

Overcoming Obstacles in Liquid Biopsy Developments for Prostate Cancer

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Abstract: Prostate cancer is one of the most common malignancies in men. Over time, it can metastasize and become lethal once it exhausts hormonal therapies and transitions into castration-resistant prostate cancer (CRPC). Several therapies have been recently approved for advanced prostate cancer, but identifying biomarkers for current treatments and searching for more effective treatments are urgently needed. Liquid biopsy is a powerful tool for isolating genetic material, proteins, and whole tumor cells from the blood. In recent decades, this technology has rapidly advanced, allowing for better insights into the pathogenesis and treatment response in different stages of prostate cancer. In this review, we summarize important clinical studies involving liquid biopsies in prostate cancer with a focus on advanced disease, notably regarding circulating tumor DNA, circulating tumor cells, and exosomes. We highlight the progress and the challenges that still exist for these technologies. Finally, we discuss promising avenues that will further expand the importance of liquid biopsy in the care for prostate cancer patients.

Keywords: liquid biopsy, circulating tumor DNA, circulating tumor cells, exosomes, advanced prostate cancer

Introduction

Prostate cancer is the second most common malignancy in men after skin cancer.¹ Although the five-year survival rate of prostate cancer is close to 100% when the disease is localized, the five-year survival rate is just 30% when prostate cancer metastases are present.² Despite hormonal therapy, metastatic prostate cancer over time invariably progresses from hormone-sensitive to castration-resistant. Metastatic castration-resistant prostate cancer (mCRPC) has a median overall survival (OS) time of 2–3 years despite treatment.³ The genetic landscape of mCRPC, which reveals many mutations including homologous recombination repair deficiency and high microsatellite instability (MSI-H),⁴ is now beginning to guide treatment selection as targeted therapies such as olaparib, rucaparib, and pembrolizumab have been approved in these settings.^{5–7} With many mCRPC treatments approved in the last decade and more on the way, there is an urgent need for real-time and tumor biomarkers to help guide the selection.⁸

A liquid biopsy is the sampling of biological fluid (most often blood, but also can involve other body fluids including urine, saliva, tears, etc.) for various tests.⁹ The liquid biopsy has become an asset in guiding cancer treatment, as it can analyze tumor characteristics in real time with serial blood draws.¹⁰ Liquid biopsy is now used to assist with diagnosing and monitoring many different cancers, most commonly via extraction of circulating tumor DNA (ctDNA), circulating tumor cells (CTCs), and exosomes.¹¹ In just the past few years, this technology has rapidly improved, making the process of obtaining a blood sample and analyzing it for cancer care and cancer progression a quick and simple routine for patients. Because prostate cancer often metastasizes to the bone,¹² liquid biopsies are a good alternative to tracking metastatic prostate cancer genetics in real time compared to constantly performing invasive procedures to obtain biopsies of bone metastases.¹⁰

This review article provides an overview of the role of liquid biopsy in advanced prostate cancer. We describe select clinically relevant data on different liquid biopsy methods currently available including ctDNA, CTCs, and exosomes. We highlight the overall limitations to these individual methods. Finally, we touch on the latest technologies that are on the horizon, along with current and future clinical trials involving liquid biopsy in prostate cancer.

The studies in this review were chosen non-systematically based on a free-hand search of the references to the most recent published systematic and comprehensive review articles discussing liquid biopsies in prostate cancer. Therefore, some smaller studies have been excluded or missed in this article.

Circulating Tumor DNA

Cell-free DNA (cfDNA) detected in the human blood plasma was first described by Mandel and Metais in 1948.¹³ In the 1970s, cfDNA in the serum of cancer patients was predicted to become an important method in guiding cancer treatment.¹⁴ Over the decades, several techniques to extract DNA have become available.¹⁵ Circulating tumor DNA (ctDNA), which refers to the subset of cfDNA derived from tumor cells, now plays a prominent role in guiding the prognosis and treatment of metastatic prostate cancer. A summary of the larger studies analyzing ctDNA in metastatic prostate cancer is shown in Table 1. Wyatt et al were the first to demonstrate concordance of matched ctDNA samples with metastatic bone or soft tissue samples in a systematic fashion from patients with mCRPC.¹⁶ The authors noted high concordance between the matched samples including 100% somatic mutations identified in tissue also present in ctDNA. In addition, the ctDNA sequencing revealed changes not detected in the paired solid biopsies, supporting the use of ctDNA for more analysis and identification of potential biomarkers. Jimenez et al similarly found biopsy specimens from metastatic sites of CRPC not able to achieve nucleic acid purity in some samples.¹⁷

The level of ctDNA and the number of mutations detected consistently correlates with more aggressive tumors that progress over time. Kohli et al followed a longitudinal prospective cohort of over 300 patients grouped into treatment-naïve metastatic hormone-sensitive prostate cancer (mHSPC), mHSPC on androgen deprivation therapy (ADT), mCRPC based on biochemical progression, and mCRPC with clinical progression.¹⁸ They noted a higher level of ctDNA was associated with a shorter time to ADT failure in mHSPC, suggesting treatment intensification early in the disease course may be warranted for mHSPC patients with higher levels of ctDNA. The overall frequency of ctDNA mutations was significantly higher in the mCRPC groups compared to the mHSPC groups. Likewise, there were more ctDNA alterations and higher levels of ctDNA detected in the mCRPC with clinical progression compared to only biochemical progression.

Some studies have focused on the androgen receptor (*AR*) gene in ctDNA with CRPC treatment resistance and OS. The *AR* transcription factor drives CRPC development and resistance to many treatments.¹⁹ Romanel et al evaluated *AR* gene aberrations from ctDNA in CRPC patients treated with abiraterone; increased *AR* copy number and mutations leading to amino acid changes of T878A and L702H were associated with abiraterone resistance.²⁰ Other studies have made similar conclusions about these mutations associated with resistance to abiraterone and enzalutamide as well and also revealed other missense *AR* mutations including F876L against enzalutamide and H874Y and T877A against abiraterone.^{21–23} Kohli et al also noted *AR* gene amplifications were associated with decreased survival in patients with CRPC.¹⁸ Annala et al analyzed the ctDNA in mCRPC patients enrolled in a Phase 2 clinical trial (NCT02125357) randomized to either abiraterone or enzalutamide and then crossed over with cancer progression.^{24,25} The authors noted that prior to the start of therapy, *AR* gene structural rearrangements truncating the ligand-binding domain in patients naïve to abiraterone or enzalutamide were linked to treatment resistance.²⁴ With serial ctDNA collections analyzed over time, more aggressive *AR* genotypes became more prevalent over the course of the trial, suggesting the potential for serial ctDNA measurements being used as a prognostic marker.²⁵

The baseline level of ctDNA fraction can be of prognostic significance prior to starting therapy as well. In a phase 2 trial (NCT02254785) comparing cabazitaxel versus abiraterone or enzalutamide in 95 mCRPC patients with poor prognosis, it was noted that baseline ctDNA level was the strongest predictor of OS.²⁶ Patients with a high average baseline ctDNA fraction had poor clinical features and a predictably poor prognosis, but patients with a poor clinical presentation with low ctDNA fraction still had good survival outcomes, suggesting ctDNA fraction at baseline may be used as a stratification biomarker.

Table I Prospective and Retrospective Studies Focused on Advanced Prostate Cancer That Incorporated Liquid Biopsy into Their Study Design and Involved at Least 40 Patients Were Highlighted in This Manuscript, in the Order of Appearance

Article	Year	Prostate Cancer Stage	Number of Patients with Liquid Biopsy Data Available	Liquid Biopsy-Related Outcomes
Circulating tumor DNA				
Kohli et al ¹⁸	2020	mHSPC and mCRPC	303	mHSPC and mCRPC had statistically significant different yields of cfDNA; ctDNA alterations may be prognostic and could have predictive and therapeutic implications
Romanel et al ²⁰	2015	mCRPC	97	AR gain, T878A and L702H mutations detected in patients taking abiraterone were more likely to have worse OS and PFS
Azad et al ²¹	2015	mCRPC	62	AR gene aberrations including F876L were associated with enzalutamide resistance and H874Y and T877A mutations were associated with abiraterone resistance
Wyatt et al ²²	2016	mCRPC	65	AR gene aberrations and copy number alterations, other DNA damage repair and PI3K pathway gene alterations detected may be associated with enzalutamide resistance
Conteduca et al ²³	2017	mCRPC	265	AR gain, T878A and L702H mutations were associated with worse PFS and OS on enzalutamide or abiraterone
Annala et al ²⁴	2018	mCRPC	202	AR gene structural rearrangements truncating the ligand binding domain, <i>BRCA2</i> , <i>ATM</i> , <i>TP53</i> mutations prior to therapy were associated with resistance to enzalutamide and abiraterone
Annala et al ²⁵	2021	mCRPC	202	Serial sample collections over the course of treatment of enzalutamide or abiraterone revealed the AR genotype evolved
Annala et al ²⁶	2021	mCRPC	95	Elevated baseline ctDNA fraction was associated with shorter time to progression and shorter OS, suggesting baseline ctDNA fraction may be a stratification biomarker
Annala et al ²⁷	2017	mCRPC	319	10/11 (91%) concordance of ctDNA and germline <i>BRCA2</i> mutations
Goodall et al ²⁸	2017	mCRPC	46	decrease in cfDNA concentration after 8 weeks of therapy was associated with longer OS; serial cfDNA analyses may guide prostate cancer care
Vandekerkhove et al ³²	2019	mHSPC	53	ADT reduced ctDNA availability in mHSPC; combination of tumor tissue and ctDNA analysis provides optimal approach for biomarker development as either one alone was insufficient to identify all genetic alterations
Circulating tumor cells				
Danila et al ⁴⁹	2007	mCRPC	120	Higher CTC numbers were associated with higher tumor burden; baseline CTC counts were predictive of survival
De Bono et al ⁵⁰	2008	mCRPC	276	Pre-treated patients with ≥ 5 CTC/7.5 mL had shorter OS by 10 months; CTC counts had higher prognostic value for OS than changes in PSA level
Goldkorn et al ⁵¹	2014	mCRPC	263	Baseline CTC counts were prognostic; rising CTC counts after 3 weeks of docetaxel treatment were associated with worse OS

(Continued)

Table 1 (Continued).

Article	Year	Prostate Cancer Stage	Number of Patients with Liquid Biopsy Data Available	Liquid Biopsy-Related Outcomes
Scher et al ⁵²	2009	mCRPC	164	High CTC counts were associated with higher risk of death; recording the CTC number every few weeks could be used to monitor disease status
Shaffer et al ⁵³	2007	mCRPC	63	Molecular profiling of CTCs in patients with ≥ 5 CTC/7.5 mL was possible, including analysis of <i>EGFR</i> , <i>AR</i> , and chromosome ploidy
Danila et al ⁵⁴	2011	mCRPC	48	<i>TMPRSS2-ERG</i> fusion analysis of CTCs did not predict treatment response to abiraterone but could be routinely performed in clinical practice
Punnoose et al ⁵⁵	2015	mCRPC	76	<i>PTEN</i> loss in CTCs had high concordance (84%) with matched fresh tissue, and was associated with worse OS
Okegawa et al ⁵⁶	2016	mCRPC	60	<i>EGFR</i> -positive CTCs in patients receiving docetaxel had shorter OS by 14.5 months
Goldkorn et al ⁵⁷	2015	mCRPC	263	High CTC telomerase activity was associated with worse OS in patients receiving docetaxel
Antonarakis et al ⁵⁹	2014	mCRPC	62	<i>AR-V7</i> detection in CTCs was associated with lower PSA response rates when receiving enzalutamide or abiraterone, shorter PFS and OS, suggesting treatment resistance
Antonarakis et al ⁶⁰	2017	mCRPC	202	Patients treated with enzalutamide or abiraterone and without CTC detection had the best prognosis, while CTC+/AR-V7+ patients had the worst prognosis
Scher et al ⁶³	2016	mCRPC	161	CTC+/AR-V7+ patients had superior OS when treated with taxane chemotherapy over abiraterone or enzalutamide, warranting prospective validation
Scher et al ⁶⁴	2018	mCRPC	142	CTC+/AR-V7+ patients had superior OS when treated with taxane chemotherapy over abiraterone or enzalutamide, while CTC+/AR-V7- patients had superior OS when treated with abiraterone or enzalutamide in a prospective blinded study
Armstrong et al ⁶⁵	2020	mCRPC	118	CTC+/AR-V7+ patients pretreatment had worse PFS and OS when receiving abiraterone or enzalutamide, but still experienced clinical benefit from subsequent docetaxel or cabazitaxel
Okegawa et al ⁶⁷	2008	mHSPC	80	Median ADT responsiveness at least 15 months longer in patients with < 5 CTCs/7.5 mL
Josefsson et al ⁷⁰	2017	mHSPC	53	Patients with <i>EGFR</i> detection in CTCs had significantly shorter PFS by 6 months while on ADT
Exosomes				
Zhu et al ⁹⁶	2021	mCRPC	52	Exosomal TUBB3 expression was associated with shorter PSA PFS in patients receiving abiraterone

Abbreviations: mCRPC, metastatic castration resistant prostate cancer; mHSPC, metastatic hormone-sensitive prostate cancer; ctDNA, circulating tumor DNA; cfDNA, cell-free DNA; AR, androgen receptor; ADT, androgen deprivation therapy; CTC, circulating tumor cell; PSA, prostate-specific antigen.

With all this potential, ctDNA analysis has been directly applied to guiding prostate cancer treatment. In one study focused specifically on mCRPC patients with deleterious germline mutations, 10 of 11 patients with germline *BRCA2* mutations had a deletion of the intact somatic allele that was detected via ctDNA, suggesting these patients with germline mutations may be prioritized for targeted therapies such as poly (ADP-ribose) polymerase inhibitors based on liquid biopsies.²⁷ In the TOPARP-A phase 2 trial (NCT01682772) evaluating olaparib in mCRPC, cfDNA sequencing for two patients with germline *BRCA2* mutations who had disease progression after initial response revealed the mechanism of the resistance to olaparib with reverting of germline and somatic *BRCA2* and *PALB2* mutations.²⁸ After eight weeks of olaparib treatment, the patients who responded had a strong decline in cfDNA levels, while non-responders had an increase in cfDNA levels ($p=0.006$). In a real-world setting, Barata et al demonstrated the feasibility of using the Guardant360 assay to identify MSI-H in ctDNA of mCRPC patients and for these select patients to receive pembrolizumab and to achieve a radiographic and prostate-specific antigen (PSA) response.²⁹ In another real-world study, for 52 patients (majority of whom had mCRPC) with *CDK12* mutations that were identified using ctDNA and/or metastatic tissue samples, the authors noted patients who received ICIs and did not receive prior chemotherapy had improved progression-free survival (PFS) compared to those who received ICIs after chemotherapy.³⁰ *CDK12* loss is associated with increased gene fusions, neoantigen burden, and T cell infiltration, which increases sensitivity of tumors to immune checkpoint inhibitors (ICIs).³¹ These and other similar studies demonstrate ctDNA and other liquid biopsies playing an increasingly important role in optimizing treatment selection in patients with advanced prostate cancer (Table 2).

Fewer ctDNA studies have focused on HSPC. Vandekerkhove et al collected ctDNA from 53 newly diagnosed mHSPC patients and noted that after ADT was started, the level of ctDNA reduced drastically (median ctDNA fraction 11% when untreated to 1.0% after treatment began).³² Along similar lines, ctDNA is still more difficult to analyze in the localized prostate cancer setting because of the significantly less tumor burden available to shed DNA into the bloodstream. In high-risk localized prostate cancer, 112 patients who had disease recurrence after radical prostatectomy detected via PSA recurrence did not have detectable levels of ctDNA.³³ In contrast, another study had ctDNA detected postoperatively in 2 of 8 patients with high-risk localized prostate cancer, although these patients may have had more aggressive disease.³⁴ This study also noted that detection of *TP53* mutations in ctDNA collected from 189 patients with high-risk localized prostate cancer was associated with a significantly shorter metastasis-free survival of over three years. As the technologies to detect extremely low levels of ctDNA improve, the use of ctDNA in predicting the aggressiveness of localized prostate cancer may become more commonplace.

Some studies have extracted cfDNA from urine samples for genitourinary cancers, which is an even less invasive method of obtaining genetic material from patients compared to isolating ctDNA from peripheral blood samples.³⁵ Casadio et al published a small proof-of-concept study involving 54 patients (29 with prostate cancer, 25 control)

Table 2 Actionable Mutations in Advanced Prostate Cancer

Genomic Mutations	Method of Testing	Treatment	Status of Therapy
AR-V7 ⁵⁹	Circulating tumor cells	Resistance to androgen axis-targeted therapies	Preliminary testing, not validated
<i>BRCA1/BRCA2/ATM</i> and other DNA repair mutations ¹²⁶	NGS on tissue germline testing on blood/saliva sample	PARP inhibitors	FDA approved, Category I
MSI-H ²⁹	NGS on tissue or ctDNA	ICIs such as pembrolizumab	FDA approved
TMB>10 mut/Mb ¹²⁷	NGS on tissue or ctDNA	ICIs such as pembrolizumab	FDA approved
PTEN loss ¹²⁸	IHC/NGS on tissue or ctDNA	PI3K inhibitors, ipatasertib or mTOR inhibitors	Promising preliminary information
<i>CDK12</i> mutation ³⁰	IHC/NGS on tissue or ctDNA	ICIs	Investigational therapy; clinical trials ongoing

Abbreviations: MSI-H, microsatellite index-high; TMB, tumor mutational burden; mut/Mb, mutation/megabase; NGS, next-generation sequencing; ctDNA, circulating tumor DNA; IHC, immunohistochemistry; PARP, poly (ADP-ribose) polymerase; ICIs, immune checkpoint inhibitors.

evaluating the possibility of using cfDNA length in urine for early detection for prostate cancer;³⁶ however, in a larger study of 131 patients (67 with prostate cancer, 64 control) performed by the same research group, the accuracy of using cfDNA was worse than using PSA for early prostate cancer diagnosis.³⁷ So far, the use of urinary cfDNA in prostate cancer is still too early to be of clinical significance.

It is evident that ctDNA complements metastatic tissue genetics and is steadily gaining momentum in ultimately replacing them to guide prostate cancer therapy selection, real-time prognosis, and therapy resistance due to the ease of obtaining serial liquid samples. Despite all the advances, ctDNA analysis is still limited by the technology in extracting the amount available from these samples and ensuring purity, especially when the cfDNA in the bloodstream is low due to low tumor burden. The half-life of ctDNA is short (1–2 hours), which limits the capture of some rare mutations and makes it difficult to determine an optimal time to collect samples.³⁸ Due to the technological and biological factors, both false-negative and false-positive results can occur.³⁹ There is extensive intratumor heterogeneity in the process of tumor evolution, and subclonal mutations and other copy number alterations may not be detected.⁴⁰ Clonal hematopoiesis can shed cfDNA during its natural aging process, which can confound cfDNA analysis as well.⁴¹ With more commercial ctDNA assays increasingly available, it is of utmost importance that their platforms can produce concordant results, as discrepancies between assays have been reported.⁴² Currently, only Guardant360 CDx and FoundationOne Liquid CDx are FDA-approved.⁴³ Notably, the Guardant360 assay has been previously reported to have some limitations in detecting insertions, deletions, rearrangements, and germline variants.⁴⁴

Circulating Tumor Cells

CTCs were first identified microscopically in the blood of a patient with metastatic cancer by Thomas Ashworth in 1869.⁴⁵ For prostate cancer, little progress was made for the next century until prostate CTCs were detected in the early 1990s via PSA mRNA in peripheral venous blood,⁴⁶ and then the technology improved to the isolation of intact prostate CTCs from peripheral blood samples.⁴⁷ In the early 2000s, studies focused on the enumeration of CTCs from peripheral blood, and later on, the studies shifted towards CTC characterization and then using CTC analyses to guide therapies. The studies in this section with advanced prostate cancer involving analysis of CTCs are also summarized in Table 1.

Moreno et al published one of the first studies that used CTC counts to predict survival in patients with metastatic prostate cancer.⁴⁸ In this pilot study of 37 patients, the authors determined a threshold of 5 CTCs per 7.5 mL of blood was associated with a decreased OS (0.70 years for patients with ≥ 5 CTCs versus more than 4 years for patients with < 5 CTCs, $p=0.002$). Another study evaluated the CTCs in 120 patients with mCRPC; there were higher CTCs for patients who had bone metastases and those who had received prior cytotoxic chemotherapy, and the number CTCs was strongly associated with decreased survival.⁴⁹ In the IMMC38 trial (NCT00133900) that enrolled 276 patients and stratified them according to favorable (< 5 CTCs per 7.5 mL) or unfavorable (≥ 5 CTCs per 7.5 mL) groups, the CTC counts were better than PSA changes at predicting OS in both pre- and post-chemotherapy treatment, suggesting CTC counts may be used as a prognostic marker for CRPC patients.⁵⁰ Similarly, from approximately 200 evaluable CTC samples in the Phase 3 trial SWOG S0421 (NCT00134056), median OS was 26 months for patients with ≥ 5 CTCs per 7.5 mL compared to 13 months for patients with < 5 CTCs per 7.5 mL (HR 2.74; 95% CI, 1.72–4.37; $p<0.001$).⁵¹ In a deeper analysis of the IMMC38 trial, the authors analyzed and trended CTC numbers at 4, 8, and 12 weeks post-chemotherapy of 164 patients and determined the CTC number as a continuous variable was prognostic for survival.⁵² All these studies provided evidence that increased CTC counts were associated with decreased OS and could provide real-time tracking for treatment responses.

Later studies beginning in the late 2000s went further by characterizing the CTCs instead of simply counting them, and they revealed their feasibility to track cancer treatment progress and to be prognostic. In one pilot study, CRPC patients with ≥ 5 CTCs per 7.5 mL blood sample had enough CTCs to run molecular profiles on these cancer cells, and analysis also revealed changes to *EGFR* and *AR* gene expressions.⁵³ Another study designed and validated a reverse transcription polymerase chain reaction (RT-PCR) assay to analyze *TMPRSS2-ERG* transcripts in CTCs for 15 of 41 CRPC patients treated with abiraterone; although this gene fusion did not predict a response to abiraterone, the study demonstrated the ease of obtaining CTCs and analyzing them for mutations.⁵⁴ Punnoose et al established concordance of CTCs with cancer tissue in terms of *PTEN* status, and *PTEN* loss in CTCs was associated with a decreased survival (HR

2.05; 95% CI, 1.17–3.62; $p=0.01$) in mCRPC patients.⁵⁵ Okegawa et al determined that EGFR status of CTCs was an independent predictor of OS in mCRPC patients who received docetaxel (5.5 months for EGFR+ CTCs vs 20.0 months for EGFR- CTCs, $p<0.001$).⁵⁶ Goldkorn et al analyzed the telomerase activity of live CTCs from the blood samples of mCRPC patients in the SWOG 0421 trial; there was a statistically significant hazard ratio of 1.14 ($p=0.001$) for OS after covariants were accounted for, so the authors determined telomerase activity could be used as a CTC-derived biomarker in future studies.⁵⁷

Many studies analyzing CTCs have focused on the androgen receptor splice variant 7 (AR-V7) mutation. This variant results in a conformational change to the androgen receptor allowing for constitutive activation and lacking the ligand-binding domain, allowing the mutant protein to maintain the downstream effects of androgen signaling.⁵⁸ Antonarakis et al were the first group to reveal AR-V7 mutations, detected in CTCs via RT-PCR assays, in 12 of 31 mCRPC patients (39%) treated with enzalutamide and 6 of 31 (19%) treated with abiraterone.⁵⁹ These patients with AR-V7 mutations had statistically significant lower PSA response rates, shorter PFS, and shorter OS compared to patients without detected AR-V7 mutations. The authors expanded their prospective study cohort to include 202 patients and achieved similar results and confirmed that patients without detectable CTCs had the best prognosis.^{59,60} This group examined CTCs for the AR-V7 mutation in 37 mCRPC patients (17 were AR-V7+) on either docetaxel or cabazitaxel; there was no statistically significant difference in PFS between AR-V7+ and AR-V7-.⁶¹ Another mCRPC study compared 16 AR-V7+ patients with 13 AR-V7- patients who received cabazitaxel supported these findings, as there was no statistically significant difference between these two groups in terms of PFS or OS.⁶² In two larger studies, one involving 161 mCRPC patients and another blinded study involving 142 mCRPC patients, the authors similarly concluded patients with AR-V7 positivity in CTCs had a better OS receiving taxane therapy over novel hormonal agents, and vice versa for patients without AR-V7 detected.^{63,64} In the prospective double-blinded PROPHECY study which used two separate AR-V7 assays to evaluate 118 mCRPC patients treated with either abiraterone or enzalutamide, patients with pretreatment CTC AR-V7 positivity had a significantly worse PFS and OS and poor PSA responses, which helps to validate the presence of AR-V7 positivity in CTCs as a negative predictive biomarker for novel hormonal agents.⁶⁵ Overall, AR-V7 positivity detected using CTCs provides a validated method to discourage therapies targeting the androgen pathway for this cohort of patients (Table 2). In a recent systematic review and meta-analysis of AR-V7 in CRPC, AR-V7 positivity was overall associated with a statistically significantly shorter OS (HR 1.98; 95% CI, 1.48–2.66; $p<0.001$) after taxane therapy, suggesting AR-V7 may also serve as a prognostic biomarker for CRPC.⁶⁶

While many studies have examined CTCs in the CRPC setting, fewer studies have evaluated CTCs in the HSPC setting. One early study involved 80 mHSPC patients noted that the patients with ≥ 5 CTCs in 7.5 mL of blood had a median time of ADT responsiveness of 17 months compared to over 32 months for patients with < 5 CTCs in 7.5 mL ($p=0.007$).⁶⁷ Another study of 33 mHSPC patients found that increased CTC counts at baseline prior to starting ADT were associated with the transition to CRPC during treatment.⁶⁸ Miyamoto et al designed a proof-of-concept study to non-invasively measure AR signaling with CTCs of HSPC before and after ADT and noted there were changes to AR signaling before and after treatment.⁶⁹ In a more recent study, Josefsson et al analyzed CTCs before and after starting ADT in 53 patients, and the mHSPC patients with detected CTCs prior to beginning therapy had a worse PFS after starting ADT (8.5 months vs NR), and EGFR positivity in CTCs had a significantly shorter time to progression while on ADT (5 months vs 11 months, $p<0.05$).⁷⁰ Similar to the setting of CRPC, increased CTC count during HSPC predicted a worse prognosis.

Like ctDNA, even fewer studies have examined localized prostate cancer and CTCs, likely due to the early stages of disease having fewer CTCs in the bloodstream. One of the earliest studies published in 2008 comparing 97 men with localized prostate cancer who received radical prostatectomy to 25 healthy controls did not find a statistically significant difference in detected CTCs between the two groups, and CTC values did not correlate with tumor volume, pathological stage or Gleason score.⁷¹ Other studies of localized prostate cancer also had small cohorts (under 100 patients) and similarly could rarely isolate CTCs and were unable to make strong conclusions.^{72–74} On the other hand, Salami et al were able to identify CTCs in 33 of 45 high-risk localized prostate cancer patients, and those with high AR expression were associated with biochemical recurrence after radical prostatectomy and metastasis.⁷⁵ A recent phase 2 clinical trial (NCT01800058) of 65 patients of treatment-naïve high-risk or locally advanced prostate cancer designed to evaluate

CTCs before, during, and after treatment was unable to make a correlation of CTC counts with OS, as <20% of patients yielded detectable CTCs and the median count was 1 CTC per 7.5 mL of blood.⁷⁶

Despite the gains, especially in the setting of mCRPC, there are several limitations to CTCs. The half-life of CTCs is estimated to be just 1–2.4 hours, and CTCs are quite heterogeneous.⁷⁷ The only FDA-approved platform since 2004 to isolate CTCs for prostate, breast, and colorectal cancers is the CellSearch system, which identifies CTCs via the epithelial cell adhesion molecule (EpCAM).⁷⁸ However, this technology does not detect many CTCs such as those with down-regulated EpCAM and mesenchymal-like CTCs, which are believed to be the tumor cells that facilitate tumor spread and contribute to metastasis.⁷⁹ Because of these limitations, several other platforms have been developed and used for different studies, but none of these have yet been approved by the FDA.⁸⁰ Different platforms likely isolate different CTC subpopulations, so an ideal platform that can capture all types of CTCs has yet to be determined. There also remain limits to the ability to comprehensively analyze CTCs, which would have the potential to tap into a wealth of knowledge regarding their genome, transcriptome, epigenome, and proteome.⁸¹

Exosomes

Exosomes are extracellular vesicles carrying contents (such as proteins, nucleic acids, lipids, etc.) surrounded by a lipid bilayer membrane and secreted by living cells. There is growing evidence suggesting that they play important roles in cancer signaling and progression and can be evaluated for early detection, diagnosis, prognosis, and therapy.⁸² Compared to ctDNA and CTCs, there is a paucity of clinical data for exosomes in prostate cancer. Large studies involving exosomes and advanced prostate cancer are lacking compared to studies involving ctDNA and CTCs (Table 1). While most of the studies are in the preclinical setting, exosomes present an exciting opportunity to inform care throughout the prostate cancer disease course.

Exosomes have been suggested to become biomarkers for the diagnosis and prognosis of prostate cancer. Differences have been identified in the content and quantity of exosomes (using proteins and microRNA) isolated from urine in patients with and without prostate cancer as a means of diagnosing cancer with high sensitivity, predicting their aggressiveness,^{83–87} and predicting recurrence after radical prostatectomy and radiotherapy.^{88,89} Similarly, studies have also evaluated the contents of exosomes isolated from blood samples as well.^{90–92} The potential utility of exosomal assays may help to prevent overdiagnosis and overtreatment by stratifying patients and identifying those who will benefit from aggressive interventions. McKiernan et al designed the ExoDx Prostate (IntelliScore) urine exosome gene expression assay, which quantifies the expression of three genes (PCA3, ERG, and SPDEF) from urine in patients with equivocal PSA level in order to better identify high-risk localized prostate cancer.⁹³ This successful assay remains the only exosome-based liquid biopsy test approved by the FDA for any malignancy so far.⁹⁴ Ultimately, biomarkers based on exosomes may be established to distinguish between benign and malignant prostate diseases as an alternative to invasive biopsies.

Clinical studies have evaluated exosomes in the setting of CRPC as well. Huang et al identified high levels of plasma exosomal miR-1290 and miR-375 to be associated with significantly worse OS (7.2 months vs 19.3 months, $p=0.0045$) in a cohort of 100 CRPC patients.⁹⁵ Zhu et al determined exosomal TUBB3 mRNA positivity to be associated with poor PSA PFS in mCRPC patients taking abiraterone (7.9 months vs 11.0 months, $p=0.014$), and increased copies of TUBB3 was even worse than fewer copies of TUBB3.⁹⁶ Del Re et al isolated plasma-derived exosomal RNA and found 14 of 36 CRPC patients with AR-V7 positivity who received either enzalutamide or abiraterone had both decreased median PFS (3 months vs 20 months, $p<0.001$) and median OS (8 months vs NR, $p<0.001$).⁹⁷ According to the authors, this was an easier method of detecting AR-V7 status compared to using CTCs, as extracting and processing exosomes is cheaper and less labor intensive, and exosomes bypass many of the limitations of CTCs such as molecular heterogeneity the limitations of CTCs (as mentioned in the previous section).

Exosomal assays have several limitations. While exosomes can be collected non-invasively from several bodily fluids and their structure ostensibly protects contents well and long enough for collection and analysis,⁹⁸ techniques for capturing a quantity necessary to unlock prognostic information are not always successful.⁹⁹ After collection, there are no standardized methods for classifying, isolating, and characterizing exosomes.¹⁰⁰ Due to similar-appearing material like lipoproteins that resemble exosomes, these can contaminate samples and generate false positive and negative

results.¹⁰¹ Suboptimal methods for capturing and analyzing exosomes and their contents affect the results of individual studies, but currently non-standardized assays prevent reliable comparison between studies, even if they are conducted with samples from patients in similar disease settings.¹⁰²

Future Directions

As described above, the non-invasive current technologies can generate actionable diagnostic and prognostic information across the disease spectrum in prostate cancer, but there are still several limitations to overcome. Most of the clinical research in liquid biopsies have been limited to observational studies. Currently, liquid biopsies still fall short in the ability to be a validated method in monitoring cancer progression, and they have not yet definitively provided predictive biomarkers for patients to determine optimal therapy selection. From the available options, there is an urgent need to prospectively test and standardize the assays to best guide cost-effective personalized prostate cancer care. In the meantime, as techniques steadily improve, further methods can be gathered to improve patient outcomes. Examples include applying liquid biopsy to study common cancer features such as cfDNA quantification, telomere activity, and methylation activity, which are still in their infancy but will likely contribute to determining more useful biomarkers to guide treatment and prognosis.^{103,104}

Total plasma cfDNA quantification may be a useful biomarker in predicting response to treatment and survival, as a high concentration of cfDNA in patients with CRPC suggests a poor outcome.¹⁰⁵ Two phase 3 trials involving taxane therapy in mCRPC patients, FIRSTANA (NCT01308567) and PROSELICA (NCT01308580), reported that baseline cfDNA concentration was an independent prognostic biomarker in patients with mCRPC, associated with shorter radiographic PFS and OS while on taxane therapy, and that a decline in cfDNA concentration was associated with taxane therapy response.¹⁰⁶ A similar study measured cfDNA levels for mCRPC patients prior to and during treatment with either abiraterone or enzalutamide; patients with higher levels of cfDNA at baseline had worse outcomes, but interestingly a rise in cfDNA levels at 4 weeks of therapy was associated with better outcomes.¹⁰⁷ Notably, this study also found a moderate positive correlation between total cfDNA and ctDNA fraction; even after adjusting for the ctDNA fraction, the cfDNA was still independently associated with clinical outcomes, supporting the possibility that cfDNA quantification may be a negative prognostic biomarker independent of ctDNA.

Telomere length is critical to replicative immortality (one of the hallmarks of cancer),¹⁰⁸ and its association with prostate cancer remains of interest given conflicting findings. There have been prior studies evaluating the telomere DNA content from prostate tumor tissue itself, showing shortened telomeres were associated with a worse clinical outcome and increased disease progression.^{109,110} Unfortunately, recent studies analyzing telomere length of leukocytes obtained from peripheral blood samples and its association with prostate cancer have revealed conflicting conclusions.^{111–115} These studies continue to provide support that via liquid biopsies, leukocyte telomere length may provide further risk stratification in localized and advanced prostate cancer patients.

Liquid biopsies also have shown promise in revealing epigenetic data more easily during the progression of prostate cancer. Studies have successfully determined the feasibility of capturing the methylome of treatment-naïve prostate cancer via comparison of urine and blood plasma to tissue biopsies.^{116,117} Studies have also evaluated the methylation changes of CRPC patients as well, providing more potential biomarkers for more aggressive forms of disease, treatment prognosis and survival.^{118–120}

Building upon individual assays, Hodara et al created a proof-of-principle study in multiparametric assays by combining information from tumor-relevant cfDNA, AR-V7 from cfRNA, CTC DNA, and CTC count all from blood samples, as well as genomic information from lymph node biopsies, in 20 mCRPC patients to generate longitudinal patient genetic profiles with potential prognostic value.¹²¹ Combined cfDNA and cfRNA profiling performed in 67 mCRPC patients showed that concurrent DNA and RNA *AR* aberrations were associated with poor treatment outcomes, further supporting the concept of further molecular insights gained from the complementary analysis of both nucleic acids.¹²² The strategy of multi-omics analysis in prostate cancer, integrating multiple pieces of data including proteomics, lipidomics, metabolomics from liquid biopsy and tissue biopsy, will synergistically provide further clues for better management of these patients.¹²³

With all these options, liquid biopsies may one day have the potential to rival tumor biopsies as a selection tool for clinical trials.¹²⁴ Several prostate cancer clinical trials involving liquid biopsies are recruiting and active (Table 3). These

Table 3 Active and Recruiting Prostate Cancer Clinical Trials Incorporating Liquid Biopsies into Their Study Design, Based on Free-Hand Search in Clinicaltrials.gov Conducted April 2022

Clinical Trial	Phase	Prostate Cancer Stage	Number of Prostate Cancer Patients	Primary End Point	Liquid Biopsy End Point
Circulating tumor DNA					
NCT01942837	2	mCRPC	66	AR mechanisms of resistance to enzalutamide	ctDNA analysis for mechanisms of AR resistance and correlation to enzalutamide response
NCT02854436 (GALAHAD)	2	mCRPC	289	ORR	ctDNA detection of DNA repair anomalies
NCT03230734 (RAPSON)	2	mCRPC	70	Health-related quality of life clinical benefit	Identification of predictive biomarkers
NCT03899467	2	mCRPC	60	Dosage and toxicity of proxalutamide	ctDNA analysis of AR for exploratory biomarkers
NCT04015622 (PROTRACT)	2	mCRPC	100	PFS	Correlation of specific ctDNA-based genomic alterations to treatment response
NCT04343885 (UpFrontPSMA)	2	mHSPC	140	Undetectable PSA rate at 12 months	Prognostic and predictive biomarkers
NCT01411345	2/3	Localized	80	PSA response rate	Relationship of ctDNA to tissue biomarkers and initial complete biochemical response
NCT03824275	2/3	Unspecified	129	Positive predictive value of 18F-DCFPyL PET/CT scan	ctDNA characterization and correlation of levels with disease burden
NCT03903835 (ProBio)	3	mCRPC	750	PFS	Treatment adjustment based on ctDNA levels
Circulating tumor cells					
NCT01942837	2	mCRPC	66	AR mechanisms of resistance to enzalutamide	CTC level as marker of response
NCT01972217	2	mCRPC	158	PFS, adverse events, dose-limiting toxicities	CTC response rate
NCT02312557	2	mCRPC	58	PSA response	CTC levels
NCT02703623	2	mCRPC	196	OS, adverse events, AR response marker signature, PSA and CTC levels	CTC levels
NCT02854436 (GALAHAD)	2	mCRPC	289	ORR	CTC response rate at 8 weeks post-baseline
NCT03050866 (CABA-V7)	2	mCRPC	140	PSA response	CTC response
NCT03148795 (TALAPRO-I)	2	mCRPC	128	ORR	CTC counts

(Continued)

Table 3 (Continued).

Clinical Trial	Phase	Prostate Cancer Stage	Number of Prostate Cancer Patients	Primary End Point	Liquid Biopsy End Point
NCT03230734 (RAPSON)	2	mCRPC	70	Health-related quality of life clinical benefit	Identification of predictive biomarkers
NCT03419234	2	mCRPC	210	PFS	AR-V7 status change in CTCs
NCT03568188 (FOCALE)	2	Localized	170	Controlled disease	CTC number reduction
NCT03899467	2	mCRPC	60	Dosage and toxicity of proxalutamide	CTC analysis for exploratory biomarkers
NCT04592237	2	Metastatic	120	PFS	CTC response rate
NCT01411345	2/3	Localized	80	PSA response rate	Relationship of CTCs to tissue biomarkers and initial complete biochemical response
NCT04983095 (METRO)	3	mHSPC	114	Failure free survival	Identification of predictive biomarkers
Exosomes					
NCT03824275	2/3	Unspecified	129	Positive predictive value of 18F-DCFPyL PET/CT scan	Exosome characterization and correlation of levels with disease burden

Notes: Studies were repeated if they fit in more than one category. Combined Phase I/2 trials and trials enrolling less than 40 patients with prostate cancer were excluded. **Abbreviations:** mCRPC, metastatic castration-resistant prostate cancer; mHSPC, metastatic hormone-sensitive prostate cancer; ORR, objective response rate; PFS, progression-free survival; OS, overall survival; ctDNA, circulating tumor DNA; CTC, circulating tumor cell; PSA, prostate-specific antigen.

trials aim to use liquid biopsies to analyze treatment response, to find novel biomarkers, to detect prostate cancer earlier, and more.

Conclusion

Liquid biopsies have made great strides in prostate cancer in terms of being more commercially available, having the ability to offer predictive and prognostic value, and easily extracting genetic information from tumors. Approved assays to detect and quantify cfDNA and ctDNA have allowed for better selection of the effective prostate cancer therapies based on detectable genetic aberrations. CTC counts have become a prognostic marker in advanced prostate cancer, and the AR-V7 status of CTCs has been suggested to be both a negative predictive biomarker for novel hormonal agents and a negative prognostic marker. Novel ideas to incorporate information gained from analyzing exosomes, leukocyte telomere length, DNA methylation and multiparametric assays are still work in progress but hold promise to add to the wealth of available information to optimize prostate cancer care. The technology continues to improve, resulting in more powerful assays and the ability to more accurately diagnose prostate cancer without invasive biopsies, to optimize patients for select treatments, and to predict treatment responses and progression. Because of the rapidly advancing pace, no set guidelines exist, such as the specific liquid biopsy assay to use and whether different liquid biopsies can be combined or used in which order, to further advance patient care. Overcoming these challenges and ultimately having them be more integrated into clinical practice are a major focus for this upcoming decade.¹²⁵

Abbreviations

mCRPC, metastatic castration-resistant prostate cancer; mHSPC, metastatic hormone-sensitive prostate cancer; micro-satellite instability-high, MSI-H; ctDNA, circulating tumor deoxyribonucleic acid; cfDNA, cell-free deoxyribonucleic

acid; CTC, circulating tumor cell; ADT, androgen deprivation therapy; AR, androgen receptor; PSA, prostate-specific antigen.

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