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Using circulating tumor cells to inform on prostate cancer biology and clinical utility

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Abstract

Substantial advances in the molecular biology of prostate cancer have led to the approval of multiple new systemic agents to treat men with metastatic castration-resistant prostate cancer (mCRPC). These treatments encompass androgen receptor directed therapies, immunotherapies, bone targeting radiopharmaceuticals and cytotoxic chemotherapies. There is, however, great heterogeneity in the degree of patient benefit with these agents, thus fueling the need to develop predictive biomarkers that are able to rationally guide therapy. Circulating tumor cells (CTCs) have the potential to provide an assessment of tumor-specific biomarkers through a non-invasive, repeatable “liquid biopsy” of a patient’s cancer at a given point in time. CTCs have been extensively studied in men with mCRPC, where CTC enumeration using the Cellsearch® method has been validated and FDA approved to be used in conjunction with other clinical parameters as a prognostic biomarker in metastatic prostate cancer. In addition to enumeration, more sophisticated molecular profiling of CTCs is now feasible and may provide more clinical utility as it may reflect tumor evolution within an individual particularly under the pressure of systemic therapies. Here, we review technologies used to detect and characterize CTCs, and the potential biological and clinical utility of CTC molecular profiling in men with metastatic prostate cancer.

Keywords

Androgen receptor; biomarker; castration resistant prostate cancer; EpCAM; liquid biopsy; microfluidic; PSA

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Declaration of interest

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Introduction

Prostate cancer is the most common non-cutaneous cancer in men and the second most common cause of cancer-related death in the United States, with 29 480 deaths in 2014¹. Six therapies improve overall survival (OS) in men with metastatic castration-resistant prostate cancer (mCRPC), including the taxane chemotherapeutics docetaxel and cabazitaxel, the hormonal agents abiraterone acetate and enzalutamide, the immunotherapeutic sipuleucel-T and the bone-targeting radiopharmaceutical radium-223²⁻⁷. However, many men with mCRPC do not respond to these therapies. For example, prostate-specific antigen (PSA) response to enzalutamide or abiraterone in the pre-chemotherapy mCRPC setting is 60–70%. Approximately 30–40% of patients have no response to these agents with respect to PSA levels (primary resistance), and among patients who initially have a response to enzalutamide or abiraterone, virtually all eventually acquire secondary resistance^{3,6,8}. Furthermore, cross-resistance between enzalutamide and abiraterone is clinically evident: for instance, PSA response to treatment with abiraterone after enzalutamide, or vice versa, is reduced by nearly half, with most responses lasting only a few months⁹⁻¹¹. The primary cause of resistance is genetic alterations in the androgen receptor (AR) gene that re-activate the AR pathway. Additional genetic lesions in *PI3K*, *RAS*, *MYC*, *WNT* or genes in DNA repair pathways may also contribute to resistance¹². Recent data suggest that bypass from AR blockade can be mediated by activation of the glucocorticoid receptor (GR), which drives expression of AR target genes¹³. In addition, emerging data suggest that certain AR variants (i.e. AR-v7) that lack the ligand binding domain may not only convey resistance to abiraterone acetate and enzalutamide, but may also promote taxane resistance given that these variants do not require microtubule-dependent AR nuclear translocation¹⁴. Understanding the molecular mechanisms that underlie the development of resistance in men with mCRPC may permit the rational selection of therapies that are better able to address these resistance mechanisms. CTCs present an opportunity to carry out non-invasive real-time tumor sampling.

Hematogenous metastasis of solid tumors involves migration and invasion of carcinoma cells from the primary tumor into blood vessels, circulation in the bloodstream, dissemination to distant sites, extravasation and colony establishment in metastatic niches. CTCs are tumor cells released from the primary tumor or metastatic site into the periphery, and are believed by many researchers to be essential in the hematogenous spread of malignancy and establishing metastases¹⁵⁻¹⁷. CTCs can be detected and captured via different technologies from peripheral blood, which is in contrast to metastatic biopsies which require an invasive procedure that may not be possible in certain locations or present too high a risk. Therefore, the ability to collect and analyze CTCs from peripheral blood for tumor-specific molecular aberrations is an attractive alternative to standard biopsies. In addition, with the continuous evolution of tumors, which involves genetic and epigenetic alteration of cancer cells and tumor heterogeneity, primary tumors and individual metastases likely provide a limited snapshot of the molecular status of a given cancer in a given patient at that time. CTCs could provide real-time and sequential “liquid biopsy” for patients with cancer, and CTC biomarker analyses from peripheral blood can be conducted repeatedly to

allow real-time monitoring of cancer progression and response to therapies in patients who have sufficient CTCs.

Recent studies have demonstrated that CTC molecular analysis is feasible and may provide important information on therapeutic targets and drug resistance mechanisms in patients with carcinoma, including prostate cancer^{18–27}. The goal of CTC molecular profiling is to identify and select therapeutic targets, and to match individual patients with therapies designed to address the molecular lesions present (accurate medicine). In addition, longitudinal assessments of CTC biomarkers may permit the changing of therapy as cancer evolves or undergoes treatment selection. The application of novel next-generation sequencing technologies in the area of CTC molecular characterization, in combination with development in CTC detection technologies, should provide important areas of growth and clinical utility for the personalized treatment of men with prostate cancer and many other cancers.

Currently, the Cellsearch® platform is the only FDA-approved CTC detection method in patients with metastatic breast, prostate and colorectal cancer. The platform, which isolates CTCs from whole blood using an epithelial cell adhesion molecule (EpCAM)-based ferromagnetic antibody, defines a CTC to be a nucleated (determined by DAPI staining) cell larger than 4 µm in diameter that lacks the common leukocyte marker CD45, and expresses cytokeratins¹⁵. Using the EpCAM capture reagent coupled with three biomarkers, CTCs are reliably defined in patients with a range of solid tumors, but are absent in normal individuals^{15,28,29}. Enumeration of CTCs has been shown to be prognostic for overall survival in many tumors including breast, colorectal and metastatic prostate cancer³⁰. Unlike PSA changes, CTC flare (enumeration surge after starting chemotherapy) has not been observed to date, and CTC enumeration changes may occur earlier than PSA declines, with some studies suggesting improvements in survival association with early CTC changes as compared with PSA declines³⁰. The measurement and enumeration of CTCs in cancer also plays a critical role in the early diagnosis of metastatic disease, in prediction as it relates to systemic therapy selection, in risk-stratification for clinical trials or clinical practice or as a surrogate biomarker for decision-making in research studies or clinical practice. CTC characterization may also offer use as a pharmacodynamic biomarker in drug development for rapidly assessing drug activity.

Circulating tumor cells (CTCs) provide real-time and easy access to tumor cells; however, there are limitations with CTC studies. One major limitation is the lack of detection of CTCs in many men despite the presence of progressive, mCRPC (>50% non-detection rate)^{30,31}. Some CTCs may lack epithelial biomarkers entirely³². This CTC heterogeneity may partly explain the escape of detection of CTCs in CRPC and other solid tumors using the standard epithelial antigen based technology. Detecting CTCs with high sensitivity and specificity is an important goal of CTC studies in prostate cancer and other solid tumors. Improvements in CTC capture by novel capture antibodies (e.g. mesenchymal antigens), negative selection methods and novel CTC chip designs that improve CTC yield, and improved CTC molecular profiling technologies will help further exploration in CTCs and its implication in metastatic prostate cancer.

This review is mainly focused on existing CTC capture or isolation technologies, methods for the molecular characterization of these CTCs from men with metastatic prostate cancer and the biological and clinical utilities of these approaches (Figure 1).

Methods: CTC isolation approaches and molecular characterization

CTC isolation approaches

A variety of techniques for CTC isolation have been studied, each has specific advantages and limitations, and most methods have suffered from a lack of robust clinical data to inform on clinical utility. The rarity of CTCs is the key technical challenge for CTC capture, with some men having no CTCs evaluable for profiling despite metastatic disease. Methods that are more sensitive may additionally suffer from low specificity (false positives) due to the isolation of other cell types rarely found in the circulation, such as endothelial cells, CD45 negative leukocytes or circulating mesenchymal or bone marrow derived stem cells^{33,34}. In addition, the CTCs derived from different types of tumors have variations in size, shape and immunophenotyping (technique used to study the proteins expressed by cells). Even CTCs from the same origin have heterogeneity in morphology and immunophenotype³⁵. Therefore, the accurate detection of CTCs based on morphology and immunophenotype is challenging. Here, we summarize the current CTC capture and detection technologies, and their advantages and limitations.

CTC enrichment based on biological properties—Due to their rarity in peripheral blood, most capture techniques require CTC enrichment prior to detection. There are multiple approaches to enrich CTCs based on physical or biological properties that distinguish CTCs from other circulating cells (Table 1). After enrichment, the CTC fraction will likely contain a substantial amount of leukocytes and therefore CTC characterization is needed to distinguish CTCs from leukocytes and other circulating normal cells. The different approaches for CTC enrichment are based on the different properties of CTCs that distinguish them from the normal hematopoietic cells (and other rare circulating cells such as endothelial cells and mesenchymal stem cells), including different biological properties and physical properties.

Immunomagnetic-based CTC enrichment assays: Immunomagnetic-based assays are based on the identification of cells with antibodies against specific antigens for positive selection, or against leukocyte antigens for negative selection. Positive selection with an antibody that recognizes EpCAM is most commonly used and the only currently FDA approved CTC assay. EpCAM is a transmembrane glycoprotein which is consistently expressed by epithelial derived tumor cells, but not by leukocytes. Anti-EpCAM antibody is coupled to magnetic ferrous beads and the resulting CTC– antigen–antibody complex is isolated subsequently by being exposed to an external magnetic field. CTCs are then detected immunocytologically.

CellSearch[®] (Janssen Diagnostics, LLC) is the only FDA-cleared CTC isolation technology¹⁵. Detection by CellSearch[®] is dependent on EpCAM expression on CTCs, which are subsequently identified as nucleated cells positive for cytokeratin 8, 18 or 19 expression and negative for leukocyte antigen CD45 expression by immunofluorescence

staining. This CTC detection technology has been widely used in prostate cancer research. CellSearch[®] is highly reproducible between laboratories and the results are stable for samples shipped for as long as 72 h¹⁵. In general, a threshold of 5 cells per 7.5 ml peripheral blood has been used to estimate prognosis. However, the relatively low yield of CTCs recovered with this method limits the ability to further refine prognosis among men with mCRPC and low CTC counts. There are several explanations for this lack of CTC detection, including loss of rare cells through multiple capture and purification steps, the strict characterization definitions and the inefficient magnetic separation of labeled cells throughout a large population of unlabeled cells. There are also other limitations with CellSearch[®]. For example, captured CTCs lose their viability after fixation, which is the step required for immunofluorescence-based detection, and thus it is not possible to culture collected cells or proceed to functional studies. In addition, review and interpretation of the CellSearch[®] data are somewhat subjective and CTCs may be called based on the operator's subjective decision and interpretation of cell morphology, size, fluorescent intensity and the presence of apoptosis; thus, a valid result requires a trained pathologist or technician. Attempts have been made to develop an automated Cellsearch[®] approach⁶⁶, which may reduce the variability and turnaround time for analysis while preserving prognostic significance. Further evaluation of this automated method in prospective studies is warranted.

In addition to these technical issues that may lead to the under detection of CTCs, some CTCs exhibit evidence of epithelial plasticity and have low EpCAM expression; instead, they possess a mesenchymal or stem-like phenotype^{35,67}. These cells would not be detected by CellSearch[®] if EpCAM was completely lost⁶⁷. To address this problem, our lab developed a cadherin-11 (OB-cadherin)-based ferrofluid capture method³³. In this platform, we enrich CTCs using an OB-cadherin antibody and captured nucleated cells identified by expression of beta-catenin, which is commonly expressed in both epithelial and mesenchymal cell types, and lack of CD45. Indeed, we have identified CTCs in some men with bone-metastatic CRPC, which lack cytokeratin and are able to be captured with an OB-cadherin ferrofluid. These cells have a different morphologic appearance when compared with EpCAM-captured CTCs, yet in some cases have been shown to be clonally derived from the epithelial population, sharing common genetic lesions such as PTEN loss and the TMPRSS2-ERG fusion gene³³. We are also currently developing other modified CTC platforms using novel ferrofluids with CTCs enriched by expression of N-cadherin, O-cadherin or c-MET as a means of identifying potentially important subpopulations of disseminating tumor cells. Actin bundling protein, plastin 3, a novel marker for CTCs undergoing the epithelial mesenchymal transition (EMT) that is not expressed in blood cells, could recognize both epithelial and mesenchymal CTCs and may overcome the limitations of CTC capture platforms that only detect either epithelial CTCs or mesenchymal CTCs^{68,69}. CTCs captured based on expression of plastin 3 are associated with poorer prognosis of colorectal cancer⁶⁹, but this method has not yet been evaluated in prostate cancer. Finally, recent data suggest that cell surface vimentin may be commonly expressed in mesenchymal CTCs in multiple solid tumor types, and may be useful to examine changing CTC phenotypes in prostate cancer¹⁶².

Other immunomagnetic-based systems, such as the AdnaTest (AdnaGen, Langenhagen, Germany), MagSweeper device (Stanford University, Stanford, CA), immiscible phase filtration platform VeriFAST (University of Wisconsin, Madison, WI) and the GILUPI cell *in vivo* collector, are all EpCAM expression dependent^{36–39}. The AdnaTest system combines immunomagnetic enrichment of epithelial cells using a cocktail of several antibodies against EpCAM, HER2, EGFR and other proteins with a polymerase chain reaction for cancer or prostate cancer specific transcripts to detect CTCs³⁹. In a recent predictive study by Antonarakis and colleagues, investigators used a modified Adnatest capable of detecting the AR variant v7 in CTC enriched samples from men with mCRPC, and demonstrated potential clinical utility without the need for enumeration⁸. In this study, AR-v7 expression correlated strongly with prior abiraterone or enzalutamide exposure, and the presence of AR-v7 was strongly associated with poor outcomes and low response rates to either agent. This study suggested that men with mCRPC who harbor an AR-v7 dominant CTC population should not be treated with these agents and should consider other systemic options⁸.

The MagSweeper device uses magnetic rods to collect CTCs that bind to anti-EpCAM-antibody coated magnetic beads, while non-specifically bound blood cells are released through a controlled shear force produced by movement of the magnetic rods in wash buffer³⁸. MagSweeper was reported to be able to gently extract live CTCs with high purity from unfixed, unfractionated blood^{38,71}. The VeriFAST technique uses magnets to selectively and rapidly move the EpCAM positive cells bound to paramagnetic particles between immiscible liquids, where only the cells bound to immunomagnetic beads can cross between phases to enable rapid isolation of CTCs⁴⁸. VeriFAST avoids the multiple transfer or wash steps required in many other CTC isolation methods, which can cause loss of rare cell populations. This approach is currently under evaluation prospectively in CRPC for the characterization and enumeration of CTCs. The GILUPI cell detector uses a functionalized structured medical Seldinger guidewire (FSMW) coated with a chimeric monoclonal antibody directed to EpCAM to collect CTCs *in vivo* during blood passage. The FSMW is inserted through a standard venous cannula for the duration of 30 min while the patient rests. After removal, CTCs are identified by immunocytochemical staining of EpCAM and/or cytokeratins and staining of their nuclei and counted. This approach is being tested in early stage high risk prostate cancer as a prognostic biomarker through the European TRANSCAN multicenter trial⁷². All above discussed systems are subject to the problem of a lack of capturing CTCs that have mesenchymal or stemness phenotypes and/or lack of EpCAM expression. Furthermore, these methods have not been prospectively studied in large cohorts of men with PC, and the clinical utility is lacking.

Microfluidic device based CTC enrichment assays: With the improvement in microfluidic engineering over the past years, innovative microfluidic devices have been rapidly developed for efficient CTC isolation. Microfluidic devices (chips) for CTC enrichment are also dependent on the CTC's biological characteristics with specific cell surface antigen expression. ^{HP}CTC-Chip consists of 78 000 microposts coated with anti-EpCAM antibodies to capture EpCAM-expressing CTCs coming into contact with the microposts as blood flows through the microfluidic chip⁴¹. ^{HB}CTC-Chip is a second

generation chip and consists of microfluidic channels etched in herringbone patterns, which induce formation of microvortices as blood flows through the chip and therefore increases the contact time between cells and the channel wall coated with anti-EpCAM antibodies. CTC Chips are not limited to EpCAM capture. Instead, the capture antibodies used to coat microfluidic channels could be tailored to target different CTC specific antigens, e.g. non-epithelial markers^{41,73}.

Different from the positive selection based microfluidic devices discussed above, the third generation CTC-Chip, CTC-iChip, is a device that uses both a positive selection and negative selection strategy to purify CTCs independent of antigens present on the tumor-cell surface⁴². The CTC-iChip enriches CTCs through three steps: first, a size-based hydrodynamic sorting removes red blood cells and platelets using a laminar flow microfluidic device; second, the chip aligns the remaining cells in a single file in the flow channel; last, magnetophoresis to remove antibody coated magnetic bead labeled cells, either CTCs (positive selection) or leukocytes (negative selection). The advantage of the negative selection mode of CTC-iChip is the ability to collect unlabeled CTCs, which are assumed to be mixed population of both epithelial and mesenchymal/stem-like or antigen negative CTCs⁴². The limitation of this device is that large clumps of tumor cells may be filtered out, and very small (<8 micron) stem-like CTCs may flow with the leukocytes. Prospective studies are required to determine the clinical utility of measuring CTCs by the iChip; however, initial publications suggest the ability to molecularly profile CTCs that are collected for mutational analysis, and thus this method may enable predictive medicine⁷⁴.

The NanoVelcro microfluidic device incorporates anti- EpCAM-antibody-coated silicon nanowires with an overlaid polydimethylsiloxane chaotic mixer to generate vertical flows, and enhances contacts between CTCs and the capture substrate⁴³. The NanoVelcro device has demonstrated its consistency for CTC enumeration in metastatic prostate cancer and established that continuous monitoring of CTC enumeration could be employed to examine disease progression and to follow prostate cancer patients' responses to different treatments⁴³.

Another microfluidic device, the geometrically enhanced differential immunocapture (GEDI), combines an anti-prostate specific membrane antigen (PSMA) antibody with a 3D geometry to capture CTCs while minimizing non-specific leukocyte adhesion⁴⁶. This GEDI microfluidic device was directly compared with CellSearch[®] and demonstrated a 2–400 fold higher sensitivity⁴⁶. Another advantage of this device compared with CellSearch[®] is its independence of EpCAM expression, and the potential ability to grow CTCs *ex vivo* on the chip and to molecularly profile CTCs on the chip for biomarker analysis, such as AR or microtubule biomarkers⁷⁵.

The microfluidic cell concentrator (MCC) is another microfluidic device to enrich CTCs by negative selection. In this device, CTCs are negatively selected after bulk erythrocyte and hematopoietic cell removal with the OncoQuick buffycoat isolation method, followed by removal of peripheral hematopoietic blood cells, identified as CD45+ cells. MCC will then process the cell suspensions following pre-processing steps for enrichment and downstream processing³⁶. This method does not rely on positive selection based on surface markers;

therefore, it enables the isolation of EpCAM negative cells. In addition, isolated cells through this device are free of antibodies or other tethering molecules.

Ephesia CTC-chip and IsoFlux are two CTC isolation platforms, which combine magnetic bead technology and microfluidic devices. Ephesia CTC-chip uses columns of biofunctionalized superparamagnetic beads self-assembled in a microfluidic channel onto an array of magnetic traps prepared by microcontact printing⁴⁷. IsoFlux uses immunomagnetic beads coated with EpCAM antibody to target CTCs, then, the sample passes through a microfluidic device that contains an isolation zone to capture CTCs on the upper surface of the cartridge in an externally applied magnetic field⁵¹. These two systems combined the advantages of microfluidic cell sorting, notably the application of a well-controlled, flow-activated interaction between cells and beads, and those of immunomagnetic sorting, notably, the use of well-characterized antibody-bearing beads. Other microfluidic platforms which have not been explored in prostate cancer are summarized in Table 1.

Functional-based CTC enrichment assay: CTCs could also be enriched by approaches dependent on the viable CTCs functions, e.g. invasiveness and secretion of specific proteins. A functional enzyme-linked immunosorbent epithelial spot (EPISPOT) assay was reported to be able to detect prostate-specific antigen (PSA) secreting CTCs from men with metastatic prostatic carcinoma⁷⁶. In another study, CTCs were detected in 59% of patients with metastatic breast cancer using the EPISPOT assay⁴⁹. CTC enumeration through the EPISPOT assay in this study was evaluated on a large cohort of metastatic breast cancer patients and demonstrated prognostic relevance of the presence of viable CTCs⁴⁹. The EPISPOT assay offers several advantages compared with other CTC isolation approaches: because the secreted specific proteins are immunocaptured by the membrane in the immediate vicinity of the cells before being diluted in the culture supernatants, EPISPOT has a greater resolution than that of flow cytometry and immunometric assays. The EPISPOT assay enumerates only viable functional CTCs targeted by the proteins they secrete. However, the limitations of this PSA EPISPOT assay are relatively low yield for CTCs, the dependence on certain secreted proteins and the requirement of 48 h cell culture.

The cell–adhesion matrix (CAM)-based Vita-AssayTM (Vitatex, Stony Brook, NY) is another functional-based CTC isolation assay, which isolates CTCs from metastatic prostate cancer patients using the propensity of tumor cells to be able to invade into collagenous matrices⁵². Friedlander et al. compared CTC recovery efficiency of Vitatex versus CellSearch[®] by isolating and enumerating CTCs simultaneously from 23 men with mCRPC using the Vitatex and CellSearch[®]. This study reported that more CTCs were recovered using the CAM platform than the CellSearch[®] platform, and the CAM platform allowed for the detection of CTC clusters, CTCs expressing EMT and stem-cell markers⁷⁷. The advantages of Vitatex are its independence of the status of EpCAM expression on CTCs, and that it allows for the capture of epithelial-like CTCs and CTC clusters, as well as for those not expressing epithelial markers.

There are, however, limitations with all of these functional based approaches. These methods are dependent on CTC viability under the artificial *in vitro* cell-culture conditions,

and are also dependent on the assumption that these specific culture conditions are sufficient to recapitulate the *in vivo* biological behavior of CTCs.

Aptamer-based CTC enrichment assays: Aptamers are single-stranded RNA or DNA molecules that bind to a specific ligand. Aptamers have been demonstrated in multiple studies to be able to target extracellular membrane proteins on cancer cells, e.g. prostate-specific membrane antigen (PSMA), human epidermal growth factor receptor 3 (HER-3), RET, tenascin-C and muc1⁷⁸. Therefore, incorporation of aptamer technology with microdevices has important potential implications for CTC isolation. Highly efficient capture and enumeration of low abundance prostate cancer CTCs using PSMA aptamers immobilized to a polymeric microfluidic device have been reported^{55,79}, however, there is little published clinical data to date on this approach.

Negative enrichment: The majority of CTC enrichment approaches are based on positive selection. However, positive selection is based on the expression of tumor associated cell surface antigens and this approach encounters the problem of tumor heterogeneity and may miss a subpopulation of CTCs. Negative selection of CTCs by depletion of leukocytes is an alternative approach for CTC enrichment to avoid this problem. Magnetic beads binding to CD45⁺ leukocytes could remove leukocytes and negatively select CTCs^{80,81}. Bi-specific antibodies against antigens on leukocytes and erythrocytes will induce the formation of large multicellular rosettes to help remove hematologic cells from the blood sample by Ficoll density centrifugation⁴⁹.

In the EPIC platform^{50,82}, nucleated cells are plated onto glass slides and subjected to immunofluorescence staining for specific markers, e.g. pan-cytokeratin (CK), CD45 and AR, and CTCs can then be identified by fluorescent automated scanners. In this manner, EpCAM and/or CK negative cells may be identifiable based on additional biomarkers. Prospective evaluation of the EPIC System's clinical utility using AR-specific and other molecular probes in the context of novel hormonal and other systemic therapies in CRPC is ongoing. The CTC-iChip and MCC discussed earlier are examples of microfluidic devices to enrich CTCs by negative selection^{36,42}.

Negative selection does not rely on surface markers, therefore, it is believed valuable to harvest all possible CTCs without biases relevant to the properties of surface antigens on the CTCs. However, under such a negative cell isolation strategy, the CTC purity may be compromised. Not all CD45⁺ cells in the blood are tumor cells, e.g. circulating endothelial cells are CD45⁺.

CTC Enrichment based on physical properties—CTCs have physical properties that can help distinguish them from normal peripheral blood cells, e.g. electric charge, size, deformability, elasticity and density (Table 1). The advantage of physical property-based CTC enrichment is to permit CTC separation without labeling. A microfiltration platform, e.g. ISET[®] (Isolation by Size of Epithelial Tumor cells) system (RARECELLS, France), is an approach to CTC isolation by size, based on the assumption that CTCs are larger than leukocytes⁵⁷. CTCs are enriched by filtering blood through membranes with pores 8 μ m in diameter, followed by staining of cells retained on the filter for cytomorphological

examination or immunocytochemistry. This size-based platform has the advantage of ease to use and independence of specific cell surface protein expression. The disadvantage of the ISET system is that the size of CTCs varies and a subset of CTCs may be smaller than leukocytes⁸³. A prospective trial of 60 patients with metastatic carcinomas of breast, prostate and lung, compared CellSearch[®] and ISET directly, and demonstrated concordant results between two assays⁸⁴. Slanted spiral microdevices are novel microfluidic devices with a trapezoidal cross-section for ultra-fast, label-free enrichment of CTCs based on the larger CTC size compared with hematologic cells. In this device, the smaller hematologic cells migrate along the Dean vortices (pairs of counter-rotating vortices) towards the inner wall, and then back to the outer wall again; in contrast, the larger CTCs stay along the microchannel inner wall due to additional strong inertial lift forces⁵⁸. There are other size-based CTC enrichment platforms, e.g. MetaCell[®], ScreenCell and VyCAP^{60–62}. All these size-based platforms are subjected to the limitation of variable CTC sizes with some smaller than leukocytes. The Metacell approach has been studied in 55 men with localized prostate cancer and demonstrated that a subset (~30%) of patients were able to generate stable CTC cultures that exhibited proliferative potential, which did not correlate with Gleason score or T stage in the patients. The long-term clinical significance of these findings, however, is unknown and CTC culturing has not yet been associated with clinical outcomes⁸⁵.

Dielectrophoresis separates CTCs from peripheral blood cells based on intrinsic differences in the polarizability and electrical properties between CTCs and normal peripheral blood cells. ApoStream[™], an example of a dielectrophoretic device, was reported to have linearity of recovery of viable cancer cells independent of their EpCAM expression level, and avoided the step of antibody labeling and enables the isolation of minimally modified CTCs for future analysis⁶³. In one study of patients with metastatic prostate cancer the ApoStream CTC enrichment platform isolated a greater number of CTCs from eight patients compared to CellSearch[®], and all cell counts obtained by the ApoStream technique were higher than CellSearch[®]. These results indicated that ApoStream platform is well suited for detection and recovery of CTCs, including the tumor cells missed by CellSearch[®]⁸⁶.

Multiple other physical properties based on CTC enrichment technologies have been explored. The DEPArray[™] technology is a CTC isolation assay, which combines microfluidic technology and a dielectrophoretic approach^{64,87}. Density-based cell separation, for example differential migration according to difference in buoyant density, is utilized by the Oncoquick CTC enrichment system (Greiner Bio One, Frickenhausen, German)⁵⁶. A photoacoustic flow cytometry system was reported to be able to detect melanoma CTCs by exploiting the broadband absorption spectrum of melanin within CTCs⁶⁵. Recent advancements in the development of elastomer (polymer having both viscosity and elasticity) microparticles bound to target tumor-specific antigens permit the acoustic separations of rare cells based on these properties⁸⁸. Cancer cells are more deformable compared with red blood cells (RBCs) and white blood cells (WBCs) and are able to squeeze through very small pores. Using a reverse-selectivity approach, an elasticity-based microfluidic device consisting of a large number of channels to reduce shear stress on each cell was reported to be able to detect CTCs from metastatic renal cell cancer patients with an efficiency of more than 78%⁵⁹. However, the importance of these devices in prostate cancer needs to be further validated.

CTC detection after enrichment—After enrichment, the CTC fraction will still contain a substantial amount of leukocytes, which are a major source of contamination for downstream high-throughput molecular studies. CTC detection is needed in many cases to distinguish CTCs from leukocytes to prevent false-positive signals following the initial enrichment step. After enrichment, CTCs can be detected by different techniques, including protein-based detection, nucleic acid-based and telomerase activity based-detection (Table 2).

Protein-based CTC detection: Cytometric approaches permit protein-based detection of CTCs, do not require cell lysis, and allow subsequent morphological identification of CTCs and molecular characterization. Cytometric approaches involve two major technologies: digital microscopy and fluorescence-activated cell sorting (FACS). Digital microscopy uses a computerized microscope with an image processing system that allows for the automatic screening of samples on the basis of nuclear and cell surface characteristics. The Cellsearch[®] method utilizes this step for manual or automated screening of cellular events. Flow cytometry identifies cells labeled with fluorochrome tagged antibodies after activation by the corresponding wavelength, which enables the analysis of thousands of cells quickly⁸⁹. FACS has been used by many investigators, including our group, for the enrichment and detection of CTCs for downstream molecular characterization⁹³. FACS is antigen-based, and multiple channels and biomarkers can be used for either positive or negative selection of cellular events. The limitations of FACS include the potential for loss of cells during processing, the impact on gene expression and RNA quality and cell viability and the marker-based requirements for CTC identification.

CellSearch[®] and many others CTC detection assays use immunofluorescence staining of CK, nuclear dye with DAPI and the common leukocyte antigen CD45 to detect CTCs by digital microscopy and differentiate CTCs from leukocytes. CTCs are defined in the Cellsearch[®] method to be CK⁺/CD45⁻/DAPI⁺ and meeting additional size and quality controls. These assays can be associated with false positive and false negative results. False-positive results are generated either by non-specific binding of antibodies to non-cancer cells in the blood, or to circulating non-tumor epithelial cells found in blood due to inflammation, tissue trauma, surgical interventions or benign epithelial proliferative diseases.

Autofluorescence may contribute to false positive staining of cells for a range of biomarkers as well. In addition, some CTCs may not express epithelial antigens. Loss of expression of CK8, 18 and 19 was reported in micro metastatic cancer cell lines and in cells undergoing an EMT^{35,94}. In men with advanced prostate cancer, loss of CK was highly associated with relapse after surgery and resistance to chemotherapy, and the prevalence of CK loss increased during bone metastatic progression⁹⁵, emphasizing that the property of stemness inherent in aggressive solid tumors may lead to the under detection of CTCs that rely on epithelial biomarkers. Therefore, some CTCs, and particularly clinically important subsets of CTCs, may be missed by these assays.

CytoTrack technology (Lyngby, Denmark) combines the virtues of flow cytometry (high capacity scanning) and scanning microscopy (detailed image analysis). It is an alternative type of flow cytometry where cells are attached to the surface of the CytoTrack disc, instead of being carried by a fluent buffer string. Fluorescently labeled cells are captured by

antibodies on glass discs and imaged for further analysis. This system eliminates the need for EpCAM preenrichment because it is based on a fluorescent scanning principle that has an extreme high capacity. The CytoTrack can scan 100 million blood cells within 1 min.

Nucleic-acid based CTC detection: Detection of specific mRNAs expressed by CTCs is an alternative to immunologic assays. Reverse transcription polymerase chain reaction (RT-PCR) is a frequently used nucleic acid-based method for CTC detection and characterization. Several studies reported that RT-PCR-based CTC detection is more sensitive than immunocytochemistry⁹⁰. As described earlier, the Adnatest CTC test, modified to detect the AR variant AR-v7 by RT-PCR, was shown to have potential predictive clinical utility in determining resistance to conventional AR-directed therapies in men with mCRPC⁸, suggesting that an RT-PCR based detection method may have clinical utility without the need for enumeration or protein characterization. RT-PCR offers high sensitivity and specificity and may be less limited by the above subjective and technical limitations inherent in protein-based assays. However, since there is no tissue specific marker in the great majority of solid tumors including prostate cancer, use of markers with poor specificity to individual tumors may result in false positives. For example, loss of PSA or PSMA is common in CTCs from men with mCRPC and RT-PCR probes against these differentiation antigens may miss important CTC events⁹⁶.

Fluorescence-assisted *in situ* hybridization (FISH) uses fluorescent nucleic acid probes to detect the presence and copy number of specific DNA sequences on chromosomes. FISH has been used to detect probes against AR, PTEN, TMPRSS2-ERG fusions and other key cancer-specific probes to permit the detection of CTCs and distinguish them from normal circulating cells. These studies have shown that CTCs may often have an underlying clonal element to them for certain probes (i.e. the TMPRSS2-ERG translocation), but heterogeneity for other events (i.e. PTEN loss)¹⁸. We have also shown that some CTCs that have lost their epithelial character may possess the same clonal FISH signature in the same individual patient, suggesting epithelial plasticity³³. A study reported high accurate detection of CTCs by FISH in prostate cancer, colorectal cancer and ovarian cancer⁸⁹. However, FISH is labor-intensive, interpretation of results can be subjective, and it requires ongoing validation work with suitable controls to account for the normal levels of FISH positivity for a given probe in normal white blood cells (such as PTEN loss in leukocytes).

Telomerase activity-based CTC detection: Telomerase is a ribonucleoprotein enzyme that synthesizes telomeric repeats on to chromosomal ends using its own RNA component as a template, and has been found to be activated in many cancer types, e.g. prostate cancer, ovarian cancer, breast cancer and non-small-cell lung cancer (NSCLC)⁹⁷. Fizazi et al. developed a CTC detection method in prostate cancer patients based on telomerase-PCR-enzyme-linked immunosorbent assay⁹². Using telomerase-specific replication, selective adenovirus for CTC detection was reported in breast cancer and gastric cancer^{98,99}. This assay was reported to be highly accurate, and in a large prostate cancer Phase III trial, CTC derived telomerase activity was prognostic for OS in a significant subset of patients¹⁰⁰. However, telomerase activity-based assays require whole blood samples to be lysed to

measure the enzyme activity. Therefore all CTCs are destroyed during the processing. Future studies demonstrating clinical utility of this detection measure are needed.

Limitations for all CTC tests: Most CTC technologies remain dependent on EpCAM or some epithelial biomarker expression by CTCs. However, for CTCs that lose, down-regulate or lack EpCAM expression, EpCAM-based capture will fail to enrich an important subpopulation of CTCs. And, cells that lose CK expression or change CK patterns may escape detection. Therefore, CTC detection by alternate tumor cell surface markers is needed, e.g. markers that are able to detect CTCs with mesenchymal or stemness phenotypes. Our lab has been working on novel CTC capture methods by using mesenchymal markers, e.g. N-cadherin, OB-cadherin and c- MET to identify potentially important subpopulations of CTCs. Meanwhile, there are also some other potential CTC surface markers to be used for capture, e.g. tumor specific CD44, protocadherin family members, cell surface vimentin and plastin 3 as potentially useful for some mesenchymal-like CTCs or neuroendocrine prostate cancer CTCs^{69,101–104}. In addition, the limited blood sample volumes available from patients limit the number of CTCs available for downstream analyses and the number of CTCs detectable in early stages of the disease. CTC capture approaches with potentially higher sensitivity and specificity are under development and may permit a greater ability for molecular and functional characterization of CTCs.

CTC molecular characterization

CTCs captured from peripheral blood provide the potential for a greater overall reflection of tumor biological heterogeneity at a given point in time than from site directed individual metastatic biopsies. CTCs may be analyzed as populations or as single cells depending on the context and scientific question. Pooled CTC analyses offer the potential for assessment of the dominant circulatory clone at a given time point in a specific patient and permit the tracking of clonal selection during systemic therapies. Individual CTC profiling offers the ability to reconstruct complex evolutionary trees of tumor molecular changes from the primary or metastatic sites and within the circulation, and may offer the ability to detect rare resistant clones before they become dominant²⁴.

The importance of CTC studies is not limited to detection and enumeration. Successful molecular characterization of CTCs could provide a real-time assessment of cancer metastasis biology, tumor biomarkers and avoid the necessity of repeated invasive biopsies. CTC biomarkers may reflect the tumor resistance and viability to treatment, and interrogation of the molecular profile of CTCs for expression of protein biomarkers, genetic variants and gene expression provides opportunities to use this information to monitor therapy and detect emerging resistance¹⁰⁵. With the development of novel technologies, there has been a rapid development in CTC high-throughput molecular assessments at various levels (protein, DNA, RNA and epigenetic).

In prostate cancer, dissemination to bone, which is challenging to biopsy, is common, and tumor cell growth, biomarker expression, survival prognosis and response to treatment changes over time. While metastatic prostate cancer is thought to be a monoclonal process initially¹⁰⁶, subclonal evolution of a fraction of tumor cells into genetically distinct

subpopulations is likely to occur, with loss of protein expression and differentiation (epithelial plasticity) layered on as an additional adaptation during therapy resistance and progression³⁵. Invasive repeat biopsies are not practical for the majority of men with bone-metastatic prostate cancer. In addition, molecular profiling at different metastatic sites of prostate cancer may vary significantly^{107,108}. Therefore, blood-based CTC molecular assays may provide essential information about the current tumor biology at a given point in time, which may be assessed longitudinally in men with prostate cancer. Many studies have demonstrated the feasibility of molecular characterization of CTCs from men with metastatic prostate cancer (Table 3).

Protein level-based molecular characterization of CTCs from men with metastatic prostate cancer

Immunophenotyping, used as a common basis for CTC detection, is also used for CTC molecular characterization at the protein level and may provide important insights into the molecular biology of human metastatic prostate cancer. For example, in a pilot study, dual staining of captured CTCs from men with metastatic prostate cancer for PSA and the cell division marker Ki67, indicated a broad range for the proportion of proliferating cells among CTCs (1–81%), and an increased Ki67 proliferative index in CTCs was associated with resistance to castration therapy⁷³. PSA loss and gain of PSMA expression in CTCs also has been found to correlate with progression to castration resistance, although there is great heterogeneity between patients and loss of both proteins is possible⁹⁶. AR protein nuclear localization by immunofluorescence staining was investigated in CTCs derived from patients with CRPC and the result demonstrated a significant correlation between AR cytoplasmic sequestration and clinical response to taxane chemotherapy¹⁹. Visualization and measurement of microtubule bundling in CTCs were performed by immunofluorescence staining in CTCs captured by the geometrically enhanced differential immunocapture (GEDI) microfluidic device from men with CRPC. The results demonstrated that visualization and measurement of microtubule bundling in CTCs could be used to monitor the drug-target engagement of docetaxel chemotherapy; this suggested a novel-mechanism of action of taxanes in reducing AR transport, which could be useful in predicting the response of docetaxel in individual patients⁴⁶. Nagy et al. reported a platform of CTC molecular analysis using multiplex Quantum Dot immunofluorescence staining and FISH procedures with anti-AR, -ERG and -PTEN antibodies and 5' *ERG*, 3' *ERG*, *PTEN* and *Cen10* probes, respectively, on an automated slide-staining platform (Ventana Medical Systems, Inc., Tucson, AZ)¹⁰⁹. As others have shown ERG status by FISH in CTCs to be associated with response to abiraterone acetate¹⁸, there may be some clinical utility in measuring ERG status at the protein level. This method offers a high-sensitivity, multiplex molecular characterization of critical CTC biomarkers in mCRPC patients and might assist oncologists to identify which patients with mCRPC are likely to respond to combination therapy with targeted PI3K/AKT inhibitors and anti-androgens/Cyp17 inhibitors.

We have used protein biomarkers in CTCs to characterize a panel of epithelial plasticity biomarkers in CTCs from men with mCRPC, and found the common expression of N-cadherin, vimentin, OB-cadherin and CD133 in CTCs from these men, as well as individual cells that have lost E-cadherin and gained N-cadherin expression or possessed dual expression,

suggesting phenotypic plasticity³⁵. These findings provided evidence for plasticity during CRPC progression, and have been validated by others using other RNA-based methods¹¹¹.

In summary, protein expression by CTCs may provide a useful biomarker for metastatic prostate cancer biology and have clinical utility if linked to specific therapeutic decisions. For example, assessment of AR status (N- or C-terminal) may be useful in selecting patients for AR-directed therapy, and assessment of the glucocorticoid receptor (GR) may be useful in determining one potential mechanism of enzalutamide resistance¹³. Additional methods to improve on the number of protein biomarkers that can be reliably assessed in individual CTCs are needed.

DNA or RNA level-based molecular characterization of CTCs from men with metastatic prostate cancer—Screening tumors for genomic aberrations (mutations, translocations and copy number variation) is essential for understanding tumor progression and resistance development for guiding specific clinical therapies. Characterization of specific mutations, gain or loss of genes or genomic regions and changes in gene expression patterns in CTCs from men with metastatic prostate cancer has been reported to be feasible and useful by many studies. FISH, RT-PCR, aCGH (array comparative genomic hybridization) and high throughput genome or exome sequencing have been reported to reveal genomic aberrations in CTCs from men with prostate cancer^{22,24,112,113}.

Cytogenetic studies based on FISH have been widely utilized in CTC analysis in prostate cancer. AR copy number changes assessed by FISH were complemented in CTCs from men with CRPC²³. Detection of the TMPRSS2-ERG gene fusion in CTCs from men with prostate cancer was reported using FISH and RT-PCR⁷². Using multicolor-based FISH on CTCs from CRPC patients, one study demonstrated AR copy number gain, PTEN loss and rearrangement of ERG in CTCs¹⁸, and a potential clinical association between response to abiraterone and ERG amplification in CTCs. Amplification of MYC has also been reported from CTC studies in prostate cancer by FISH¹¹⁰.

RT-PCR is highly sensitive and specific, and can detect the expression of individual genes even at the single cell level. Therefore, RT-PCR is widely used in CTC-enriched blood for the study of cancer biomarkers. Using global gene expression profiling with microarray and quantitative RT-PCR of CTC specific expression of selected genes, Smirnov et al. demonstrated that gene expression profiles of CTCs may be used to distinguish normal donors from advanced cancer patients with metastasis¹¹⁴. Mutations in the AR gene were detected in CTCs from patients with metastatic prostate cancer using PCR amplification and direct sequencing²³. RT-PCR has been used for the study of EMT related genes in CTCs from prostate cancer patients and identified a heterogeneous pattern of expression in EMT-related genes¹¹¹. The TMPRSS2-ERG fusion transcript was detected by RT-PCR from CTCs captured by the microvortex-generating herringbone- chip from patients with metastatic prostate cancer²⁶. There are limitations to the RT-PCR analysis of CTCs due to the fact that CTC-enriched fractions still contain leukocytes, which interfere with CTC-specific gene expression profiling and create a lower signal to noise ratio and reduce the ability to observe less common RNA events. Efforts to improve upon the purity of CTCs

through novel detection/ capture approaches should facilitate improved downstream RNA studies.

Whole genome amplification (WGA) and gene copy number analysis via aCGH have been utilized in CTC genomic studies in a variety of cancers, including prostate cancer. High-level copy number gains in the AR locus were reported in CTCs from mCRPC patients^{25,27}. In our lab, we successfully analyzed DNA of CTCs from four men with mCRPC by aCGH and revealed loss of AR copy number gains, MYCN copy number gain and ABL1/2 copy number gain in enzalutamide resistant mCRPC patients¹¹⁵. In this study, we found that loss of genomic AR copy gain and gain of the MYCN region developed during enzalutamide resistant visceral progression and was observed with longitudinal CTC profiling. Additional common gains and losses of known oncogenic pathways were also commonly observed in our study.

With the rapid development of next generation sequencing and the ability to perform single cell whole genome sequencing, CTCs could provide a non-invasive source for genomic DNA and RNA for whole exome or genome sequencing and analysis²². Lohr et al. developed a modular set of protocols for census-based whole-exome sequencing (WES) and confident calling of somatic single nucleotide variants (SSNVs) from prostate CTCs²⁴. Their results demonstrated that WES could provide a window into the genetic analysis of metastatic prostate cancer and the evolutionary progression of metastatic disease from a small locus in the primary cancer, and this may provide a potential use in the clinic²⁴. Our lab has also sequenced the whole exome of CTCs from a man with enzalutamide resistant CRPC and revealed many SSNV and insertion/deletions (INDELS), whose importance in enzalutamide resistance needs further study (manuscript in preparation). An essential point for all of these methods is the need for adequate internal controls including matched leukocytes to determine the somatic nature of the genomic changes versus germline changes, validation of WES variants by Sanger sequencing and mechanistic studies to determine the validity and relevance of genomic findings to the clinical care of patients.

In addition to DNA sequencing, RNA sequencing of CTCs has also been reported in breast cancer¹¹⁶. The authors identified changes in epithelial and mesenchymal target genes during response and progression in women with metastatic breast cancer, suggesting the importance of this plasticity to therapeutic response. Yu et al. reported single-molecule RNA sequencing of CTCs from an endogenous mouse pancreatic cancer model and identified Wnt2 as a candidate gene enriched in CTCs²⁷. RNA sequencing on single CTCs isolated from patients with metastatic prostate cancer and on single prostate cancer cell line LNCaP cells spiked into the blood of healthy donors were reported¹¹⁷. These results demonstrated that RNA-sequencing is feasible to be carried out on small numbers of CTCs isolated from men with mCRPC.

Epigenetic level based molecular characterization of CTCs from men with metastatic prostate cancer: CTCs have also been used in cancer epigenetic studies. Comprehensive profiling of whole genome DNA methylation status at CpG sites were performed on CTCs from CRPC and the result demonstrated that CTCs epigenetically resemble CRPC tissue

taken at autopsy²¹. Larger studies of the CTC epigenome and how it changes over time during systemic therapy and metastatic dissemination in prostate cancer are needed.

With the sparse number of CTCs, robust and accurate genetic profiling of CTCs is challenging. Most genetic studies of CTCs are done on DNA or RNA extracted from enriched CTCs, which is contaminated by WBC wild-type DNA or RNA, and which may lead to misclassification of epigenetic signatures. Single CTC genomic analysis overcomes this limitation but this is technically and financially daunting in high numbers from patients. Despite the challenges of pure CTC capture, limited CTC enumeration and complex downstream processing appears feasible by select laboratories. The development of a platform that allows isolation of highly pure individual CTCs will offer opportunities to advance understanding of gene expression in individual CTCs to be used in clinical setting.

Biologic utilities of CTCs

Molecular profiling of CTCs can help elucidate the mechanisms involving invasiveness, aggressiveness, plasticity, tumor dissemination and metastasis in prostate cancer. CTCs may also provide a source of phenotype and tumor functionality, and may provide a source of renewable tumor tissue itself that may have clinical utility.

Metastasis in prostate cancer—Tumor initiating cells are cancer cells that are thought to have stem cell-like properties and are capable of initiating tumor growth^{118–120}. To develop metastasis, the tumor initiating cells have to survive passage through the circulation and then be able to exit the circulation and invade into the microenvironment of metastatic sites¹²¹. The tumors cells travel through the circulation as CTCs, which likely are continually repopulated by the metastases themselves. Therefore, CTC research could facilitate the identification of dominant tumor metastasis initiating cells, and offer the prospect of understanding these initiating cells' functions. Only three groups to date have reported success in the ability to culture CTCs from patients with metastatic cancer^{101,122,123}. These cultures permitted drug sensitivity analyses of cultured CTCs that could be used to predict clinical response and benefit for a wide range of agents. Further studies are needed to help optimize CTC cultures and profiling for drug sensitivity testing.

An additional method to culture CTCs from men with mCRPC is the organoid culture method, based on isolation of single cells and then using a growth-factor-based method established for colorectal cancer to isolate and propagate stem-like cells. In one study, a single CTC culture was developed using organoid methods in a patient with mCRPC who had >100 cells in 8mL of blood, and this organoid culture recapitulated the histology and molecular genetics of the patient's primary tumor¹²⁴. These data suggest that organoid culturing methods may provide a useful framework for the functional and genomic characterization of CTCs from men with mCRPC, but larger studies are needed to test this suggestion.

EMT in metastatic prostate cancer—Epithelial mesenchymal transition (EMT) plays essential roles in mesoderm development, and in wound healing and fibrosis^{125,126}. EMT has been hypothesized to play a critical role in the cancer metastasis process¹²⁷. The hypothesis is that tumor cells transition from epithelial to mesenchymal at migration, then

revert back to epithelial at site of distant metastasis (Figure 2). Though the concept of EMT is still a topic of debate in cancer, expression of mesenchymal markers in tumor tissue has been reported to be a poor prognostic factor in multiple cancers, including prostate cancer^{129–134}. Real-time analysis from CTCs should provide evidence for this process. The heterogeneous expression of both epithelial and mesenchymal markers on CTCs support previously reported partial EMT rather than “none or all”^{35,131}. Mesenchymal markers, including N-cadherin, O-cadherin, vimentin, twist, fibronectin, serpin peptidase inhibitor, have been explored in CTC EMT studies. Table 4 summarizes the reported evidence of EMT in different tumors including prostate cancer through CTCs studies.

Several groups reported the up-regulation of EMT markers in CTCs. In our study, N-cadherin and O-cadherin were shown to be commonly expressed in EpCAM-captured CTCs from mCRPC patients (Figure 3), which indicate the existence of transition of epithelial to mesenchymal phenotype³⁵. Expression of mesenchymal and stem cell markers in CTCs from a metastatic breast cancer patient was reported to be related to therapy resistance and metastasis development^{139,135}. Chen et al. demonstrated that a subset of EMT-related genes (e.g. *PTPRN2*, *ALDH1*, *ESR2* and *WNT5A*) were expressed in CTCs of CRPC, but less frequently in a small cohort of castration sensitive prostate cancer¹¹¹. This finding suggested that increased expression of EMT related genes in CTCs is associated with mCRPC, and these unique EMT related gene signatures may provide a new opportunity for patient stratification and personalized treatments. Given the association of EMT with taxane resistance and chemoresistance in general, the implications of these findings for improved outcomes with docetaxel in the metastatic castration- sensitive setting are intriguing¹⁴⁰.

The CTC studies in EMT have been limited by the fact that most CTC capture technologies are dependent on epithelial marker expression (e.g. EpCAM). With the development of new CTC isolation technologies and improved CTC molecular profiling technologies, functional characterization of CTCs will help to elucidate the EMT process *in vivo* and clarify the cancer metastasis mechanisms.

Clinical utility of CTCs

In addition to biological utility, CTC analysis has the potential to be useful as a platform for clinical biomarkers. In the clinical setting, CTCs may provide prognostic, predictive, pharmacodynamic, or surrogate uses in specific therapeutic disease states in prostate cancer, from localized disease and metastasis prevention to mCRPC and novel therapy development.

Prognostic biomarkers—A prognostic biomarker reflects disease outcome independent of therapy. CTC enumeration has been proven to be prognostic in several types of metastatic solid tumors, e.g. breast cancer, colorectal cancer, bladder cancer, NSCLC, SCLC and prostate cancer^{30,136,141–146}. CTC enumeration was reported to be an accurate and independent predictor of overall survival in mCRPC and it led to FDA clearance of CellSearch® for the evaluation of CRPC³⁰. Multiple studies demonstrated the independent prognostic role of CTC enumeration in CRPC: before treatment, four or fewer cells per 7.5 ml of blood were related to favorable prognosis, whereas five or more cells per 7.5 ml of

blood were associated with an unfavorable prognosis. In addition, a decrease in the CTC counts to less than five after treatment was associated with improvement in OS^{30,145,146}. Favorable and unfavorable CRPC groups, stratified by CTC number, had more than 10 months difference in overall survival³⁰. Median overall survival in patients with unfavorable CTC counts at 2–5 weeks after initiation of the new chemotherapy regimen was >50% shorter than in the individuals with favorable CTC counts at this time point³⁰. The independent prognostic relevance has been confirmed in a randomized phase 3 trial of men with mCRPC treated with docetaxel± abiraterone, in which CTCs were found to provide additional discriminatory value over PSA and other prognostic factors¹⁴⁷.

CTCs may also be useful as part of a biomarker panel in determining prognosis, and may complement other prognostic factors, such as visceral disease, PSA and pain³³.

A CTC and (lactate dehydrogenase) LDH biomarker panel was able to clearly separate survival outcomes in men with mCRPC treated with abiraterone acetate in the phase 3 postdocetaxel mCRPC trial¹⁴⁸, suggesting a role for post-treatment prognostic monitoring.

Potential predictive biomarkers—Predictive biomarkers are biological/molecular determinants or clinical parameters, which are associated with sensitivity (positive prediction) or resistance (negative prediction) to specific therapies. In CRPC, a number of prognostic biomarkers are available to guide risk stratification, however, there are no confirmed or validated predictive biomarkers. Aside from their use as prognostic biomarkers in CRPC, the potential of CTCs to predict the response to treatment is especially attractive. The feasibility of detecting predictive molecular changes in CTCs could be applied as a non-invasive method to select patients who should receive or avoid a specific therapy. For example, detection of mutations in epidermal growth factor receptors (EGFR) in CTCs from NSCLC provides predictive value for EGFR-directed therapy in NSCLC¹¹². Demonstration of EML4-ALK fusion by FISH testing in CTCs from NSCLC patients can guide the treatment of NSCLC with ALK inhibitors^{149,150}. The presence of AR-v7 in CTCs may indicate the lack of benefit from novel hormonal therapies. Antonarakis et al. reported that none of the mCRPC patients with detectable AR-V7 in CTCs responded to enzalutamide or abiraterone, which was defined as a reduction in serum PSA levels of 50% or more⁸. These findings suggest a potential predictive value of AR-V7 in determining resistance to enzalutamide and abiraterone in CRPC patients, but this needs to be validated by large-scale prospective studies.

Molecular profiling of CTCs could help to discover predictive biomarkers in prostate cancer and to facilitate therapeutic decision making and individualization in treatment. Robust prospective randomized studies of CTCs to stratify CRPC patients are needed, and the relevance of CTC heterogeneity with treatment response and resistance development in prostate cancer needs to be unveiled. Table 5 summarizes selected potential predictive biomarkers that may be assessable in CTC studies in prostate cancer and are worthy of prospective validation.

CTCs from men with CRPC receiving taxane chemotherapy demonstrated a significant correlation between AR cytoplasmic sequestration and clinical response to taxane¹⁹. These

results indicated that monitoring AR subcellular localization in the CTCs might predict clinical responses to taxane chemotherapy, and efforts are underway to test this hypothesis in the TAXYNERGY trial (NCT01718353). Resistance to androgen deprivation therapy (ADT) in general has been linked to the presence of AR variants that have truncated or spliced C-terminal regions, in which the ligand-binding domain for androgens has been disrupted. Evaluation of AR splicing variants in CTCs could help guide hormonal therapy selection and predict response or resistance to therapy¹⁵¹. Antonarakis ES et al. reported that AR-V7 was reliably detected in CTCs from men with mCRPC and detection of AR-V7 in CTC was strongly associated with enzalutamide and abiraterone resistance including lack of PSA declines and short progression free survival in approximately 60 patients⁸. This result indicated that AR-V7 could be used as a biomarker to predict de novo or acquired resistance to androgen pathway targeted therapies. Clinical validation of these predictive biomarkers is ongoing through a PCF-Movember global treatment sciences challenge award entitled “Development of Circulating Molecular Predictors of Chemotherapy and Novel Hormonal Therapy Benefit in Men with Metastatic Castration Resistant Prostate Cancer (mCRPC)” (Clinicaltrials.gov NCT02269982). This study will help to further validate the potential predictive role of AR-V7 across a range of platforms and in the context of whole genomic CTC analysis. With the development of next generation genome sequencing, AR mutations have been identified in CTCs from CRPC patients²³. The F876L agonist-switch mutation in AR was reported to confer genetic and phenotypic resistance to enzalutamide¹⁵².

CTCs isolated from men with CRPC exhibited wide variability in Ki67 positivity, and increased Ki67 proliferative index in CTCs was associated with resistance to castration therapy⁷². Danila et al. reported that the frequency of detection of the TMPRSS2-ERG fusion in CTCs by RT-PCR from patients with metastatic CRPC was 37%, and that androgen-driven TMPRSS2-ERG fusion in CTCs is a potential predictive biomarker of sensitivity to abiraterone⁹³. AR genomic amplification and copy number gain occurring under the selective pressure of androgen deprivation therapy have been documented in CTCs from men with CRPC and have potential predictive value for sensitivity to second-generation AR antagonists^{18,93}. PTEN loss in CTCs from men with CRPC was reported and the status of PTEN loss in CTCs may be predictive for patient response to small molecule inhibitors of phosphatidylinositol-3-kinase (PI3K)/PTEN¹⁸. Visualization and measurement of microtubule bundling in CTCs from CRPC patients can be used to monitor the drugtarget engagement of docetaxel and might be useful in predicting the effectiveness of docetaxel in individual CRPC patients⁴⁶. The recently successful evaluation of docetaxel in the metastatic castration sensitive population (ECOG CHAARTED trial) suggests that CTC-based biomarkers of taxane sensitivity (such as EMT biomarkers, which can promote chemoresistance or AR-v biomarkers) may be able to identify the reason for improved outcomes in these men prior to the onset of castration resistance¹⁵³.

The amount of neuroendocrine differentiation in prostate adenocarcinoma increases with disease progression and predicts resistance to androgen deprivation therapy¹⁵⁴. MYCN amplification is seen in 40% of neuroendocrine prostate cancer (NEPC) and 5% of prostate adenocarcinoma, respectively, and has been found to induce a neuroendocrine phenotype in prostate cells^{154,155}. By aCGH analysis, MYCN gene copy number gain in CTCs from an enzalutamide resistant CRPC patient was demonstrated by our lab¹¹⁵. The result indicated

that CTC assessments of MYCN expression might play a predictive role in CRPC response to enzalutamide.

With the development of novel CTC capture technologies and next generation sequencing, we anticipate a time when oncologists will detect the disseminating tumor burden as well as rapidly select targets and effective therapies through blood-based CTC analysis. Rapid assessments of risk/benefit can be performed after brief therapeutic trials without the need to wait for radiographic evidence. However, this clinical application requires that all of the detection and characterization tools described above in this review be matched with prospective clinical trials testing this clinical utility.

CTCs as a potential surrogate biomarker in CRPC—A surrogate biomarker is a laboratory measurement or physical sign that is used in therapeutic trials as a substitute or intermediate for a clinically meaningful endpoint that is a direct measure of how a patient feels, functions or survives and is expected to predict the full effect of the therapy on the gold standard endpoint¹⁵⁶. The gold standard for most phase 3 trials in CRPC remains overall survival; however, this endpoint is increasingly challenging to obtain given the number of newly approved agents and increasing survival times and cross-over effects. A surrogate marker is intended to substitute for overall survival and help to provide early decision-making at a trial level for the discovery of potentially active agents, and at the individual patient level for the detection of patients with a poor prognosis who need intensified or alternative treatments, or for responding patients who should remain on therapy.

Circulating tumor cell (CTC) enumeration and kinetics appear to be good candidates as OS surrogate biomarkers and are under intense investigation across multiple phase 3 trials in CRPC of abiraterone, enzalutamide, ipilimumab and other agent classes. The surrogate role of CTC enumeration in metastatic prostate cancer was evaluated in the phase III COU-AA-301 trial, which was the first phase III study to prospectively assess CTCs as a surrogate biomarker as part of a regulatory qualification process¹⁴⁵. CTC conversion, defined as converting from unfavorable (CTC ≥ 5) to favorable (CTC < 5) counts was predictive of OS as early as 4 weeks after treatment¹⁵⁷. In this study, the incorporation of CTC count changes with serum LDH demonstrated a level of individual level surrogacy for OS by correlating well with survival. Proof of CTC enumeration surrogate role requires reproduction in large clinical trials and future trials are needed to further evaluate the CTC based surrogate developed from COU-AA-301. Confirmation of the surrogate role of CTCs would help to speed up approval of novel therapies using CTC number as surrogate for OS, by increasing the efficiency and reducing the cost of novel therapeutic drug development and eliminating the OS induced bias introduced by treatment in a post-trial setting.

CTC molecular analysis for new therapeutic development—The molecular characterization of CTCs can be useful as a pharmacodynamics measure of drug effect or in selection of patients for various clinical trial designs. CTC analysis could permit the assessment of early efficacy or failure in clinical trials, as well as target engagement, which could lead to significant savings in drug development. For example, in phase I trials, novel markers in mCRPC could speed up the trial by facilitating predictive biomarker-driven

patient selection and surrogate biomarker-driven early read outs of novel drug effects. Meanwhile, access to CTC molecular profiling may offer a real-time sampling platform for pharmacodynamic studies, which allows for real-time monitoring of the drug effect on CTCs at different dose levels to determine pharmacokinetic/pharmacodynamics correlations and avoid repeated primary tumor or metastasis biopsies.

There are several challenges in the use of CTCs in new therapeutic development in CRPC. First, many men with metastatic prostate cancer lack CTCs. With the development of novel CTC technologies, more sensitive CTC capture devices may address this limitation. Second, only a limited number of CTCs are able to be detected and captured from peripheral blood while different CTC phenotypes likely exist. The ability to culture CTCs *ex vivo* for drug sensitivity testing may overcome this limitation but has not yet been demonstrated in prostate cancer^{101,122,123}.

Beyond CTCs

Cell free circulating tumor DNA (ctDNA) has been reported in various tumors including prostate cancer and is reported to be associated with unfavorable outcomes¹⁵⁸. ctDNA is believed to originate from apoptotic or necrotic tumor cells from primary tumors, metastatic lesions or CTCs^{158,159}. ctDNA has been detected in plasma and serum from prostate cancer patients, and the detection of increased DNA levels and tumor-specific DNA sequences may provide diagnostic and prognostic information^{160–162}. A higher level of ctDNA was reported to be associated with metastatic versus localized prostate cancer¹⁵⁸. CTC characterization and ctDNA analysis are complementary to each other. ctDNA analysis has the great advantage of easy collection and high throughput batched or real-time analysis, and provides the advantages of simplicity and sensitivity. However, its limitation is the restriction to measurable DNA aberrations and the uncertain source of ctDNA from viable vs. dying cells. CTC analysis can provide additional information, e.g. cell morphology, immunocytochemical phenotype, viability and the ability to reveal multiple molecular aberrations within the same cell. Further improvements in DNA sequencing technologies would allow improved genome analysis and associated gene discoveries by using both ctDNA and CTCs.

Conclusions

There is great promise in using CTCs as a platform for personalized medicine in advanced prostate cancer. The rate-limiting step for widespread use of CTCs is the lack of robust and high throughput CTC capture technologies and the lack of prospective studies on clinical utility. CTC molecular analysis is an exciting research area in CRPC and holds great promise for novel biomarker development and novel therapies development. CTC studies provide extraordinary depth in analysis of whole cell, DNA, RNA or protein based tests, and allow for cancer heterogeneity analysis for development of individualized therapies. Currently, many promising technologies for CTC isolation and analysis are ongoing, which will allow widespread use of CTCs in prostate cancer research and patient treatment. Finally, cell free methods to isolate genomic DNA and RNA are under evaluation and may provide additional information beyond that available in CTCs¹⁶³.

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Abbreviations

ADT	androgen deprivation therapy
AR	androgen receptor
CRPC	castration resistant prostate cancer
CTC	circulating tumor cell
ctDNA	cell free circulation DNA
DAPI	4',6-diamidino-2-phenylindole
EMT	epithelial mesenchymal transition
EpCAM	epithelial cell adhesion molecule
EPISPOT	epithelial spot
FSMW	structured medical Seldinger guidewire
FACS	fluorescence-activated cell sorting
FISH	fluorescence assisted <i>in situ</i> hybridization
GEDi	geometrically enhanced differential immunocapture
GR	glucocorticoid receptor
ISET	isolation by size of epithelial tumor cells
MCC	microfluidic Cell Concentrator
mCRPC	metastatic castration resistant prostate cancer
OS	overall survival
PSA	prostate specific antigen
PSMA	prostate specific membrane antigen

Glossary

EMT	a process by which epithelial cells lose their cell polarity, and epithelial specific cell–cell adhesion and cell–matrix adhesions, and gain properties commonly found in mesenchymal cells, such as invasiveness
Overall survival	the length of time from either date of diagnosis or start of treatment for a disease until death

Predictive biomarker	a biomarker that identifies the likelihood of benefit from a specific therapy
Prognostic biomarker	a biomarker that reflects disease outcome independent of therapy

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Figure 1.

Schematic illustration of the three major steps of a CTC assay. Due to CTC rarity in peripheral blood, most capture techniques require CTC enrichment, followed by detection.

(1) There are multiple approaches to enrich CTCs based on physical or biological properties that distinguish CTCs from other circulating cells. (2) After enrichment, CTCs can be detected by different techniques, including protein-based detection, nucleic acid-based and telomerase activity-based detection. (3) Successful molecular characterization of CTCs could provide a real-time assessment of cancer metastasis biology, tumor biomarkers such as mutations or epigenetic signatures or gene expression levels and avoid the necessity of repeated invasive biopsies.

Figure 2.

Epithelial plasticity during prostate cancer dissemination. Reproduced from Bitting et al.¹²⁸.

Due to genetic or epigenetic changes, normal prostate cells begin to grow un-controllably, a premalignant process known as prostate intraepithelial neoplasia (PIN). In response to signaling from the surrounding stroma, some of these cells undergo an epithelial–mesenchymal transition (EMT) and invade through the basement membrane. These invasive cells enter the bloodstream and may exist as epithelial circulating tumor cells (CTCs), mesenchymal CTCs, or CTCs with a dual phenotype. Upon exiting the vasculature, disseminated tumor cells (DTCs) may sit dormant or undergo apoptosis. Other DTCs undergo a mesenchymal–epithelial transition (MET) and grow as detectable macrometastases. In prostate cancer (PC), bone metastases are typical and are initially AR dependent, progressing through a range of AR mutations or splice variants and other oncogenic and tumor suppressor mutations. Visceral metastases are atypical, are variably AR dependent, and generally involve loss of an epithelial phenotype (EP) and are enriched for a neuroendocrine or anaplastic phenotype. EP is not clearly linked to the process of lymph node metastasis; instead, nodal metastases likely involve other forms of invasion or migration.

Figure 3.

Co-expression of epithelial and mesenchymal proteins in CTCs from men with metastatic castration resistant prostate cancer (mCRPC). Reproduced from Armstrong et al.³⁵. All panels represent merged images derived from phase/DAPI, CD45/DAPI, CK/DAPI and either vimentin (Vim)/DAPI, N-cadherin (N-cad)/DAPI expression or O-cadherin (O-cad)/DAPI as indicated. Shown are examples of (A) a leukocyte with CD45 expression, (B) a CTC with no vimentin expression, (C) a CTC with vimentin expression, (D) a CTC with N-cadherin expression, (E) 3 CTCs, 2 with N-cadherin expression (arrowheads), (F) a CTC with O-cadherin expression and a nearby leukocyte and (G) an additional CTC with O-cadherin expression. Scale bars represent 20 nm and were added from an image taken at identical magnification and resolution. Control cells were assayed in parallel at the same time of CTC collection and analysis with each set of patient samples and are shown in the Supplementary material.

Table 1

CTC enrichment based on biological properties or physiological properties.

CTC enrichment	Mechanism	Examples	Selected references
Biological properties-based			
Immunomagnetic-based	Enrichment with magnetically labeled antibody	CellSearch AdnaTest MagSweeper VeriFAST GILUPI MACS® MicroBeads	36–40,70
Microdevices	Microfluidic cell sorting	^{HP} CTC-Chip ^{HB} CTC-Chip CTC-iChip NanoVelcro GEDI OncoCEETM Biofluidica LiquidBiopsy® Ephesia CTC-chip	26,41,42,43–46,47
Negative selection	Hematologic cells depletion	CTC-iChip Microfluidic Cell Concentrator (MCC) EPIC platform Multicellular rosettes	48,42,49,50
Combination of immunomagnetic technology and microdevices	Combined enrichment with magnetically labeled antibody and microfluidic cell sorting	Ephesia CTC-chip IsoFlux	47,51
Functional based	PSA secretion by CTC Invasion of CTCs into collagenous matrices	EPISPOT assay Cell-adhesion matrix (CAM)-based Vita-Assay™	52,53,54
Aptamer based	Aptamer binding to cell surface specific protein	Aptamer modified microfluidic device	52,55,50
Physical properties based			
Density based cell separation	Differential migration according to difference in buoyant density	Oncoquick	56
Size or elasticity separation	Isolation based on size and/or deformability	ISET Slanted spiral microdevices MetaCell® ScreenCell VyCAP Elasticity-based microfluidic device	57,58,59,60–62
Electric charges	Different polarizability and electrical properties	ApoStream™ DEPArray	63,64
Photoacoustic resonance	Photoacoustic resonance	Photoacoustic flow cytometry	65

Table 2

CTC detection by protein based, nucleic acid based or telomerase activity based technologies.

CTC detection	Detection principle	Advantages	Limitation	Selected references
Protein-based CTC detection				
Immunofluorescence staining	Antigen expression	Many parameters can be simultaneously measured	Low throughput and loss of CTC viability	30
Flow cytometry	Antigen expression	Fast	Dependent on antigen expression; and decreased CTC viability	89
Nucleic acid-based CTC detection				
RT-PCR	Expressed cell specific makers	High sensitivity	High frequency of false positive	90
FISH	DNA sequence detection	Highly accurate	Labor intensive	91
Telomerase activity based CTC detection				
Telomerase-PCR-enzyme-linked immunosorbent assay	Telomerase activity	Highly accurate	All CTCs are destroyed during whole blood lysis	92

Table 3

CTC characterization through molecular profiling studies in prostate cancer.

Molecular profiling	Target	Molecular studies	Selected reference
Protein level	PSA and Ki-67	Immunofluorescence	73
	AR localization	Immunofluorescence	19
	Microtubule dynamics	Immunofluorescence	46
	Multi-target: AR, ERG, and PTEN	Immunofluorescence	109
DNA and RNA levels	AR copy number	FISH	23
	TMPRSS2-ERG gene fusion	FISH	73,93
	Multi-target: ERG break-apart, AR copy number, and regional deletion of PTEN	Multi-color FISH	18
	MYC amplification	FISH	110
	AR gene mutation	PCR and direct sequencing	23
	Whole genome	aCGH	25
	Whole exome	WES	24
	Target RNA (e.g. Wnt2)	single-molecule RNA sequencing	27
Epigenetic level	Telomerase activity	qPCR-based telomeric repeat amplification	100
	DNA methylation	Methylation array	77

Table 4

Evidence of EMT by CTC studies in solid tumors including prostate cancer.

Cancers	EMT markers	Selected references
Prostate	Cytokeratins, vimentin, N-cadherin, O-cadherin, CD133	35
Prostate	Insulin like growth factor, epidermal growth factor receptor, forkhead box P3, transforming growth factor beta 3	111
Breast	Cytokeratins, vimentin, twist	135
Breast	Cytokeratins, vimentin, N-cadherin, O-cadherin	35
Breast	Panel of 7 epithelial and 3 mesenchymal genes by RNAish	116
NSCLC	E-cadherin, vimentin, N-cadherin	136
NSCLC	Cytokeratins, vimentin	137
SCLC	E-cadherin, vimentin, N-cadherin	136
SCCHN	Cytokeratins, vimentin, N-cadherin, CD44	138
Multiple solid tumors	Cell surface vimentin	103