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Quantitative and Qualitative Analysis of Blood-based Liquid Biopsies to Inform Clinical Decision-making in Prostate Cancer

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Abstract

Context: Genomic stratification can impact prostate cancer (PC) care through diagnostic, prognostic, and predictive biomarkers that aid in clinical decision-making. The temporal and spatial genomic heterogeneity of PC together with the challenges of acquiring metastatic tissue biopsies hinder implementation of tissue-based molecular profiling in routine clinical practice. Blood-based liquid biopsies are an attractive, minimally invasive alternative.
Objective: To review the clinical value of blood-based liquid biopsy assays in PC and identify potential applications to accelerate the development of precision medicine.
Evidence acquisition: A systematic review of PubMed/MEDLINE was performed to identify relevant literature on blood-based circulating tumor cells (CTCs), circulating tumor DNA (ctDNA), and extracellular vesicles (EVs) in PC.
Evidence synthesis: Liquid biopsy has emerged as a practical tool to profile tumor dynamics over time, elucidating features that evolve (genome, epigenome, transcriptome, and proteome) with tumor progression. Liquid biopsy tests encompass analysis of DNA, RNA, and proteins that can be detected in CTCs, ctDNA, or EVs. Blood-based liquid biopsies have demonstrated promise in the context of localized tumors (diagnostic signatures, risk stratification, and disease monitoring) and advanced disease (response/resistance biomarkers and prognostic markers).
Conclusions: Liquid biopsies have value as a source of prognostic, predictive, and response biomarkers in PC. Most clinical applications have been developed in the advanced metastatic setting, where CTC and ctDNA yields are significantly higher. However, standardization of assays and analytical/clinical validation is necessary prior to clinical implementation.

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Patient summary: Traces of tumors can be isolated from blood samples from patients with prostate cancer either as whole cells or as DNA fragments. These traces provide information on tumor features. These minimally invasive tests can guide diagnosis and treatment selection.

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1. Introduction

Recent studies have provided insight into the molecular landscape of prostate cancer (PC), identifying prognostic biomarkers, actionable targets, and drug resistance biomarkers. The difficulty of obtaining suitable tumor material for molecular testing is one of the reasons hampering clinical implementation of genomic profiling. Primary prostate tumor biopsies, although routine, are often scant in yield, and fixation procedures impact DNA quality. Biopsies of osteoblastic metastatic lesions, on the contrary, are technically challenging and distressing for the patient. Moreover, PC evolves over time as a consequence of therapy-induced selective pressure. Secondary resistance to standard-of-care androgen receptor (AR) targeting agents often involves genomic changes in a polyclonal manner that may be relevant for the selection of subsequent lines of therapy. A single tumor biopsy, from either primary or metastatic lesions, is limited in its ability to capture spatial heterogeneity, especially for repeated longitudinal assessments. Indeed, primary PC is among the most spatially heterogeneous and clonally complex cancer types [1].

The concept of a liquid biopsy encompasses the analysis of tumor material present in a bodily fluid [2]. This material can exist as biomolecules (e.g. circulating tumor DNA [ctDNA], RNA, proteins, and mitochondrial DNA), circulating tumor cells (CTCs), or extracellular vesicles (EVs). Liquid biopsies have emerged as an attractive way to study tumor molecular landscapes in a minimally invasive manner, allowing for real-time snapshots of the overall tumor burden. Additionally, liquid biopsy–based biomarkers could serve as early endpoints in clinical trials to expedite drug development [3,4].

We review current knowledge of blood-based liquid biopsy components, their impact on clinical decision-making in PC, opportunities for accelerating precision medicine, and the challenges of implementing such tests in clinical practice.

2. Evidence acquisition

A systematic review of the PubMed/MEDLINE database was performed to identify literature on CTCs, ctDNA, and EVs in PC, published between 2005 and July 2020. Articles involving CTCs, ctDNA, and EVs in blood from PC patients were selected, and references cited within them were also considered.

3. Evidence synthesis

3.1. Circulating tumor cells

CTCs are cancerous cells from primary or metastatic lesions that either have been passively shed or have actively migrated from the tumor into the circulatory system. They can be found as single cells or clusters, the latter having a higher metastatic potential [5].

The half-life of CTCs in circulation is short (<1–2.5 h) [5]. The relatively small number of CTCs in blood remains a challenge for a comprehensive molecular analysis. CTC load increases with disease progression, being very low or near zero for most localized tumors. Typically, CTCs are isolated from a peripheral blood sample of 7.5–10 ml. Nevertheless, some studies have successfully pursued peripheral blood aphaeresis to increase the CTC yield [6]. It remains unclear, however, whether all tumor foci and lesions are represented in the CTC yield.

There are different strategies facilitating CTC isolation from blood samples, based on distinct physical or biological characteristics of CTCs (Table 1). Biological criteria–based methods for CTC isolation rely on selecting cells that express specific antigens (positive selection) and disregarding those that express other antigens (negative selection). This strategy is based on immunoaffinity, using antibodies targeting surface markers of epithelial cells such as EpCAM (CD326), for positive selection, while disregarding normal blood cells based on leukocyte markers such as CD45, CD16, or CD66b. The Food and Drug Administration (FDA)-approved CellSearch system (Menarini-Silicon Biosystems, Castel Maggiore, Italy) relies on a positive immunoaffinity assay based on EpCAM expression, followed by a semi-automated visual identification process based on immunofluorescence. It defines a CTC based on the presence of a DAPI-positive nucleus; lack of expression of CD45; expression of the epithelial cell markers EpCAM and cytokeratins (CKs) 8, 18, and/or 19; and a diameter of >4 μm [7]. However, several studies have demonstrated that expression of epithelial cell markers varies in CTCs (ie, EpCAM-low cells) [8], indicating that a proportion of CTCs may be missed if selection is based only on EpCAM expression. Therefore, other platforms use EpCAM-independent methods, such as characterizing all nucleated cells and identifying CTCs based on specific tumor-associated protein expression (i.e. CK8 and AR) and cell morphology [7,9]. CTCs can also be isolated, leveraging their distinct physical properties as different deformability, density, surface charge, and size compared with nontumoral circulating cells. Several microfluidic devices using this approach have been developed (Table 1) [8,10,11].

DNA and RNA from CTCs can serve as proxies for tumor genomic characterization. Single CTC studies allow for fine dissection of intratumor heterogeneity [12], which might help in understanding therapy resistance; however, single-cell characterization is far from being clinically applicable. Clonal genome-wide copy numbers can be ascertained relatively inexpensively from single nucleotide polymor-

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Table 1 – Isolation platforms for CTCs in PC

Technology	CTC definition	Application in PC
Antibody-based positive selection		
CellSearch	EpCAM+, CD45–, CK8+, CK18+, CK19+, DAPI+	[57]
Adna Test	EpCAM+, PSA+/PSMA+/EGFR+	[15]
CTC-Chip	EpCAM+, specific antigen+	[89]
CTC-iChip	>3.8 μm size, EpCAM+	[90]
IsoFlux	CD45–, DAPI+, PanCK+, or EpCAM+	[91]
MagSweeper	EpCAM+, CD45–, DAPI+	[10,92]
CellCollector	EpCAM+, CD45–, PanCK+, PSA+, Hoechst+	[74]
NanoVelcro	EpCAM+, CD45–, PanCK+, morphological verification	[93]
Antibody-based negative selection		
EasySep	CD45–	[94]
RosetteSep	CD45–, CD66b–, glycophorin A–, density	[74]
EPISPOT	CD45–, CD66b–, glycophorin A–, PSA+, FGF2+	[74]
CTC-iChip	CD45–, CD16–, CD66b–	[90]
Selection-free		
Epic Sciences	PanCK+, CD45–, DAPI+, AR+	[9]
AccuCyte	DAPI+, PanCK+, CD45–, CD66b–, CD11b–, CD14–, CD34–, EpCAM+	[95]
Selection based on distinct physical properties		
ApoStream	Dielectrophoretic field flow	[96]
Celsee Diagnostics	>7.5 μm, deformability	[97]
ISET	≥8 μm	[95]

CTC = circulating tumor cell; EGFR = epidermal growth factor receptor; PC = prostate cancer; PSA = prostate-specific antigen; PSMA = prostate-specific membrane antigen.
Positive (+) and/or negative (–) expression of different capture/detection antigens, or physical properties, is used as a criterion for CTC isolation by different technologies or platforms.

phism arrays or low-pass whole-genome sequencing (lpWGS). These lower-resolution approaches can also identify features of genomic instability associated with aggressive phenotypes, such as large-scale transitions [13]. Despite the small amount of input material obtained from CTC samples, whole-exome sequencing (WES) approaches to identify mutations have been proved to be feasible [10,14].

The study of aberrant AR transcripts in CTCs, particularly those derived from AR splice variants, has attracted notable attention due to its potential clinical relevance for AR-targeting agents [15,16]. Interrogation of specific transcripts using in situ padlock probes is an opportunity for targeted transcriptomic approaches [17]. Beyond AR, multiplex assays enable comprehensive profiling of tumor transcriptomics from CTCs. These range from multiplex quantitative polymerase chain reaction approaches [18,19] to single-cell RNA-seq analysis [11]. In addition, methylome analysis in PC CTCs has generated profiles that resemble those derived from metastatic biopsies [20]. Lastly, protein expression in CTCs can also serve as a putative predictive biomarker. For instance, the detection of nuclear versus cytoplasmic AR-V7 has been correlated with a response to AR signaling inhibitors (ARSi) [21]. Another example is prostate-specific membrane antigen (PSMA) expression in CTCs [22], which could be relevant for the development of PSMA-based radiopharmaceuticals [23].

3.2. Circulating tumor DNA

Cell-free DNA (cfDNA) comprises short DNA fragments (<200 bp) shed into the circulation from apoptosis or necrosis of normal and tumor cells. In healthy individuals, cfDNA fragments have a dominant peak at 167 bp, supporting a model where cfDNA is associated with the nucleosome core particle and linker histones [24]. In cancer patients, tumor-mutated alleles can be observed in DNA fragments shorter than nucleosomal DNA [25]. In the bloodstream, cfDNA has a short half-life estimated between 16 min and a few hours [26].

As both normal and tumor cells shed DNA into the blood, tumor DNA is diluted into circulating nontumoral DNA primarily coming from hematopoietic cells [24]. The subset of cfDNA arising from a tumor is known as ctDNA or “cfDNA tumor fraction” [27]. The dilution of ctDNA in nontumoral cfDNA is a significant confounding factor, and tumor fraction is likely to be a more reliable biomarker [26]. Several preanalytical conditions are required to maximize cfDNA yield and quality [28], as depicted in Fig. 1. Once cfDNA is isolated and quantified, inference of the tumor fraction relies on computational analysis of the frequency of reads that carry tumor-specific aberrations (e.g. point mutations, copy number alterations, and genomic rearrangements). Fragments of cfDNA originated in the tumor tend to have smaller sizes than those of nontumoral cfDNA. Hence, size selection of cfDNA can enrich for tumor content in ctDNA analysis [25]. This specific fragmentation pattern of cfDNA has also been postulated to be cancer-type specific and could potentially be helpful for cancer diagnosis [29].

Tumor fraction in cfDNA usually increases in later disease stages and with a higher tumor burden. For example, in two studies of metastatic castration-resistant PC (mCRPC), the median ctDNA fraction was in the range of 15–20%, although interpatient variability was high [30,31].

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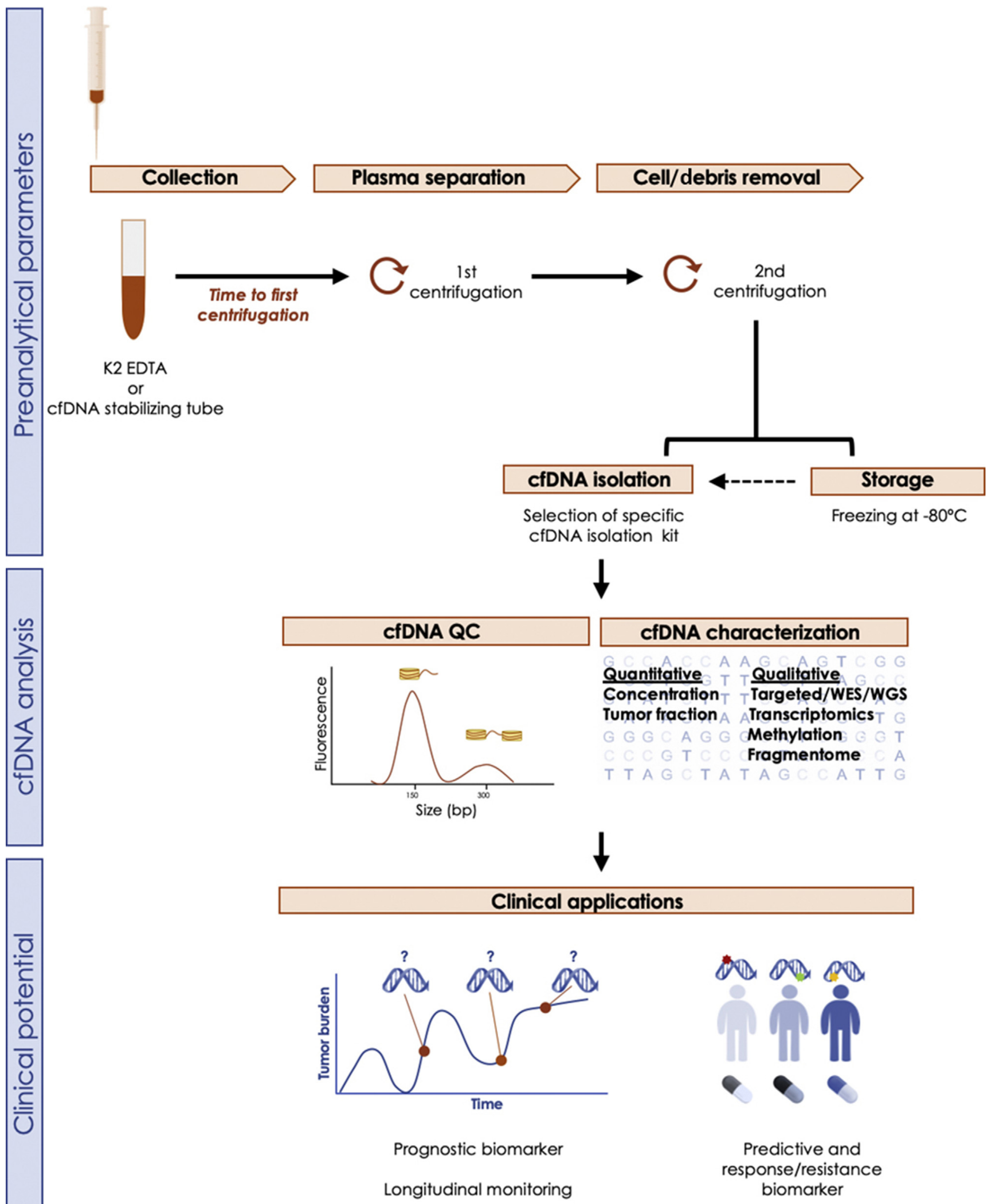

Fig. 1 – Workflow depicting preanalytical, analytical, and postanalytical steps for blood-based cfDNA studies. After venipuncture, blood is collected in a tube containing anticoagulants (EDTA and citrate are preferred to heparin). The time from sample acquisition to processing is critical, as cfDNA degrades within few hours. To overcome this problem, tubes containing different DNA stabilizers are available; the use of these tubes is particularly relevant in large multicenter studies with centralized analysis, or in general when the sample is not processed at the point of collection. A two-step centrifugation process is recommended to separate the plasma component, from which the cfDNA will be extracted. If the cfDNA is not extracted immediately, plasma can be stored at −80 °C for prolonged periods, although repeated freeze-thawing cycles compromise cfDNA quality by increasing the amount of nontumor DNA contamination. After cfDNA isolation, quality control (QC) testing to assess cfDNA concentration and fragment size is performed prior to characterization. cfDNA = cell-free DNA; WES = whole-exome sequencing; WGS = whole-genome sequencing.

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The lpWGS uses copy number ratios to calculate tumor fraction, with a lower bound of detection of about 3%. However, this method might result in false-negative results for copy number–quiet tumors [32,33]. Targeted sequencing approaches, on the contrary, are a more affordable strategy to deliver high-read depths at specific regions in the genome but assume a priori that at least one somatic mutation would be present within a targeted region to infer ctDNA proportion; the probability of detecting mutations increases with the size of the targeted regions.

Highly sensitive assays, such as droplet digital polymerase chain reaction (ddPCR), can detect point mutations with sensitivity ranging from 0.001% to 1%. These assays can detect AR mutations [34] and copy number gains [35]. However, its application is limited to the analysis of individual or a small set of known mutations (multiplexed ddPCR). These can be particularly useful for longitudinally monitoring tumor adaptation to targeted therapies, especially for hotspot mutations or for mutations previously determined by larger-scale sequencing.

Using targeted sequencing, Wyatt et al [36] showed good concordance between ctDNA and metastatic tissue biopsy for alterations in selected PC driver genes. Others have also used WES on ctDNA showing high agreement with tissue biopsies, although WES requires a higher minimum tumor fraction, probably above 10% [30,37]. Identification of low-frequency events, such as subclonal mutations, is more likely to be masked in samples with a low tumor fraction. Indeed, inference of the clonal versus subclonal origin of a mutation requires capturing enough ctDNA alleles in order to define clonality thresholds. By allocating sequencing depth to fewer genomic regions, targeted sequencing allows the study of low allele frequency events.

Methylation profiling of cfDNA is likely to be more sensitive than somatic gene alteration profiling for the detection of ctDNA, since there are millions of methylation marks available to profile but only a few thousand somatic gene alterations [38]. Recent studies in tumor [39] and cfDNA [40,41] samples have identified methylation-based PC subtypes and changes during disease progression.

3.3. Extracellular vesicles

EVs are secreted vesicles with a lipid bilayer and a typical size between 50 nm and 1 μm. Regarding their origin, EVs usually derive from the plasma membrane (e.g. microvesicles) or, alternatively, have an endosomal origin (e.g. exosomes). Some recent works have also described smaller (exomeres, ~35 nm [42]) and larger (oncosomes, 1–10 μm [43]) EVs with important roles in cancer. The International Society for EVs (ISEV) recommends that EVs should be classified according to: (1) size (small [<100 nm], medium [100–200 nm], or large [>200 nm] EVs), (2) biochemical composition (e.g. CD63+/CD81+), or (3) cell of origin [44]. EVs can contain proteins, lipids, metabolites, RNA (mRNA and miRNAs), and DNA as cargo. EVs play a key role in cell-to-cell communication during cancer progression and metastasis, as well as in triggering immune responses [45–47]. In addition, EVs have been associated with metastasis or relapse in cancer patients and can serve as diagnostic and prognostic markers; these can also be used for detecting therapeutic targets [47]. To date, few studies have investigated the relevance of blood EVs in PC [48,49], with most evidence coming from urine-derived EV studies [50,51]. EV size poses a significant challenge to the accuracy and reliability of their isolation and quantification [52]. Ultracentrifugation is currently considered the gold standard method, but to increase specificity, additional techniques such as filtration, density gradients, and chromatography can be implemented [44]. Different approaches have been used for the molecular characterization of EVs and their cargo, including transcriptomic analysis (ddPCR, real-time PCR, and RNA-seq) for the analysis of prostate-specific antigen (PSA), PCA3, ERG, AR, or AR-V7 [50,53,54] as well as WGS of larger EVs [43].

3.4. Moving liquid biopsies toward clinical management of PC

3.4.1. Quantitative prognostic and response biomarkers to accelerate drug development

Drug approval in the metastatic PC (mPC) setting is based on improvements in overall survival (OS) and/or radiographic progression-free survival (rPFS) [55]. CTC and cfDNA/ctDNA kinetics have value as prognostic and response biomarkers in mPC, offering faster readouts for clinical trials and allowing to accelerate the development of the most promising drugs.

Pioneering work by Cristofanilli et al [56] demonstrated the prognostic value of CTC enumeration in breast cancer, with an optimal threshold of five or more CTCs in 7.5 ml of blood, using the CellSearch system. In prostate cancer, two studies in patients prior to initiating chemotherapy demonstrated that (1) patients with five or more CTCs per 7.5 ml of blood, referred to as an “unfavorable profile” or a “high CTC count”, had shorter OS (prognostic biomarker), and (2) achievement of a decrease in CTC counts after therapy initiation (fewer than five CTCs per 7.5 ml; CTC conversion) correlated with OS (response biomarker) and was a stronger predictor than PSA changes [57,58]. Using the COU-301 registration trial of abiraterone as a model, Scher et al [59] confirmed the value of CTC counts and their changes over time, as prognostic and response biomarkers. CTC counts combined with lactate dehydrogenase (LDH) levels were shown to be a surrogate of OS at the individual patient-level, endorsing their use as intermediate biomarkers in mPC clinical trials. In a retrospective meta-analysis including data from five independent randomized clinical trials, Heller et al [60] demonstrated that CTC0 (change from detectable to undetectable CTCs) and CTC conversion consistently achieved higher C-index than percentage PSA decreases to discriminate OS, supporting that CTC kinetics could outperform PSA changes as a response biomarker.

Since then, CTC counts have been incorporated as a biomarker of response in several phase II trials, including the proof-of-concept studies for olaparib in PC [61], where CTC kinetics strongly correlated with rPFS. Further studies have also shown that relative changes in CTC counts (>30% decrease from baseline) could be potential surrogate endpoints [62]. Moreover, CTC kinetics could assist in therapy switch decisions as indicators of disease progres-

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sion; increases in CTC counts after 10–12 wk of therapy significantly correlate with reduced rPFS and OS [63,64]. Of interest, large tumor–derived EVs expressing the same cell surface capture markers as CTCs (i.e. EpCAM) can be co-isolated with them and studied in platforms such as CellSearch. Enumeration of these EpCAM + EVs may have a prognostic value in mCRPC, to further stratify patients with favorable CTC counts [49]. Additional studies are needed to confirm how the combination of different liquid biopsy approaches can improve patient stratification.

The cfDNA yield, and in particular the ctDNA fraction, increases as cancer progresses, in association with markers of overall tumor burden (PSA, LDH, and alkaline phosphatase). In an analysis of 571 patients from the FIRSTANA and PROSELICA phase III trials of taxane-based therapies [65], cfDNA baseline levels were an independent prognostic factor of rPFS and OS. In addition, absolute and relative changes in cfDNA levels on therapy correlated with PSA responses. Similarly, in the TOPARP-A trial, a 50% drop in cfDNA levels on olaparib therapy strongly correlated with rPFS and OS [66].

In a randomized trial of abiraterone acetate versus enzalutamide, ctDNA fraction was quantifiable by WES and deep targeted 72-gene panel sequencing. Higher ctDNA fractions (>30%) were associated with clinical markers of tumor burden, including PSA, LDH, and alkaline phosphatase. Tumor fraction was prognostic, with ctDNA >30% presenting the worst rPFS, followed by a fraction between 2% and 30%, and patients with no detectable ctDNA experiencing longer times to progression [30]. In the hormone-naïve mPC setting, ctDNA levels appeared to diminish rapidly during the initial weeks of androgen deprivation therapy [67].

3.4.2. *Clinical applications in localized prostate cancer*

Current models for estimating the risk of relapse after definitive local therapy rely on pretreatment serum PSA abundance, International Society of Urological Pathology grade on biopsy, and clinical T category. Different biomarkers (i.e. PTEN status, TP53 mutations, and cribriform histology) and tissue-based molecular signatures (i.e. Decipher and Oncotype DX) have been proposed to improve patient stratification in localized PC, and in some cases these have been included in clinical guidelines, but their impact on therapeutic decision-making is still limited [68]. Tools supporting precise stratification of localized PC are needed, particularly in the case of longitudinal monitoring of patients on active surveillance protocols. Several studies have demonstrated the presence of disseminated cancer cells, particularly in the bone marrow [69,70], among patients with localized PC, providing the rationale for studying disease dissemination via liquid biopsies.

Davis et al [71] studied the presence of CTCs in patients with localized PC ($n = 97$) with the CellSearch platform. CTCs were detected in a similar proportion of biopsy-positive patients (21%) to a control cohort of negative biopsy patients (20%); moreover, when present, counts were low (fewer than one to three CTCs per sample). In a more recent study, Salami et al [72] identified CTCs using the Epic Sciences platform in 33/45 (73%) patients with high-risk localized PC prior to receiving treatment. Biochemical recurrence was associated with higher baseline AR-positive CTC counts. Xu et al [73] showed that identification of CTCs and CTC-RNA–based signatures could improve detection of clinically significant PC. Kuske et al [74] combined three independent CTC assays (CellSearch, CellColector, and EPISPOT) and found a cumulative positivity rate of 81% in patients with nonmetastatic high-risk PC; however, only 21% harbored five or more CTCs per 7.5 ml of blood. This work suggests that composite biomarker assays might increase our capacity to interrogate liquid biopsies in localized PC. In sum, the small number of CTCs in the blood of patients with clinically localized PC makes potential clinical applications challenging.

Similarly, the representation of ctDNA in early disease settings seems extremely low, challenging any downstream applicability for clinical testing, although the presence of ctDNA in patients with localized PC has been demonstrated in studies of methylation [75], allelic imbalance [76], and LOH [77]. The most comprehensive series to date came from Hennigan et al [78], in which no significant tumor fraction was detected by lpWGS or targeted sequencing, even in patients with high preprostatectomy serum PSA levels who subsequently recurred.

Lastly, the field of tumor EVs in blood as PC biomarkers remains relatively unexplored. Park et al [48] used PSMA expression to enrich for tumor-derived EVs from patients with either benign prostatic hyperplasia or localized PC tumors. Interestingly, concentration of PSMA-positive EVs increased from low- to high-risk PC.

3.4.3. *Liquid biopsies for precision use of AR targeting agents*

Resistance to AR targeting agents typically emerges through multiple alterations affecting AR activity. Liquid biopsy can be repeated over time and represents an attractive opportunity for biomarker stratification for more precise ARSi use.

AR amplification and mutations can be detected in ctDNA, and are associated with worse OS, PFS, and PSA response rate [31,34,79]. Carreira et al [80] showed that longitudinally acquired plasma samples allow monitoring of tumor dynamics and emerging drug resistance mechanisms.

Antonarakis et al [81] provided proof-of-concept evidence for the clinical value of AR-V7 detection in CTCs. Up to 39% of mCRPC patients treated with enzalutamide and 19% with abiraterone had AR-V7 positive CTCs; the presence of AR-V7 in CTCs was associated with lower PSA response and shorter biochemical progression-free survival (bPFS). This was further validated in a larger prospective study where mCRPC patients were classified as CTC–, CTC+/AR-V7–, and CTC+/AR-V7 +. PSA response rates, bPFS, and OS were shorter in patients positive for CTCs, and even shorter in those with AR-V7+ CTCs [15]. AR-V7 status likely offers both prognostic and predictive information. In particular, nuclear-specific localization of AR-V7 in CTCs was found to impact OS significantly in pre-ARSi blood CTCs but was not associated with differential response to taxanes [21]. The presence of AR-V7 in CTCs was further validated as an independent predictor of poor outcome in the PROPHECY

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multicenter prospective trial using two isolation approaches (AdnaTest and Epic Sciences assay) [82]. Del Re et al [83] used plasma-derived exosomal RNA to detect AR-V7, with AR-V7 exosome-positive patients having a worse prognosis and shorter response to treatment.

AR profiling in CTCs and ctDNA from the same patient offers complementary information that could aid in ARSi treatment decision. However, the polyclonal nature and coexistence of multiple AR aberrations (copy number changes, splice variants, and mutations) limit the negative predictive value of each assay separately [16].

3.4.4. *Predictive biomarkers for targeted therapies*

The use of ctDNA as a predictive biomarker offers a potential advantage over tissue-based biopsies because ctDNA may comprise material shed by different metastatic lesions [41]. In a randomized phase II trial of ARSi in mCRPC [30], detection of *TP53* mutations, *DDR* gene alterations, or *AR* amplification in ctDNA was associated with worse outcome. Other studies have confirmed a poor prognosis in patients with *TP53* alterations in ctDNA [84].

Several clinical trials in mPC are now using panel-based cfDNA next-generation sequencing (NGS) to enrich their populations for testing targeted agents. Phase II trials of PARP inhibitors, such as TRITON2 (rucaparib) or GALAHAD (niraparib), allowed recruitment based on *DDR* gene alterations in ctDNA. In the TOPARP-A trial of olaparib, a good correlation in *DDR* mutation status between plasma and tumor was observed, with ctDNA detecting reversion mutations in *BRCA2* and *PALB2* upon secondary resistance [66]. Detection of clinically-relevant DDR gene deletions in samples with low ctDNA fraction remains a difficult task that any assay to be implemented in clinical practice needs to address. Relevant to registration trials of AKT inhibitors in PC, recent work by Herberts et al [85] identified *AKT1* and *PIK3CA* mutations in ctDNA. In addition, the detection of microsatellite instability and tumor somatic hypermutation in ctDNA is associated with *MMR* gene defects, and could be relevant to patient selection for immune checkpoint inhibitors [27,86].

Overall, although promising, challenges remain when using ctDNA to identify tumor mutations in the clinical setting. For example, a study comparing two different commercially available panels revealed discordant results, probably due to different coverage of the panels, but also due to different sensitivities and specificities for certain alterations [87]. These results highlight the need for pursuing clinical qualification of ctDNA assays in prospective trials. Umbrella studies such as PC-BETS (NCT03385655) and ProBio (NCT03903835) are now testing the clinical value of ctDNA in multiarm clinical trials.

3.5. *Perspectives and future directions*

Liquid biopsies can accelerate biomarker development for precision care in PC. As novel biomarker-driven therapies are validated, liquid biopsies also represent an opportunity to facilitate the implementation of genomic testing into community practice, where metastatic biopsies are pursued less commonly than in academic centers.

The value of CTC counts as prognostic and response biomarkers has clearly been demonstrated, offering a surrogate biomarker for accelerating drug development and potentially guiding therapeutic decisions. However, cost and access to technology, as well as heterogeneity among studies in terms of CTC definitions and isolation platforms, have complicated the translation of CTC analysis to routine clinical testing. Preliminary studies suggest that ctDNA kinetics may also be a useful prognostic and response biomarker in clinical practice, although further qualification in clinical trials is needed. As both CTCs and ctDNA yield parallel tumor burden, applicability in localized disease may be challenging, although as ultrasensitive assays are developed, liquid biopsies might have the potential to assist in monitoring patients after radical therapy or to complement tissue-based biomarkers to improve patient stratification. The use of EVs in a clinical setting holds promise, and could complement CTC and ctDNA analyses, but faces challenges in standardization of isolation methods and downstream applications.

Identification of targetable alterations and emerging resistance biomarkers represents an attractive feature of liquid biopsies, particularly in the advanced disease setting, and could assist in the implementation of precision medicine therapeutics in PC practice. The FDA clearance of the CellSearch system for CTC enumeration was a first-in-class achievement. The recent FDA approval of the Guardant360 CDx and FoundationOne Liquid CDx as cfDNA NGS-based companion diagnostic assays represents a milestone in the field, but also a reminder that liquid biopsy assays need to be analytically validated and clinically qualified to be endorsed for routine clinical use. Other platforms of CTC characterization or cfDNA analysis, as well as assays for cfDNA-based cancer diagnosis are now at different stages of clinical validation. Some protocols allow co-isolation of CTCs and ctDNA within the same blood sample [88]; however, these are not used in clinical-grade tests.

In addition, research on novel features from liquid biopsy analytes, such as “fragmentomics” (based on ctDNA fragment sizes), tissue-of-origin analysis, and methylation profiling, could potentially be informative in earlier tumor stages. Importantly, since liquid biopsies can reveal a broader landscape of mutations in multiple analytes, integration of these complex multidimensional data into composite biomarkers is a current need and an active area of research in the field.

4. Conclusions

The field of liquid biopsies in PC has advanced exponentially over the last decade, developing prognostic and predictive biomarkers, and holding promise for a minimally invasive means of monitoring tumor evolution. Liquid biopsies could guide therapeutic decisions and accelerate the development of precision medicine in PC. However, issues relating to standardization of assay sensitivity and specificity, prospective clinical qualification of different assays, as well

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as cost and accessibility need to be addressed to endorse their implementation in routine clinical practice.

***Author contributions*:** Joaquin Mateo had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study concept and design: Casanova-Salas, Athie, Mateo.
Acquisition of data: Casanova-Salas, Athie.
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