.

## Functional analyses and culture of circulating tumour cells

Functional analysis of CTCs offers the possibility to isolate specific pathways and manipulate metabolic function in prostate cancer CTCs<sup>162–164</sup>. In one study of metabolic markers, a glucose-metabolic (GM<sup>+</sup>) CTC subtype was identified, characterized by expression of both *PGK1* and *G6PD*; in this study, an increased number of hyper-metabolic GM<sup>+</sup> 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<sup>+</sup> CTCs versus EMT CTC subtypes; epithelial AUC 0.729 and mesenchymal AUC 0.648)<sup>164</sup>. 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<sup>162,163</sup>. 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<sup>163</sup>. CD133-positive CTCs have been shown to have elevated Ki67 expression and no change in AR expression or AR cellular co-localization with nuclear markers, suggesting that CD133 expression is a marker of CTC proliferation and is independent of AR pathway activity<sup>162</sup>. These results show the 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-naive metastatic prostate cancer<sup>92</sup>. 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 in the long term<sup>92</sup>. In other studies<sup>165</sup>, organoid models have been used to attempt CTC culture in 3D. In one study, organoid culture of CTCs enriched for 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 cultures maintained for >6 months<sup>166</sup>. Validation through qRT-PCR showed that *AR-V7* was expressed in one of the CTC organoid 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<sup>166</sup>. 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<sup>165</sup>. 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 maintenance<sup>92,165,166</sup>. These studies



**Fig. 2 | Current clinical utility for circulating tumour cell detection in localized and metastatic prostate cancer.** **a**, Circulating tumour cells (CTC) have been studied in patients with localized prostate cancer (mostly stratified as ‘high risk’), with varying positivity rates (%) observed across studies. For study details, please see Supplementary Table 4. **b**, CTC analysis from blood of patients with metastatic prostate cancer can be carried out to predict treatment selection and/or response. For study details, please see Supplementary Table 3. **c**, Potential for future studies

### CTCs in metastatic prostate cancer

#### CTCs in treatment selection

- Patient groups: mCRPC
- Range of cohort size:  $N = 37\text{--}193$
- Isolation approaches include: AdnaTest and EpicSciences
- Survival outcomes: PSA response, PFS, OS
- Treatment options: ARPIs vs taxanes
- Take-home message: AR-V7-positive and AR-V7-nuclear-positive CTCs can predict increased OS on taxane therapy

#### CTCs in treatment response

- CTC cut-off:  $\geq 3$  or  $\geq 5$  CTCs/ml
- Patient groups: mHSPC or mCRPC
- Range of cohort size:  $N = 28\text{--}523$
- Isolation approaches include: CellSearch, AdnaTest, FACS, CanPatrol, Parsortix, EpicSciences
- Survival outcomes: PSA response, PFS, OS
- Treatment options: ADT, abiraterone, enzalutamide, docetaxel, cabazitaxel, taxanes, radium-223
- Take-home message: a higher number of CTCs predicts shortened survival

### CTCs in localized prostate cancer

- Studies include varying treatments (RP, RT, ADT)
- Patient groups: HRLPC
- Range of cohort size:  $N = 26\text{--}180$
- Isolation approaches include: CellSearch, CellCollector, RT-PCR, flow cytometry, EpicSciences, EPISPOT
- Range of CTC positivity: 8–80%

focused on dissemination of tumour cells via the lymphatic fluid, with possible routes including pelvic lymphatic aspiration or cisterna chyli drainage. ADT, androgen deprivation therapy; ARPI, androgen receptor pathway inhibitors; HRLPC, high-risk localized prostate cancer; mCRPC, metastatic castration-resistant prostate cancer; mHSPC, metastatic hormone-sensitive prostate cancer; OS, overall survival; PFS, progression-free survival; PSA, prostate-specific antigen; RP, radical prostatectomy; RT, radiotherapy; RT-PCR, reverse transcription polymerase chain reaction.

are also limited by the challenge of definitively identifying cultured cells as CTCs.

## Circulating tumour cells and clinical prognosis in prostate cancer

To date, research on enumeration of CTCs to predict treatment response has focused on metastatic disease (Fig. 2, Supplementary Table 3). In this study, CellSearch has been predominantly used to enumerate CTCs (typically identified as CD45<sup>+</sup>, EpCAM<sup>+</sup> and CK 8, 18 and/or 19<sup>+</sup>) and predict response to anti-androgen hormonal agents, radiotherapy and chemotherapy in patients with mCRPC<sup>167–174</sup>. In several studies, detection of  $\geq 5$  CTCs per 7.5 ml of peripheral blood in patients with mCRPC versus patients with  $< 5$  CTCs/7.5 ml blood was associated with shortened progression-free survival (PFS) and/or OS before treatment with cabazitaxel (chemotherapy) (OS 8.8 versus 28 months,  $P = 0.002$ ; PFS not significant<sup>167</sup>); before treatment with abiraterone or enzalutamide (second-line hormonal therapies) (abiraterone: PFS  $P = 0.043$ , OS  $P = 0.027$  (ref. 169); enzalutamide: PFS  $P = 0.021$ , OS  $P = 0.003$  (ref. 171)); or after the first treatment cycle with cabazitaxel and docetaxel (chemotherapies) (docetaxel: OS  $P = 0.004$ , PFS not evaluated)<sup>167–169,171</sup>. Detection of  $\geq 5$  CTCs/7.5 ml in blood was also associated with shortened PFS and OS compared with  $< 5$  CTCs/7.5 ml in patients with mCRPC before and after 12 weeks of radium-223 radiotherapy (PFS 17.1 versus 25 weeks,  $P = 0.061$ , OS 29.4 versus 57.9 weeks,  $P = 0.032$ )<sup>170</sup>. In another study, high radium-223 therapy completion rate (6-month injection course) was reported in patients

with a  $\geq 5$  baseline CTC count<sup>173</sup>. 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<sup>171</sup>.

In one study, CTC-positive status, irrespective of detection threshold ( $\geq 1$  CTC), was associated with shorter rPFS (CTC-positive versus CTC-negative,  $P = 0.043$ ) in patients with mCRPC receiving second-line hormonal therapy with abiraterone<sup>169</sup>, and with shorter OS after the first treatment cycle in patients receiving cabazitaxel chemotherapy (CTC-positive versus CTC-negative,  $P = 0.047$ )<sup>167</sup>. Importantly, in another study, the predictive accuracy of  $\geq 5$  CTCs/7.5 ml blood for OS after the 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<sup>168</sup>.

Few studies have been carried out to investigate CTC quantification in mHSPC<sup>46,175,176</sup>. Baseline CTC presence before docetaxel treatment has been shown to be independently predictive of shortened OS in patients with mHSPC<sup>46</sup>. In another study, baseline CTC presence before docetaxel treatment in patients with mHSPC was predictive of progression to mCRPC<sup>175</sup>. 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  $\geq 5$  CTCs<sup>176</sup>. 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 (AUC 0.86 versus 0.58 versus 0.67, respectively) after a 3-year follow-up<sup>177</sup>. 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 for reaching this aim.

The potential of CTCs in screening, diagnosis or treatment of localized prostate cancer has been assessed in only a small number of studies<sup>40,178–181</sup> (Supplementary Table 4). 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)<sup>181</sup>. In other studies, CTC count and enumeration of AR-positive CTCs in 49 patients with localized 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<sup>40</sup>. The incorporation of PSMA<sup>+</sup>/CD45<sup>-</sup> CTC presence 90–120 days after surgery into a clinicopathological risk score (Cancer of the Prostate Risk Assessment) 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 Cancer of the Prostate Risk Assessment score alone versus 0.86 combined with CTC presence)<sup>179</sup>. Thus, CTC detection shortly after surgery might provide an 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<sup>167–174</sup>. 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.

## Detection of the AR and AR variants in prostate cancer circulating tumour cells

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)<sup>182,183</sup>. 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<sup>184</sup> with particular attention to AR-V7, the most frequently studied AR-V<sup>153,185–196</sup>. 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<sup>197</sup>. 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% versus 61%,  $P < 0.001$ ), shorter PSA-PFS (median 1.4 versus 6.0 months,  $P < 0.001$ ), shorter clinical or rPFS (median 2.1 versus 6.4 months,  $P < 0.001$ ) and reduced OS (9.2 versus >11.9 months,  $P < 0.001$ )<sup>153</sup>. Furthermore, results from a study including 36 patients

with mCRPC receiving taxane chemotherapy or anti-androgens showed that patients with AR-V7-positive CTCs had superior PSA response rates (41% versus 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 with 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<sup>189</sup>. 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<sup>190</sup>. 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 the CTC-present/AR-V7-present group<sup>190</sup>.

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<sup>198,199</sup>. In these studies, no association was reported between CTC AR-V7 positivity and post-therapy PSA changes, time on therapy or rPFS<sup>200,201</sup>. 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<sup>191</sup>. Importantly, in another work, absence, rather than 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<sup>192</sup>. Results from further studies have shown that CTC AR-V7 was associated with reduced rPFS, PSA-PFS<sup>202</sup>, OS<sup>202,203</sup> and time to treatment failure<sup>203</sup> 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<sup>204</sup>. 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<sup>152</sup>. In this study, shorter PFS (modified AdnaTest hazard ratio 1.9, 95% CI 1.1–3.3;  $P = 0.032$  and Epic Sciences 2.4, 95% CI 1.1–5.1,  $P = 0.020$ ) and OS (modified AdnaTest hazard ratio 4.2, 95% CI 2.1–8.5 and Epic Sciences 3.5, 95% CI 1.6–8.1), were reported in patients with AR-V7-positive CTCs compared with 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<sup>152</sup>. Additionally, the Epic Sciences AR-V7 assay is limited by dependence on nuclear localization for positivity and is no longer available for purchase<sup>205</sup>. 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<sup>206–209</sup>. Small sample sizes, different isolation and detection methods, and study design or failure to reach end point could contribute to discordant results.

Most studies on CTCs have involved patients with mCRPC, but CTCs could also help to identify which patients are likely to progress from mHSPC to castrate-resistant disease. In 42 patients with oligometastatic HSPC, AR-positive CTC status assessed through immunofluorescence (Epic Sciences assay) at the baseline before radiotherapy was associated with shorter PFS than AR-negative CTC status ( $P = 0.011$ , median PFS AR<sup>+</sup> = 9.3 versus AR<sup>-</sup> = 27.1 months)<sup>210</sup>. In another study, AR-positive 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 cancer-specific survival (14.3 versus 33.0 months,  $P = 0.002$ )

than AR-negative CTC status, with all patients with AR-V7-positive CTCs dying of prostate cancer during the study follow-up<sup>211</sup>. 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<sup>205,206</sup>. This association was no longer significant ( $P = 0.55$ ) if nuclear-agnostic criteria were used<sup>205</sup>, 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 the androgen-regulated genes *GRHL2*, *HOXB13* and *FOXAI* could predict OS (median OS: not reached versus 24.8 months versus 16.2 months for 0, 1 and  $\geq 2$  transcripts, respectively;  $P = 0.0052$ )<sup>212</sup>. In another study, microfluidic capture and single-cell immunofluorescence analysis of 'AR-on (PSA<sup>+</sup> and PSMA<sup>-</sup>)', 'AR-off (PSA<sup>-</sup> and PSMA<sup>+</sup>)' and 'AR-mixed (PSA<sup>+</sup> and PSMA<sup>+</sup>)' 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<sup>213</sup>. 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<sup>213</sup>, but validation in larger cohorts is needed<sup>213</sup>. 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<sup>v</sup> detected in 2 of 17 CTCs (12%), AR-V7 in 0 of 17 (0%) and AR-v567es in 10 of 17 (59%)<sup>161</sup>. 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 versus 7.29 for patients with ARv567es-negative versus ARv567es-positive CTCs), although AR-V7 had the strongest influence in predicting outcomes (PFS 12.02 versus 8.48 months for patients with AR-V7-negative versus AR-V7-positive CTCs, HR = 0.38,  $P = 0.01$ )<sup>214</sup>. Thus, the differential detection of AR splice variants in prostate cancer CTCs could aid in 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 monitoring and conflicting results. Thus, these methods are not currently used in clinical practice. However, increasing assay sensitivity might improve the detection of AR-V7 in CTCs to predict resistance to hormonal therapies, including abiraterone and enzalutamide<sup>215</sup>.

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<sup>161</sup>. CellSearch has been shown to capture CTCs with AR amplification, whereas with ISET, only CTCs with AR gain of copies are captured<sup>34</sup>. 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<sup>216</sup>. Results from further studies of genomic paired analyses of cfDNA and CTC DNA have shown discordant AR gene rearrangements in patients with mCRPC<sup>217</sup>. These findings suggest the potential need of parallel liquid biopsy analyses to capture the heterogeneity of genetic and transcriptomic alterations.

## Conclusions

Understanding metastatic dissemination of prostate cancer is crucial to explaining 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 (CNAs and SSNVs) and transcriptomic (heterogeneous expression of EMT markers) sequencing showing intra-patient CTC heterogeneity at the single-cell level.

The prognostic significance of  $\geq 5$  CTCs/7.5 ml blood in patients with mCRPC at the baseline before treatment with chemotherapy, second-line hormonal therapy and radiotherapy 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 localized 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. 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 for gaining an 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 whole-genome sequencing 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 be aimed at improving 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 an important insight into risk stratification at diagnosis, progression, response to treatment and lethality of this common and ubiquitous disease.

Published online: 16 January 2026

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## Acknowledgements

S.M.A. would like to thank the Clarendon Fund in partnership with the St Edmund Hall Kerr-Muir Scholarship, specifically Mr. James Kerr-Muir, for their generous support. T.A. is funded by Cancer Research UK (RCCPDB-Nov23/100013) and supported by the National Institute for Health and Care Research (NIHR). I.G.M., C.M.E. and A.D.L. would like to acknowledge the John Black Charitable Foundation for support. C.M.E. would like to acknowledge support from Rosetrees. A.D.L. is funded by Cancer Research UK (C57899/A25812). Y.J.L. has received funding from Prostate Cancer UK (MA-CT20-011) to investigate the potential of using circulating tumour cells to predict prostate cancer surgical outcome. A.D.L. and R.J.B. are co-PIs of the TRANSLATE prostate biopsy trial funded by HTA (NIHR131233) and are module leads of the QUANTUM Biobank part funded by the John Black Charitable Foundation.

## Author contributions

S.M.A., T.A., D.T.C., N.M.S.R., S.B., C.S., A.D.L. researched data for the article. All authors contributed substantially to discussion of the content. S.M.A., T.A., D.T.C., N.M.S.R., R.B., S.F., W.Y., J.D., S.S., F.C.H., R.J.B., Y.J.L., I.M., C.M.E., T.M.M., A.D.L. wrote the article. All authors reviewed and/or edited the manuscript before submission.

## Competing interests

S.M.A., C.M.E. and A.D.L. have received support from BioRad through the Celselect grant. T.M.M. is on the advisory boards for Merck, Foundation Medicine, Johnson & Johnson and Pfizer. A.D.L. has received educational support from GlaxoSmithKline, Astellas, Lilly, AstraZeneca and Ipsen. A.D.L. and F.C.H. have paid roles as BJUI Editors. Y.J.L. has received support from ANGLE PLC for his circulating tumour cell study. A.D.L. has received honoraria for reviewing for European Urology and Lancet Oncology. A.D.L. has received consulting fees from AlphaSights. A.D.L. undertakes medicolegal expert witness work related to prostate cancer management in the UK. The funders had no role in the study design, data collection and analysis, decision to publish or preparation of the manuscript. The other authors declare no competing interests.

## Additional information

**Supplementary information** The online version contains supplementary material available at <https://doi.org/10.1038/s41585-025-01121-8>.

**Peer review information** *Nature Reviews Urology* thanks the anonymous reviewers for their contribution to the peer review of this work.

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