Lab on a Chip

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The role of liquid biopsies in prostate cancer management

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Liquid biopsy has emerged as a complement to invasive tissue biopsy to guide cancer diagnosis and treatment. The common liquid biopsy biomarkers are circulating tumor cells (CTCs), extracellular vesicles (EVs), and circulating tumor DNA (ctDNA). Each biomarker provides specific information based on its intrinsic characteristics. Prostate cancer is the second most common cancer in males worldwide. In men with low-grade localized prostate cancer, the disease can often be managed by active surveillance. For men who require treatment, the 5-year survival rate of localized prostate cancer is the highest among all cancer types, but the metastatic disease remains incurable. Metastatic prostate cancer invariably progresses to involve multiple bone sites and develops into a castration-resistant disease that leads to cancer death. The need to appropriately diagnose and guide the serial treatment of men with prostate cancer has led to the implementation of many studies to apply liquid biopsies to prostate cancer management. This review describes recent advancements in isolation and detection technology and the strength and weaknesses of the three circulating biomarkers. The clinical studies based on liquid biopsy results are summarized to depict the future perspective in the role of liquid biopsy on prostate cancer management.

Introduction

Prostate cancer is the second most common cancer diagnosed in 2020, accounting for 14.1% of all male cases in men and the fifth leading cause of cancer death worldwide.[1] Incidence rates of prostate cancer rapidly increased during the early 1990s due to the detection of asymptomatic disease through the adoption of the prostate-specific antigen (PSA) test.[2] The 5-year relative survival rate of prostate cancer is the highest (98%) among all cancer types.[2] Prostate cancer incidence declined from 2007 to 2014 in accordance with decreased PSA testing based on the recommendation from the US Preventive Services Task Force due to the concerns about overdiagnosis and overtreatment along with the high false-positive rate of the PSA blood test.[2] The death rate from prostate cancer has dropped in the last decade, but this trend had flattened in recent years (2013-2017), likely because of declined PSA testing, resulting in diagnosing more men when their disease had already metastasized.[2]

The 5-year survival rate in the case of distant disease is 30%.[3] Androgen deprivation therapy (ADT) is the standard treatment for metastatic hormone-sensitive prostate cancer (mHSPC).[4] ADT achieves rapid control of metastatic disease but invariably fails, and men progress to castrate-resistance prostate cancer (CRPC). Despite the recent development of systemic therapies for CRPC improving prostate cancer's survival rate, CRPC is still regarded as an incurable disease.[5]

Liquid biopsy represents a less-invasive method to trace cancer. It has emerged as a candidate to substitute an invasive tissue biopsy for more frequent and accurate sampling of cancer to enable precision medicine for each individual.[6, 7] Many studies have demonstrated that liquid biopsy can help in cancer diagnosis and prognosis as well as to monitor cancer treatment.[8-12] Circulating tumor cells (CTCs), extracellular vesicles (EVs), and circulating tumor DNA (ctDNA) are the most common circulating biomarkers drawn from a liquid biopsy. CTCs are cancer cells that are shed from tumors (primary and/or metastatic) into the vasculature. EVs are nano-sized vesicles, including exosomes and microvesicles, that play a role in intercellular communication through their molecular cargo (proteins, mRNA, microRNAs (miRNAs), and DNA). ctDNA is tumor-derived, short, fragmented DNA found in the bloodstream, reflecting cancer-related genetic changes. Each of these circulating biomarkers has unique characteristics to potentially aid cancer management (**Figure 1).**[13-19]

The PSA test has low selectivity to detect prostate cancer and monitor the disease progression itself.[20-22] Next-generation liquid biopsy utilizing various kinds of circulating biomarkers may be able to provide additional and complementary information to the PSA test and improve the accuracy in cancer diagnosis, monitoring of disease status, and providing personalized treatment options. This review will address the potential implications of liquid biopsy on prostate cancer management and delineate the current challenges and perspectives for the future.

Conventional and Microfluidic technologies for isolation of circulating biomarkers

The efficient isolation of circulating biomarkers is often the first step to perform a liquid biopsy-based clinical study. This section will describe the most widely used and representative microfluidic platform-based isolation methods for circulating biomarkers regarding their technical significance, limitations, and usability in clinical practice.

CTCs

CTC isolation methods can be categorized into label-dependent or label-free, as shown in a few examples summarized in **Table 1**. Label-dependent methods rely on

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affinity-based cell enrichment using tumor-specific antibodies such as epithelial cell adhesion molecule (EpCAM). For instance, the CellSearch® system enumerated EpCAM-positive tumor cells using magnetic nanoparticles, which was first approved by the U.S. food and drug administration (FDA) for the liquid biopsy in 2004.[23, 24] Antibody-conjugated magnetic microbeads,[23-26] microfluidic chips,[27-32] and nanostructured substrates[32] have also been developed for the efficient capture of CTCs. EpCAM capture has been used to develop CTC isolation directly from the bloodstream.[33] On the other hand, the label-free CTC isolation method utilizes the physical characteristics of CTCs from the other blood cells, such as size, density, and dielectric properties. There are various types of label-free CTC isolation technologies, including, but not limited to, size-based membrane filtration,[34-36] size-based microfluidic separation,[37-40] density gradient separation,[41] and dielectrophoresis.[42, 43]

Overall, label-dependent, affinity-based methods have advantages in providing relatively pure CTCs with fewer contaminating blood cells. Despite many clinical studies performed with these methods, inevitable limitations originated from the heterogeneous expression of a cancer-specific surface marker in CTCs. For example, lower EpCAM expressing CTCs may not be isolated as efficiently as CTCs with high EpCAM expression, resulting in poor capture efficiency and a biased interpretation. A cocktail of antibodies[25] or combination with hydrodynamics[28] has been used to overcome the drawbacks with some success.

In general, the size-based CTC isolation methods provide a faster protocol with a higher capture yield and easy-to-use platform. However, the sizes of both CTCs and other blood cells are heterogeneous, influencing the sensitivity and specificity of enumerated CTCs. Tunning the hydrodynamics in a microfluidic device may enhance the purity of size-based CTC isolation.[36, 37, 39, 40] The combination with an affinity-based method increases both purity and yield.[27] A dielectrophoresis (DEP) array chip can be added to the workflow for downstream analysis to retrieve individual cells among the heterogeneous population of CTCs.[42] On the other hand, a 'no isolation' strategy deposits all nucleated cells on glass slides for unbiased profiling of rare cancer cells.[44]

CTC isolation technologies have continuously advanced, providing an acceptable level of isolation and analysis performance, depending on the needs of investigators and physicians. There is still room for improvement. Isolation technology for the future needs to be easy to use and automated for high throughput sample processing. Complete integration from automated sample processing for CTC isolation to the final enumeration and analysis would be highly desired. Furthermore, the CTC isolation methods with unique features (e.g., efficient retrieval of intact live CTCs or sorting CTCs depending upon specific marker expression) may further discover the clinical utility of CTCs.

EVs

Various technologies have been developed to isolate EVs from other particles in biofluids. As summarized in **Table 2**, the differences in density,[45, 46] size,[47-58] surface charge,[59-62] water-solubility,[63, 64] and immuno-affinity[65-71] have been explored to provide EVs in high yield and purity. The most widely used method is ultracentrifugation (UC).[45] However, it requires high G-force and is a labour-intensive operation utilizing expensive equipment with a long process time (> 4 hours) and still results in low yield, purity, and reproducibility. In the density gradient ultracentrifugation (DG-UC) method,[46] purity is improved because protein contaminants are fractionalized into different density layers. However, the density fraction in which EVs accumulate is influenced by the gradient materials and the sample type. Therefore, the yield and reproducibility are still low, and the process is slow and labour-intensive.

Size-based isolation of EVs has also been exploited by combining various kinds of microfluidic technologies. In the size exclusion chromatography (SEC) method,[47] a column packed with porous beads with a pore size smaller than the EVs are used. The bigger particles move faster while smaller particles exit the column slowly. The sample fractions are sequentially eluted, and the faction containing EVs can be collected. The yield and purity of EVs are relatively high, but the samples are often diluted and require additional concentration. Many size-based EVs isolation methods such as ultrafiltration (UF),[48] tangential-flow-filtration (TFF),[49] Exodisc,[50-54] exosome total isolation chip (ExoTic),[55] exosome detection method via the ultrafast-isolation system (EXODUS),[56] etc. provide rapid and easy-to-use operation with higher yield than UC methods. However, contaminating particles of similar size cannot be discriminated.

The differences of the surface charge or dielectric properties are exploited in ion-exchange chromatography (IEC)[59], electrophoresis[60], and DEP[61] chips. Various methods that use hydrophilic polymers,[63] salts,[62] aqueous two-phase solutions,[64] organic solvents,[72] etc. have been developed for easy EV isolation by precipitation. These methods are rapid, cost-effective, and the EV recovery yield is higher than UC. However, the precipitating reagents and protein contaminants often precipitate together and the purity of the EVs samples is low.

The immunoaffinity-based methods can be simple and provide EVs with high purity.[65-71] Though antibodies specific to surface proteins of EVs (e.g., CD9, CD63, CD81) are commonly used, the analysis can be biased to the subpopulation of the particular EVs with positive expression of the specific proteins. Also, antibodies are expensive, and therefore preconcentration may be necessary for the analysis of large volume samples. Since it is known that EVs are highly heterogeneous, further studies are needed to discover the functions of the EV fractions with specific surface proteins.

Recent EV isolation technologies have been developed to improve isolation performance, yield, purity, usability, hands-on procedures, and processing time. The best EV isolation method can be selected depending upon the origin of the sample and downstream applications.[54] Combining EV detection technology with an isolation process is being explored to provide a robust tool for clinical applications.[73] EV isolation technology faces several challenges. Isolating pure EVs

from blood plasma remains problematic because many blood particles are similar in size and density to EVs.[50, 67, 74-79] The number of lipoproteins in blood plasma makes it difficult to isolate pure EVs,[80] affecting the downstream analysis. Also, EVs are released from all cells in the body.[81] Thus, the origin of EVs from normal versus cancer cells may be difficult to determine. Though recent studies have attempted to isolate tumour-specific EVs,[78, 82, 83] the rare portion of tumour-derived EVs may lead to an inaccurate readout in clinical studies. Furthermore, clinical setting-friendly operation and automated analysis platforms need to be developed for large-scale clinical studies.

cfDNA

Cell-free DNA (cfDNA) is short fragmented DNA of approximately 180 base pairs,[84, 85] which is shed from cells and circulates in the bloodstream. The tumour-derived cfDNA is called ctDNA, which may contain the tumour's genetic signature, e.g., point mutations, rearrangements, copy number variations, and methylation patterns.[10, 11, 86] The half-life of cfDNA is short, from few minutes to 2.5 hours,[87, 88] making it difficult to detect a rare target marker. The low concentration of ctDNA within abundant wild-type cfDNA released from numerous blood cells is an additional challenge. Therefore, efficient cfDNA isolation is essential before downstream analysis. The isolation technology of ctDNA is relatively standardized compared to EVs and CTCs. The most widely used method is based on solid-phase DNA extraction[89], and the concentration of chaotropic agents or the pH of buffer solution is optimized to enhance the isolation efficiency of short DNA. The widely used commercial kits for cfDNA isolations include QIAamp Circulating Nucleic Acid Kit (Qiagen), MagMAX™ Cell-Free DNA Isolation Kit (Applied Biosystems), and Plasma/Serum Cell-Free Circulating DNA Purification (Norgen).

A few microfluidic approaches for cfDNA isolation based on a methodology to separate the short DNA selectively enriched cfDNA from longer genomic DNA (gDNA) have recently been demonstrated.[90-92] The detection of genetic aberration via direct hybridization of target sequences in ctDNA is suitable to determine tumour progression.[93-96] The cfDNA isolation workflow has been enhanced to facilitate on-site cfDNA-based clinical studies by minimizing manual handling and reducing the possibility of sample-to-sample cross-contamination.[97-100] ctDNA-based liquid biopsies for companion diagnostics has been adapted in several clinical settings, likely as a result of reproducible and established cfDNA isolation methodology. The remaining challenges include isolating rare tumour alterations in cfDNA as well as reducing background gDNA signals.

Emerging microfluidic technologies in liquid biopsy applications

Each circulating biomarker has its own advantages and limitations to be routinely utilized in clinical settings as shown in **Table 3**. In the previous section, we discussed the pros and cons of state-of-the-art technologies to isolate various circulating biomarkers. Here, we provide our perspectives for future research by discussing a few show-case examples of the latest microfluidic platform technologies that have attempted to address the key remaining challenges in the liquid biopsy field.

CTCs

Tumours are inherently heterogeneous, and the CTCs shed from tumour sites may exhibit even more significant heterogeneity associated with their dynamic changes from epithelial to mesenchymal transition or undergoing therapeutic treatment. A single cell-level analysis is highly desired for sensitive detection and molecular profiling of rare and heterogeneous CTCs. In addition, the current biomarkers such as EpCAM do not fully represent the crucial functions of CTCs. CTCs may survive better through the alliance with other cells in blood circulation, such as immune cells or platelets, which may provide better insights for developing novel theragnostic options. Furthermore, the continuous real-time monitoring of CTCs is beneficial to unveil the dynamic change of CTC phenotypes during cancer therapy. Here, we discuss a few recent studies in CTC analysis that have investigated to unveil tumour heterogeneity using single-cell analysis techniques, to understand better the interaction with other blood cells during blood circulation, and to have real-time monitoring of CTCs undergoing cancer treatment.

Labib *et al.* developed single-cell mRNA cytometry that enabled isolation and detection of CTCs based on mRNA expression using the target mRNA-specific formation of magnetic clusters within the cells followed by on-chip magnetic cell sorting (**Figure 2a**)[101]. A microfluidic chip for multiplexed western blotting for single-CTC protein profiling has also been developed (**Figure 2b**)[102].

CTCs in the bloodstream are associated with non-malignant cells, and these CTC clusters have been proposed to have more significant metastatic potential than single CTCs.[103-106] In conventional CTC enumeration methods, however, the cells expressing epithelial markers such as EpCAM and cytokeratins but not CD45 were counted as CTCs. The dual-positive cells characterized by double staining for both epithelial and CD45 were often neglected. Remarkably, a pioneering study by Szczerba *et al.* used single-cell sequencing and demonstrated the alliance of CTC clusters with neutrophils leads to more efficient metastasis formation in xenograft mouse models (**Figure 2c**).[103]

Despite the importance of the *in vivo* studies using cancer mouse models, longitudinal CTC analysis in mice has been difficult because of the limited sample volume and rarity of CTCs. Hamza and colleagues developed an optofluidic system that can continuously collect CTCs from an unanaesthetised mouse longitudinally (**Figure 2d**).[107] This continuous monitoring system allows the measurement of real-time alteration of tumour signatures within CTCs upon tumour progression and drug response within the same mouse, facilitating future studies on the roles of CTCs in cancer management.

Overall, we believe the single-cell level omics studies to figure out the cellular heterogeneity and inter-cellular

communication would provide a good foundation for future theranostics. In addition, further development of a more user-friendly, robust, and standardized operation of CTC analysis that can be routinely practiced in clinical settings is highly desired.

EVs

EVs are highly heterogeneous in their size, source, molecular composition, and function. Not only tumour cells but also host cells shed EVs of similar size and density. Though the number concentration is much higher for EVs than CTCs, the tumour-specific EVs markers are often unknown in many cancer types. Therefore, there is an unmet need to develop breakthrough technologies for the specific detection/isolation of tumour-derived EVs (tdEV) among a large population of other kinds of nano-sized vesicles. In addition, there is no consensus for the minimum sample volume requirement or normalization methods, which must be dependent on the sample type and isolation methods. As discussed in the previous section, the field is fast evolving, and each EVs isolation technologies have their own advantages and limitations.

Here, we highlight a few emerging EVs analysis technologies that have analyzed single EVs with or without prior EV isolation steps. Using droplet microfluidics or highly sensitive sensors with tumour-specific surface markers or multifaceted cell-to-cell interaction, highly sensitive and specific characterization of tdEVs could be achieved. In addition, the biological role of tdEVs in cancer metastasis was investigated using an organ-on-a-chip.

Utilizing ultrasensitive nanoplasmonic sensors, Liang *et al.* quantified tdEV directly from a small volume (1 μL) of human plasma without an EV isolation step and demonstrated the ephrin type-A receptor 2 (EphA2)-positive EVs outperformed conventional CA19-9 in pancreatic cancer diagnosis and prognosis (**Figure 3a**).[78] Future studies with a larger sample size cohort are required to broaden the clinical utility.

Ko *et al.* recently reported a sequencing-based single EV protein profiling method using droplet microfluidics (**Figure 3b**).[108] This highly sensitive, antibody-based immunosequencing method could unveil specific proteins at the single EV level. However, the prior EVs enrichment and dilution step are required because of the limitation in sample volume that can be analyzed in a microfluidic droplet reactor. In addition, clinical samples with an unknown frequency of tdEVs were not tested yet.

Instead of using antibodies targeting specific surface proteins, Kumar and colleagues focused on a multifaceted cell-to-cell interaction to detect tumour-specific EV (**Figure 3c**).[82] Inspired by the association of CTCs with platelets, they developed a microfluidic chip functionalized with a human platelet membrane and detected specifically captured tdEVs using a small volume (1 μL) of human plasma samples. Although this chip provides benefits, including a purification-free detection of tumorous EVs from blood plasma and high detection sensitivity in a single EV resolution, the heterogeneous nature and stability of platelet membranes need to be considered for further clinical application.

Emerging evidence suggests a crucial role of tdEVs in organotrophic tumour metastasis.[109] Kim *et al.* developed a 3D human liver chip to mimic the premetastatic niche formation by breast tdEVs (**Figure 3d**).[110] The EVs isolated from cancer patients with liver metastasis induced more adhesion of CTCs to the human liver chip than EVs from healthy donors or nonmetastatic cancer patients. The *in vitro* liver-chip model demonstrated that tdEVs induce a reconstruction of the premetastatic niche. However, the associated physiological mechanisms for the roles of tdEVs in organotrophic metastasis need further investigation.

Despite active ongoing research, the currently available EV detection methods are impractical in routine clinical practice. In addition to the development of clinical-setting-friendly EV analysis platforms, novel microfluidic technologies designed to test new hypotheses regarding the roles of EVs in cancer biology have the potential to expand our knowledge on cancer metastasis and drug resistance.

ctDNA

The workflow of cfDNA-based liquid biopsy in the clinical application has been relatively standardized. Nevertheless, it is worthwhile to discuss a few unique approaches related to cfDNA analysis. Das *et al.* developed an electrochemical clamp assay that can detect circulating nucleic acids without enzymatic amplification directly from blood serum (**Figure 4a**).[96] Jin and colleagues presented a new strategy of cfDNA isolation by using a dimethyl dithiobispropionimidate (DTBP)-modified chip (**Figure 4b**).[100] Since this method does not use chaotropic reagents that may affect DNA release from noncancerous cells, it may reduce the background signal. Ou *et al.* developed an integrated system that can combine droplet generation, digital polymer chain reaction (digital PCR), and rapid droplet counting technology, which could improve the detection sensitivity significantly (**Figure 4c**).[111] Perez-Toralla *et al.* developed a droplet digital polymer chain reaction (ddPCR)-based cfDNA detection system by integrating the microfluidic extraction and droplet-emulsification on a chip (**Figure 4d**).[97]

Taken together, the automation and standardization of the workflow of cfDNA-based liquid biopsy will be essential criteria for large-scale clinical studies. Technologies for specific isolation of ctDNA and amplification-free molecular detection are highly desired for broader applications.

Clinical applications of liquid biopsy in prostate cancer

Diagnosis of prostate cancer

Pathology is the gold standard for all cancer diagnosis. To diagnose prostate cancer, though a needle biopsy is inevitable, efforts continue to answer the question of who needs a biopsy and when it is needed.[112] The current diagnostic paradigm for prostate cancer is composed of serum PSA-based screening followed by ultrasound- or other imaging-guided needle biopsies (**Figure 5**). Unlike many cancer types with high mortality, most men who present with prostate cancer are

asymptomatic, particularly in the era of PSA testing, which detects many cancers long before they are clinically apparent. However, about 50% of men who undergo biopsy after PSA screening have negative results.[113] A new diagnostic biomarker/tool that complements or could replace the PSA test for prostate cancer diagnosis should improve specificity without sacrificing sensitivity.

Early-stage or localized prostate cancer presents with very few CTCs, limiting their application in the diagnosis of prostate cancer. Thus, most of the studies of CTCs have focused on prognostic or therapeutic applications in later stages of the disease. In 2007, Nagrath and colleagues reported a microfluidic device, the CTC-chip, that enabled CTC isolation from the whole blood.[114] CTC-chip was applied to isolate CTCs from various cancer types (prostate, lung, pancreatic, breast, colon), and it demonstrated its potential diagnostic value since CTCs were detected from 100% of prostate cancer cases (7 localized, 19 aggressive prostate cancer patients), but none of the 20 healthy donors. This platform is based on antigen-specific isolation. Ozkumur *et al.* demonstrated a microfluidic CTC capture platform integrated inertial focusing module that enables both antigen-dependent and -independent isolation of CTCs continuously.[115] In this research, CRPC patients (n=41) were distinguished from healthy donors (n=13), incorporating to the number of captured CTCs. The median number of CTCs was 3.2 CTCs/ml, with 90% of CRPC patient samples having CTCs above the threshold set using the data from healthy donors. Recently, Ried and colleagues reported a study for prostate-specific diagnostic testing by combining the ISET®-CTC screening blood test and immuno-cyto-chemistry (ICC) with a prostate-specific marker.[116] Twenty-seven male patients who had not undergone cancer diagnosis were selected; among them, 25 men were positive for CTC, and two men were negative. Of the 25 men with CTCs, the 20 men who had CTC with ICC-PSA-positive markers were diagnosed with prostate cancer. Especially in this sub-cohort, the CTC-based diagnosis of cancer by the cytology-based ISET methodology matched the detection of cancer by the standard diagnostic methods. Chalfin and colleagues looked for disseminated tumor cells (DTCs) in bone marrows of localized patients undergoing radical prostatectomy. Evaluation across multiple platforms revealed that epithelial markers are non-specifically expressed in the bone marrow and DTCs were typically not detected in localized prostate cancer patients using prostate-specific markers.[117]

In contrast to CTCs, EVs have beneficial properties to be useful for diagnosis of cancer, including the abundant amount, no biased sampling issue, and well-preserved intravesicular content protected by a lipid-bilayer. Many studies have demonstrated that the biomolecules contained in EVs, including miRNA, mRNA, protein, lipid, were correlated to the presence of cancer, and these studies exhibited a diagnostic power of EV in PCa.

Previously, multiple reviews have introduced the development of ExoDx Prostate (IntelliScore) (EPI), a urine EV RNA expression test, which can predict the likelihood of having high-grade PCa[118, 119]. A prospective phase 1 adaptive utility trial demonstrated that EPI performed better than the optimized model of standard clinical parameters with an area under the curve (AUC) 0.70 versus 0.62. It has been estimated that 26% of unnecessary prostate biopsies can be avoided using an EPI cut-point of 15.6, with a negative predictive value of 89% and missing 7% of Grade group 2 PCa.[120] In 2019, it became the first EV-based liquid biopsy test granted a Breakthrough Device Designation by the US Food and Drug Administration (FDA). It is now available for clinical use in the United States. One thing to be noted is that this platform utilized the Exosome Diagnostics (St Paul, MN) EXOPRO Urine Clinical Sample Concentrator Kit to separate EVs. Given the various EV purity among different EV separation technologies, further exploration of whether the diagnostic molecules are carried by EVs or just co-separated with EVs is needed.[54, 121] Bhagirath and colleagues demonstrated miRNA-1246 might be a potential diagnostic biomarker for prostate cancer.[122] This study demonstrated that the level of urinary exosomal miRNA-1246 was higher in the group of patients, while the level of miRNA-1246 extracted from tumor tissues and cell lines was lower. Rodriguez *et al.* performed next-generation sequencing (NGS, deep sequencing) and real-time quantitative polymer chain reaction (RT-qPCR) with an independent patients cohort using miRNA samples extracted from urinary EVs of PCa and healthy controls.[123] The NGS analysis showed five miRNA (miR-196-5p, miR-34a-5p, miR-143-3p, miR-501-3p, and miR-92a-1-5p) were significantly less abundant in the group of prostate cancer patients (n=20) than healthy donors (n=9). Among five miRNA, miR-196-5p ad miR-501-3p were confirmed by RT-qPCR to be lower in PCa samples from a different patient cohort, PCa (n=28), healthy doner (HD, n=19). Worst *et al.* also recently exhibited the potential utility of exosomal miRNA as a diagnostic marker.[124] In this research, the miRNA expression profile showed miR-10a-5p ($p = 0.018$) and miR-29b-3p ($p = 0.002$), but not miR-99b-5p, were higher in plasma-derived EVs from patients with PCa compared with benign prostatic hyperplasia (BPH) controls.

Exosomal miRNA has been widely studied as the diagnostic biomarker since EVs are reported to contain more miRNA than the other RNA molecules such as mRNA. The application of miRNA as a diagnostic marker has been limited due to lack of specificity. Thus, many studies have emerged to investigate EV-mRNA as a diagnostic and prognostic biomarker for prostate cancer management. For instance, Woo *et al.* demonstrated the performance of GATA2-mRNA to distinguish PCa patients (n=117) from healthy donors (n=48) among GATA2, PCA3, and TMPRSS2-ERG (GAPT-E) tested in the cohorts.[125] The other biomolecules contained in EVs were also investigated to determine their value in diagnostics. Sunkara *et al.* reported several protein markers (PSA, PSMA, HSP90, EpCAM, EGFR1) that were higher in PCa patients (n=43) compared to healthy donors (n=30).[51] Among them, HSP90 showed the highest correlation to distinguish the patients from the healthy donors. Skotland and colleagues performed a lipidomic analysis of urinary exosomes in prostate cancer patients (n=15) and healthy controls (n=13) and reported the combinations of the lipid species, phosphatidylserine (PS) 18:1/18:1,

lactosylceramide (d18:1/16:0), and PS 18:0-18:2 distinguished the two groups with 93% sensitivity and 100% specificity.[126]

cfDNA has been widely studied as a potential diagnostic marker since it has several benefits from its intrinsic properties, such as being less affected by the spatial heterogeneities of cancer. Theoretically, cfDNA could enable a diagnosis of cancer with a 100% detection rate since tumor cells secrete or release cell-free DNA. Particularly, with the detection technologies for analyzing cfDNA rapidly advancing, the role of cfDNA in the early diagnosis of cancer has the potential to become more significant as the detection limit of cfDNA becomes more sensitive and specific.

The clinical study by Constancio and colleagues demonstrated that DNA methylation in cfDNA might enable less invasive screening of PCa.[127] The PCa panel (FOXA1me, RARβ2me, RASSF1Ame, and GSTP1me) depicted 72% sensitivity and specificity in terms of PCa detection. Brait *et al*. studied the diagnostic power of the selected gene combinations for Pca, which resulted in a combination DNA methylation marker panel consisting of MCAM, ERα, ERβ offered 75% sensitivity and 70% specificity.[128] Especially, the MCAM marker in cfDNA distinguished between early-stage PCa cases (T1C, n= 60) and healthy donors (n=30) with 66% sensitivity and 73% specificity, suggesting the epigenetic markers exhibited in cfDNA may be an early diagnostic marker for PCa.

Not only blood samples but also urine samples can be used for circulating DNA analysis. Brikun and colleagues demonstrated the DNA methylation markers in urine cfDNA, may be useful for PCa diagnosis.[129, 130] The predictive performance of 32 positive markers was tested in terms of PCa detection sensitivity and selectivity. The result demonstrated that the use of 10 of 32 positive markers showed the best performance for PCa diagnosis, with yields of 93% of sensitivity and 77% of specificity from first voided (FV) urine.

Besides, a cell-free RNA such as miRNA in blood has also been widely studied to find the efficient diagnostic marker for PCa. For example, Urabe and colleagues demonstrated that the combination of two miRNAs (miR-17-3p and miR-1185-2-3p) showed 90% of both sensitivity and specificity.[131] In detail, the discovery cohort (41 PCa, 41 NPBx (negative prostate biopsy), 41 HD) was used for identifying 18 candidate miRNAs, and the training cohort (384 PCa, 100 NPBx) and the validation cohort (384 PCa, 100 NPBx) were used to investigate the best model for diagnosis of PCa.

To summarize, studies utilizing liquid biopsies to improve the diagnosis of prostate cancer have mainly focused on differentiating cancer from non-cancer, which is the first step of developing a diagnostic tool (**Table 4**). However, biomarkers that cost-effectively improve PSA performance are still needed.

Prognosis of prostate cancer

For any patient who is diagnosed with PCa, clinical management decisions are often made based on a determination of risk. It is known that some patients with the indolent disease may not need any treatment but can be safely followed with active surveillance (AS) strategies. Other patients may need intervention, but their disease can still be cured by local therapy alone (radical prostatectomy or radiotherapy), while a small group of patients will inevitably recur despite optimal local therapy and eventually die of PCa.[132, 133] It the ultimate goal of any prognostic model to determine which risk group a patient belongs to enable the appropriate treatment of patients with aggressive disease as early as possible while preventing overtreatment from those with indolent diseases. The current risk stratification systems are based on several key clinical and pathological features, including clinical tumor stage (cT stage), Gleason score (GS), serum PSA, etc.[112] However, there is still significant heterogeneity in prognosis in the same patient population using different published schemes.[133, 134] The potential reasons include, but are not limited to, the inherent inaccuracy in determining cT stage and GS, as well as the limited volume of tissue samples obtained from a conventional biopsy which may be insufficient to reveal the tumor heterogeneity.[135, 136] In recent years, many efforts have been made in the liquid biopsy field to overcome these shortages and explore better ways to predict the prognosis of PCa (**Table 5**).

In PCa, most of the CTC studies have been conducted among patients with metastatic disease and/or CRPC with or without treatment.[137] And CellSearch is still the only FDA-approved test for CTCs to monitor treatment in men with PCa. For example, de Kruijff and colleagues demonstrated that the baseline CTC count was independently associated with poor progression-free survival (PFS) and overall survival (OS) in metastatic castrate-resistance prostate cancer (mCRPC) patients treated with cabazitaxel.[138] Heller and colleagues evaluated the added value of CTC numbers to a standard prognostic model containing albumin, lactate dehydrogenase (LDH), PSA, hemoglobin, and alkaline phosphatase (ALK) for mCRPC patients and found adding CTC enumeration produced a more accurate prediction of OS.[139] Besides CTC numbers, the RNAs or proteins in CTCs have also been widely studied, and several CTC-based molecular markers (i.e. EGFR, PSMA, WNT5a, AURKA, BMP7, synaptophysin, and EMT phenotypic markers, etc.) have been found associated with prognosis of patients with mPCa.[140-144]

It would be beneficial to determine the prognostic value of CTCs in patients without clinical metastasis. Xu and colleagues found the presence of CTCs in localized PCa before treatment was significantly associated with a higher GS, risk group and clinically significant PCa.[145] Miyamoto and colleagues established a CTCL score by digital quantitation of prostate-derived transcripts in CTCs, the preoperative elevation of which can predict microscopic cancer dissemination to seminal vesicles and/or lymph nodes in patients with localized PCa.[146] Pak and colleagues demonstrated that CTCs were detected in 36% of patients with undetectable serum PSA after surgery and the postoperative CTC detection was independently associated with an increased risk of biochemical recurrence (BCR).[147] Similarly, Friedlander and colleagues found among patients with high-risk localized PCa, the ones with detectable postoperative CTCs showed a trend toward shorter recurrence-free time.[148] One common challenge in studying CTCs in non-metastatic patients is that both the detection rates and

detected CTC numbers based on a limited volume of a peripheral blood sample could be very low.[149, 150] To solve this problem, diagnostic leukapheresis has been used as a new sampling method, and *in vivo* CTC capture devices have also been developed. Both strategies have made it possible to reliably and comprehensively study CTCs in patients with relatively low tumor burdens.[151-154]

The advantages of EVs as PCa biomarker are that they can be released by all living cells, regardless of the disease stage and tumor malignancy, and that urine, especially post-digital rectal examination (DRE) urine, is an ideal sample type enriched in PCa derived EVs.[119, 155-157] Connell and colleagues generated four prostate urine risk (PUR) signatures based on post-DRE urine-derived EV RNA profiles.[158] In their validation cohorts, PUR-4 status predicted the presence of clinically significant intermediate- or high-risk disease (AUC = 0.77, 95% CI 0.70–0.84). In a separate AS cohort, PUR-4 group had a significant association with time to progression (IQR HR 2.86, 95% CI 1.83–4.47; P < 0.001). The other urine-based EV RNA test that received the Breakthrough Device Designation more recently is the Sentinel™ Prostate Test, which utilizes the urinary EV small non-coding RNAs (sncRNAs) for diagnosis and risk stratification with high sensitivity and specificity.[159] Besides urine, the prognostic value of plasma EVs has also been studied. Joncas and colleagues found that detectable levels of AR-V7 mRNA in plasma EVs isolated from patients with CRPC were associated with a shorter time to progression.[160] One thing to be noted is that despite satisfactory clinical validation, there is a diversity of EV separation methods used in these studies. Given the various EV purity among different EV separation technologies, further exploration of whether the prognostic molecules are carried by EVs or just co-separated with EVs is needed.[54, 156]

Similar to CTCs, it is challenging to obtain sufficient ctDNA from patients with localized PCa. Hennigan and colleagues demonstrated that ultra-low-pass whole-genome sequencing (WGS) and targeted resequencing did not detect ctDNA in plasma acquired before surgery or before recurrence in 112 localized PCa patients.[161] In metastatic PCa, Choudhury and colleagues developed a method using a computational tool to estimate tumor fractions in cfDNA through copy number alterations detected by sparse WGS.[162] They found the tumor fraction was associated with metastasis location, positively correlated with ALK and negatively correlated with hemoglobin in patients with CRPC. Kohli and colleagues found ctDNA-based TP53 mutations, RB1 loss, and AR amplification correlated with poorer survival in mCRPC, and mutations in multiple DNA repair genes (ATM, BRCA1, BRCA2, CHEK2) were associated with shorter time to ADT failure and survival in mCSPC.[163] Sonpavde and colleagues demonstrated a higher number of ctDNA alterations, ctDNA-based AR alterations, and amplification of MYC and BRAF were associated with worse failure-free survival and/or OS.[164] Epigenetic changes/markers in ctDNA have also been explored.[165, 166] Beltran and colleagues demonstrated that ctDNA methylation was reflective of methylation patterns observed in biopsy tissues and was capable of detecting CRPC neuroendocrine differentiation-associated epigenetic changes.[166] However, even in metastatic PCa, the congruence among samples from the same patient tested by different platforms has brought up some concerns. Torga and Pienta demonstrated very low congruence for same patient-paired samples in 2 CLIA-certified commercially available tests, which implies that patients could potentially receive different treatments depending on the cfDNA platform and patients may need a significant tumor burden to have reliable results.[167] Besides cfDNA, cfRNA has also been studied as prognostic markers for PCa. Souza and colleagues evaluated the mRNA levels of eight genes in preoperative cell-free plasma samples from 60 localized PCa patients by RT-qPCR.[168] They found the combination of GOLM1, NKX3-1 and TRPM8 was able to identify high risk PCa cases (sensitivity=85%, specificity=58%), yielding a better overall performance compared with the biopsy GS and serum PSA.

Precision medicine and treatment response monitoring

Compared to a decade ago when docetaxel was the first and only life-prolonging agent for mCRPC, the "armamentarium" now holds many new weapons against mPCa. Several next-generation hormonal agents (abiraterone, enzalutamide, apalutamide, etc.), cabazitaxel, and sipuleucel-T have been proved to prolong the OS of mPCa.[5, 169] Radium-223 dichloride (radium-223) is used in symptomatic patients with bone metastases but no known visceral metastases.[170] More recently, poly (adenosine diphosphate ribose) polymerase (PARP) inhibitor (olaparib, rucaparib, etc.) and immune checkpoint inhibitor (ICI) have been used in selected patients with certain genetic features.[171-173] However, despite the robust progress in therapeutics development, mPCa remains incurable. Due to the tumor heterogeneity, which inevitably leads to the primary and secondary resistance to all these therapeutics, each of these agents can only benefit a certain subgroup of patients for a certain period of time.[5] In this case, it is critical to predict and monitor the treatment response for PCa patients in the metastatic phase (**Table 6**).

The dynamic changes of CTC quantity have been widely adopted as a way to evaluate the treatment response or disease progression ("CTC response" and "CTC progression") in clinical trials of new therapeutics for mPCa.[174-176] Lorente and colleagues found in mCRPC patients with baseline CTCs <5 treated with abiraterone or chemotherapy, the increase of CTC number during the first 12 weeks of treatment was associated with significantly worse OS, independent of baseline CTC numbers and established clinical variables.[177] Similarly, Heller and colleagues evaluated the value of CTC number decrease in assessing treatment response in five prospective randomized phase 3 trials that enrolled a total of 6,081 patients. They found both CTC0 (≥1 CTC at baseline, 0 at week 13) and CTC conversion (≥5 CTCs at baseline, ≤4 at week 13) are robust and meaningful response endpoints for early-phase mCRPC clinical trials, and they have higher discriminatory power for OS than serum PSA.[178] Bono *et al.* reported that the FDA-approved test for CTCs to predict prognosis and monitor treatment response in patients with CRPC.[137] Patients in unfavorable CTC (≥ 5 CTC/7.5mL at baseline) group, 125 among 219 (57%) had shorter OS (median OS, 11.5 versus 21.7 months; Cox hazard

ratio, 3.3; P < 0.0001). While many NGS-based cfDNA tests are commercially available, there are system-dependent variability and standardization is necessary to ensure clinical benefit of personalized medicine. For example, Torga et al. reported very low congruence for the paired samples from the same patients in 2 CLIA-certified commercially available test.[167] Therefore, despite potential benefits of liquid biopsy tools, patient's clinical features (e.g. response evaluation criteria in solid tumors [RECIST]) and radiological assessment (e.g. bone scan) are still considered much more important.

Besides monitoring treatment response, since CTC and ctDNA are reflective of molecular features of the tumor, they have emerged as minimally invasive tools to predict the treatment effect, potentially guiding personalized precision medicine. Lack and colleagues performed exome sequencing of tumor tissue and CTC samples and found 62% of CTC mutations were shared with castrate-resistant disease, either alone or with treatment-naïve disease.[179] Similarly, Faugeroux and colleagues also observed shared somatic mutations between CTCs and matched metastasis biopsies, and found a small number of CTCs were sufficient to represent 1/2 to 1/3 of the mutations in the matched metastasis biopsy.[180] Wyatt and colleagues performed targeted sequencing across 72 clinically relevant genes in 45 plasma cfDNA samples and demonstrated that 75.6% of cfDNA samples had a ctDNA proportion greater than 2%, in which all somatic mutations identified in matched metastatic tissues were concurrently present.[181] Vandekerkhove and colleagues conducted a similar ctDNA analysis among patients with mCSPC and reported the concordance for mutation detection in matched samples was 80%.[182]

Androgen receptor splice variants (AR-Vs) are one of the key mechanisms contributing to abiraterone and/or enzalutamide resistance. Many alternatively spliced AR-Vs lack the C-terminal ligand-binding domain, but retain the transactivating N-terminal domain, leading to constitutive activation in the absence of ligands.[183] Since 2014 when Antonarakis and colleagues first reported a significantly worse outcome in patients treated with abiraterone or enzalutamide who harbored AR-V7 in their CTCs, AR-V7 has been the most promising and well-studied biomarker in liquid biopsy.[184] The original assay was based on the AdnaTest, followed by RT-qPCR. In recent years, Scher and colleagues demonstrated CTCs expressing AR-V7 protein localized to the nucleus could also identify mCRPC patients with an improved OS on taxane-based chemotherapy relative to abiraterone and enzalutamide.[185] In a further multicenter validation study, the authors showed in high-risk mCRPC patients with positive nuclear-localized AR-V7 in CTCs, the patients treated with taxanes had superior OS relative to those treated with AR signaling (ARS) inhibitors (median OS, 14.3 vs. 7.3 months; hazard ratio, 0.62; 95%CI, 0.28-1.39; P = .25).[186] In a multicenter, prospective blinded study (the PROPHECY study), Armstrong and colleagues validated the prognostic significance of baseline CTC AR-V7 by using both the AdnaTest CTC AR-V7 mRNA assay and the Epic Sciences CTC nuclear-specific AR-V7 protein assay. The results showed the AR-V7 detection by both assays was independently associated with shorter PFS and OS, and the observed percentage agreement between the two AR-V7 assays was 82%.[187] Besides CTCs, AR-V7 has also been detected in EVs. Del Re and colleagues assessed AR-V7 in plasma-derived EVs by highly sensitive ddPCR and found median PFS was significantly longer in patients with AR-V7$^-$ EVs compared to those with AR-V7$^+$ EVs (20 vs. 3 mo, p < 0.001).[188] Strati and colleagues compared the expression patterns of AR-FL, AR-V7, and AR-567es in CTCs and paired plasma-derived EVs in patients with mCRPC and found all AR variants were expressed in higher levels in CTCs than in paired EVs (AR-FL: 92.3% in CTCs vs. 76.9% in EVs; AR-V7: 49.3% in CTCs vs. 7.7% in EVs; AR-567es: 23.2% in CTCs vs. 3.8% in EVs).[189, 190] Woo *et al.* detected higher AR-V7 and lower AR-FL expression in urinary EVs isolated from 4 mL of urine of patients with CRPC (n=14) than patients with hormone-sensitive prostate cancer (n=22).[52]

Recent years have witnessed an increasing and widespread valuation of ctDNA in PCa precision medicine. Multiple studies demonstrated that a decline in cfDNA concentration was independently associated with a better outcome among mCRPC patients treated with either taxane-based chemotherapy, ARS inhibitor, or PARP inhibitor.[191-193] Several studies found *AR* and *PIK3CA* amplification were associated with primary resistance to ARS inhibitors and poor outcome.[193, 194] Somatic alterations in *TP53*, previously linked to reduced tumor dependency on AR signaling, were also independently associated with rapid resistance to abiraterone and enzalutamide.[195] mCRPC patients harboring homologous recombination repair (HRR) defects are good candidates for PARP inhibitors, and secondary resistance can be associated with HRR restoration, including *BRCA2* mutation reversion.[196, 197] Multiple studies have demonstrated that many tumor tissue somatic DNA repair mutations, as well as post-treatment mutation reversion can be detected in cfDNA, supporting the role of cfDNA-based liquid biopsies as a predictive and resistance monitoring biomarker in mPCa treated with PARP inhibitor.[192, 198] On the other hand, previous studies have found PCa harboring DNA mismatch repair defects (MMRd) and mutational loss of cyclin-dependent kinase 12 (CDK12) may respond favorably to ICIs.[199-201] Recently, multiple studies have demonstrated the potential role of ctDNA in reliably detecting these genetic features, thus guiding the patient selection for immnunotherapy using a minimally invasive liquid biopsy tool.[202, 203]

Conclusion and outlook

The current diagnostic paradigm and treatment strategies for PCa has been depicted in **Figure 5**. Though liquid biopsy has shown great potential to improve the current practice, there are still many unmet needs. To complement the PSA test for prostate cancer screening, new assays with high specificity are needed. After PSA screening, the key challenge is how to further select the best candidates for needle biopsy in order to avoid such an invasive test in non-cancer population. Once PCa has been diagnosed, new assays for risk stratification are needed to complement the current model. For patients with

low-risk disease, AS may be the best option, but both physicians and patients need more confidence in defining who has "real" low risk disease and when to switch to definitive treatment. For the patients who receive definitive therapies to treat their primary tumors, there is an approximately 70% of chance to be cured, while the remaining patients will eventually develop BCR. New tests are needed to help predict the 30% who may benefit from more intensive early treatment. Even though metastatic PCa is still incurable, successful personalized treatment can prolong a patient's life. However, since an increasing number of new drugs and reagents have been approved in recent years, more precise and detailed assays are needed to help select the best treatment and monitor the response for patients with mPCa.

Besides that most of the tests mentioned in this review need to be further validated by well-designed clinical trials, there are some common limitations need to be aware of. First, the sample sizes/patient numbers in many liquid biopsy studies are very small (e.g. n<50). It is understandable that it is difficult to try a new platform on a large cohort directly, but this reminds us that we need to be very cautious when we interpret the data and findings. Secondly, though many studies emphasized that cfDNA and EVs had a much better application in diagnosis/prognosis than CTCs because of their high abundance, the real proportion of cancer-derived cfDNA or EVs in the circulation remains unknown, which is related to the key question, specificity, especially for the diagnosis of prostate cancer. For both CTC and EV studies, it is important to mention the separation methods when reporting the data, due to the huge diversity and a lack of consensus in their separation. Several studies have demonstrated that some EpCAM-positive cells in the circulation may be irrelevant to cancers, as well as that a large proportion of separated "EVs" may be other components (e.g. lipoproteins) from the biospecimens[54], which, to some extent, explains the suboptimal reproducibility of liquid biopsy research.

Despite the fact that a large number of microfluidic devices have been developed for the liquid biopsy application, the conventional methods are still preferred and used for most clinically relevant studies, as described in the clinical application sections of this manuscript. The microfluidics field should be aware that the clinical field has no choice but to be cautious about using a new platform that is not yet fully validated and standardized. In addition, CellSearch is still the only FDA-approved liquid biopsy test for PCa, though the ExoDx Prostate EPI test was granted in 2019 as a Breakthrough Device Designation by the FDA. Although emerging microfluidic technologies have recently come close on the stage, the microfluidic device and clinical fields need to keep closely collaborating together toward the same goal, to overcome prostate cancer as well as all cancers.

Author Contributions

C.-J. Kim and L. Dong contributed equally to this work. C.-J. Kim, L. Dong, S. R. Amend, Y.-K. Cho, and K. J. Pienta conceived the research and prepared the manuscript. All authors read and corrected the manuscript.

Conflicts of interest

Y.-K. Cho is an inventor of the patents on CD-CTC™ and Exodisc, which are licensed to Clinomics (Ulsan, Korea) and Labspinner (Ulsan, Korea), respectively. All other authors have nothing to disclose.

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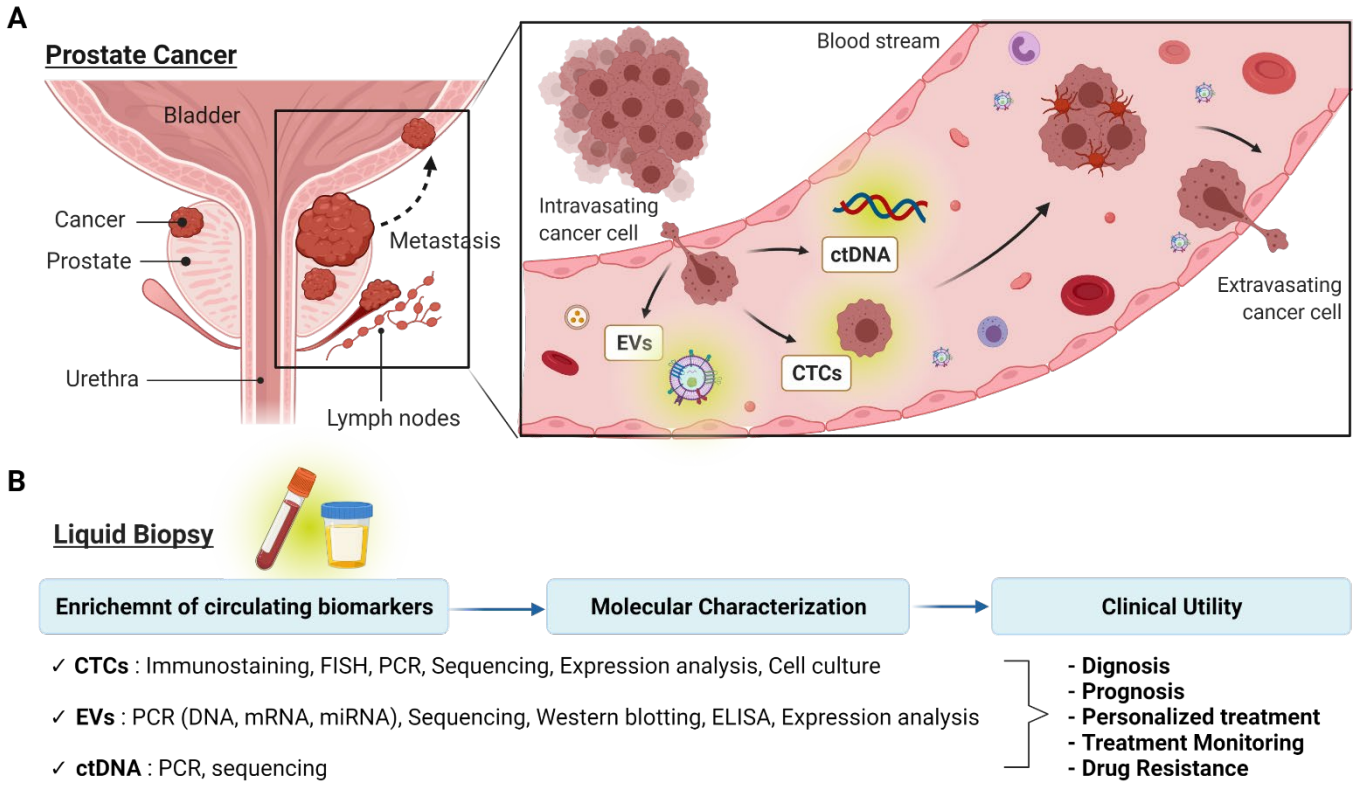

Figure 1. Schematic illustration of the liquid biopsy in prostate cancer management. (A) Circulating tumor cells (CTCs), extracellular vesicles (EVs), and circulating tumor DNA (ctDNA) are found in bloodstream and used for liquid biopsy biomarkers. **(B)** Molecular analysis of various kinds of circulating biomarkers can provide precision medicine for highly personalized disease management.

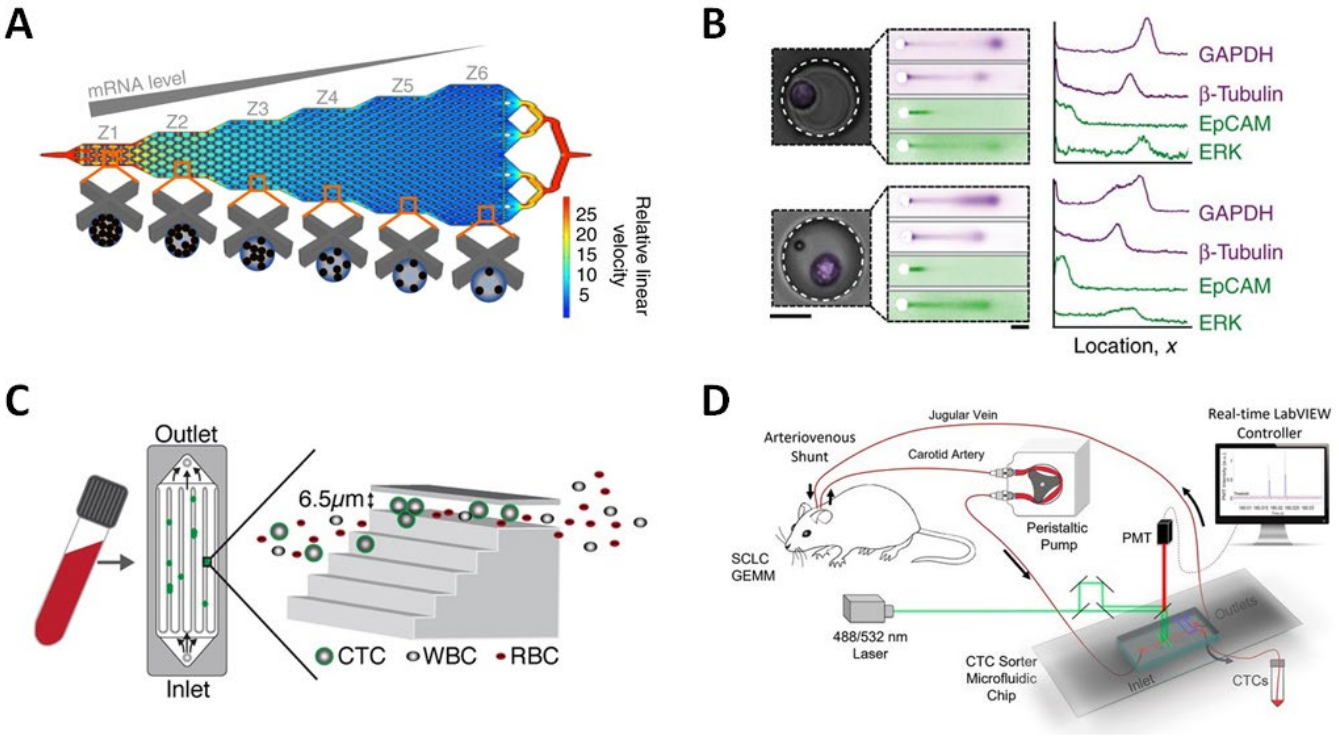

Figure 2. Emerging microfluidic technologies in liquid biopsy application. (A) Single-cell mRNA cytometry,[101] **(B)** Multiplexed single-cell western blotting on a chip,[102] **(C)** Application of Parsortix® to study the alliance of CTC clusters with neutrophils affecting to metastasis formation,[103] **(D)** Optofluidic system to continuous CTC isolation and simultaneous target CTC sorting.[107] Panels A,B,C were adapted with permission from Springer Nature. Panel D was adapted under the copyright (2019) National Academy of Sciences.

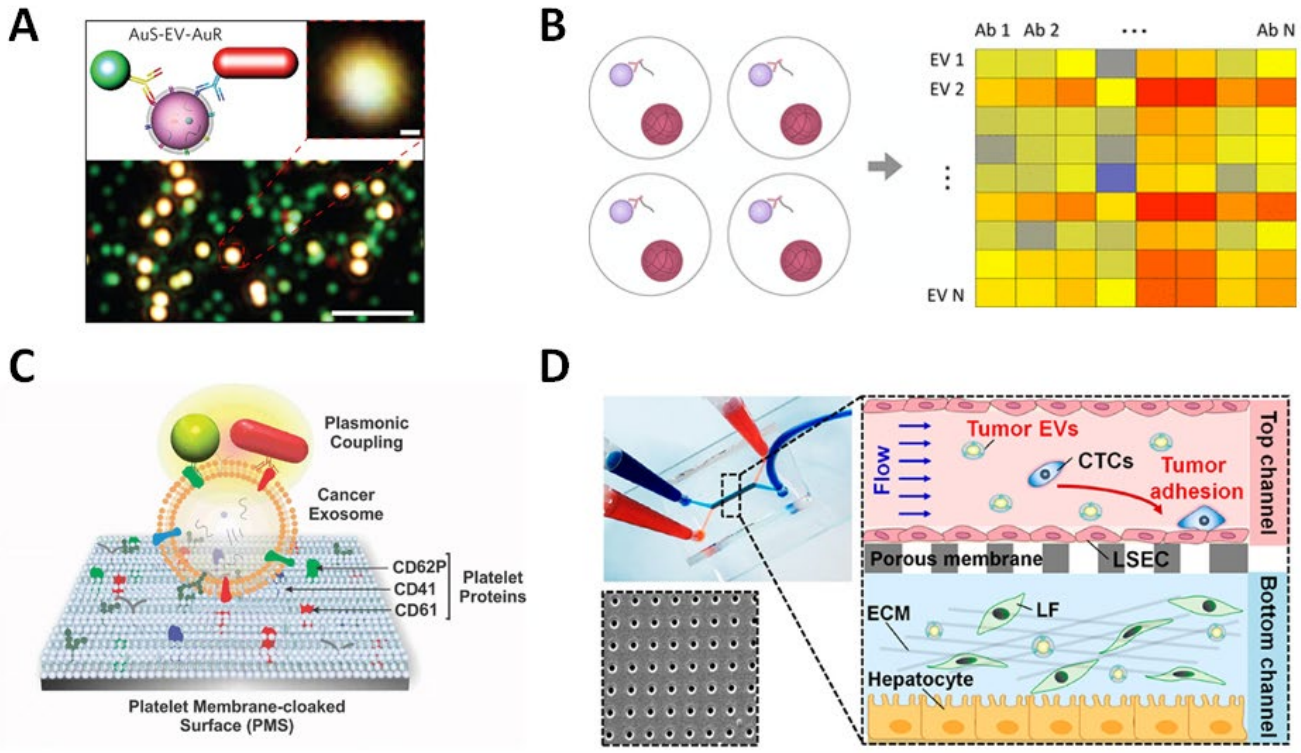

Figure 3. Emerging microfluidic technologies in liquid biopsy application. (A) Nanoplasmon-enhanced scattering (nPES) assay for the detection of the plasma-derived tdEV,[78] **(B)** Sequencing-based single EV protein profiling method using droplet microfluidics,[108] **(C)** Microfluidic chip functionalized with human platelet membrane for the tumour-specific EV detection, [82] **(D)** Three-dimensional human liver chip to mimic the premetastatic niche formation by breast tdEVs.[110] Panels A was adapted with permission from Springer Nature. Panel B and D were adapted with permission from ACS Publications. Panel C was adapted with permission from John Wiley & Sons.

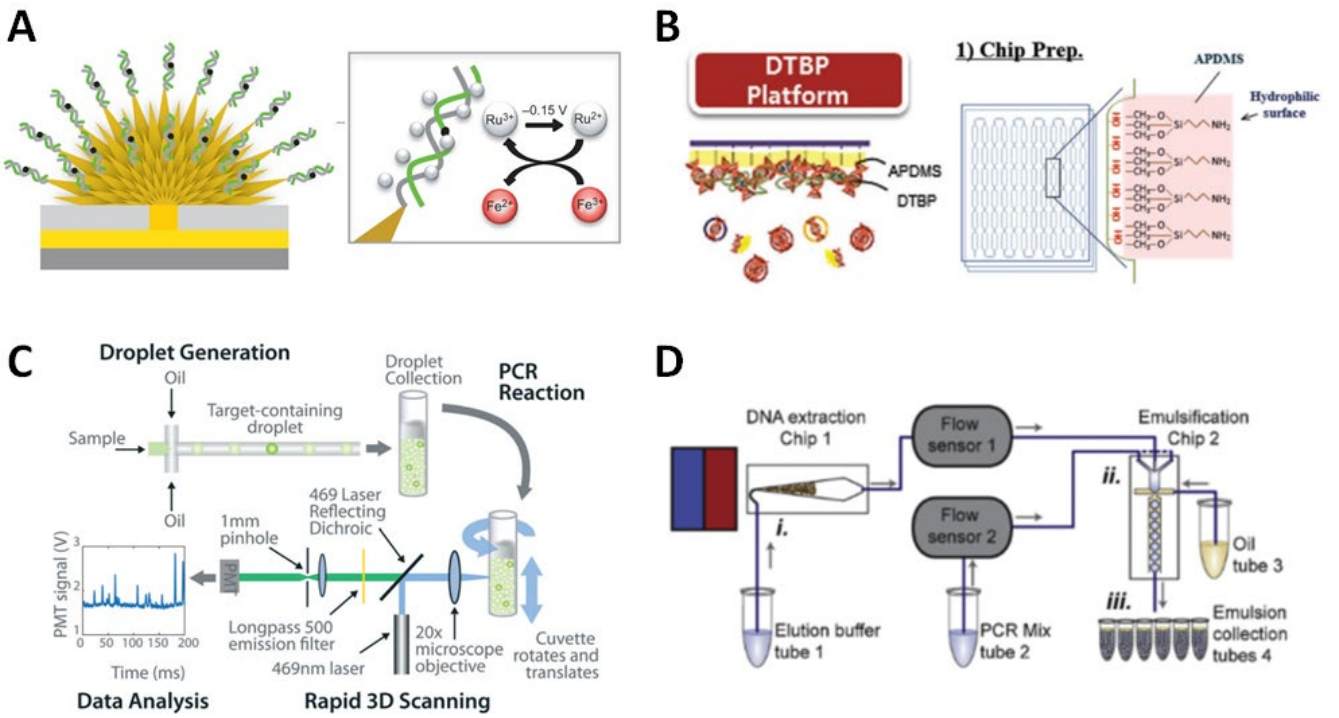

Figure 4. Emerging microfluidic technologies in liquid biopsy application. (A) Electrochemical clamp (PNA clamps) assay using a chip-based sensor for circulating nucleic acid detection,[96] **(B)** Dimethyl dithiobispropionimidate (DTBP) based cfDNA isolation,[100] **(C)** Integrated comprehensive droplet digital detection (IC3D) digital PCR system,[111] **(D)** ddPCR-based cfDNA detection system by integrating the microfluidic cfDNA isolation and droplet-emulsification on a chip.[97] Panels A was adapted with permission from Springer Nature. Panel B was adapted with permission from John Wiley & Sons. Panel C was adapted with permission from Royal Society of Chemistry. Panel D was adapted with permission from Elsevier.

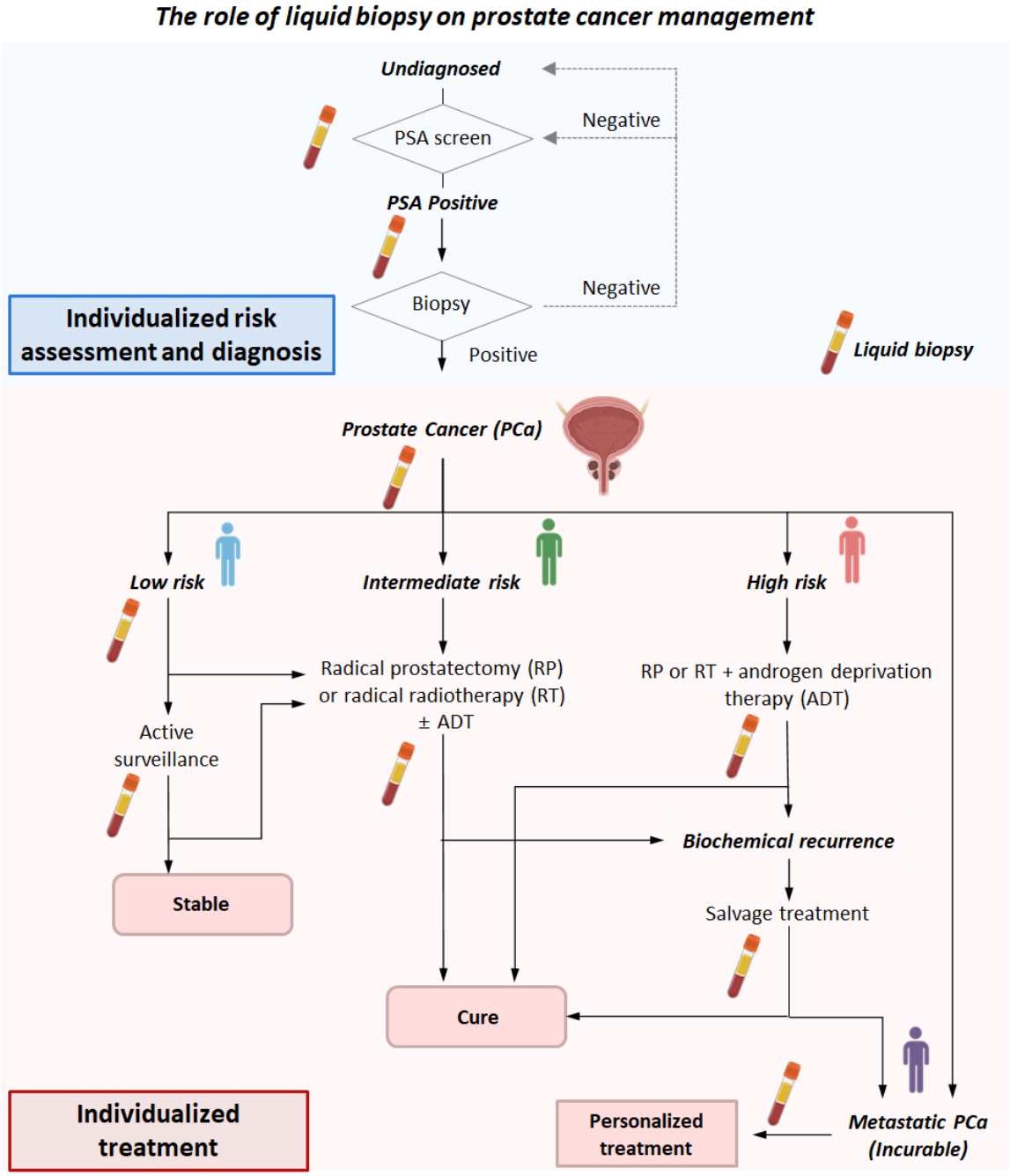

Figure 5. The potential role of liquid biopsy on prostate cancer management. In the diagnostic phase, to complement PSA test for prostate cancer screening, new assays with high specificity are needed. After PSA screening, liquid biopsy can potentially help further select the best candidates for needle biopsy in order to avoid such an invasive test in non-cancer population. After the prostate cancer has been diagnosed, liquid biopsy-based new assays for risk stratification can complement the current model. For patients with low-risk disease, active surveillance is the best option. Liquid biopsy can potentially help answer the question of who has "real" low risk disease and when to switch from active surveillance to definitive treatment. For the patients who receive definitive therapies to treat their primary tumors, there is an approximately 70% of chance to be cured, while the remaining patients will eventually develop biochemical recurrence. Liquid biopsy-based assays are needed to help predict that "30%", thus more intensive or systemic treatment can be given as early as possible. At last but not least, metastatic prostate cancer is still incurable, however, successful personalized treatment can largely prolong patient's life. More precise and detailed assays are needed to help select the best treatment and monitor the response for patients with metastatic prostate cancer.

Table. 1. Examples of commercially available CTC isolation platforms

Methodology	Device (Company)	Isolation principle	Remarks	Ref.
Label-dependent CTC isolation technologies				
Magnetic particles	CELLSEARCH® (Menarini-Silicon Biosystems)	Immunomagnetic sorting	EpCAM-coated ferrofluid nanoparticles; FDA approved	23, 24
	AdnaTest (Qiagen)	Immunomagnetic sorting	CTC enrichment by Ab[a] cocktail (EpCAM, MUC-1, etc.) coated microbeads followed by RT-PCR	25
	MACS system (Miltenyi Biotec)	Immunomagnetic sorting	CTC enrichment or depletion of leukocytes using Abs[a]-coated microbeads and flow cytometry analysis	26
Microfluidic chips	CTC-iChip (Janssen Diagnostics, in progress)	Size + Immunoaffinity (DLD[b]-inertial focusing-magnetophoresis)	Positive and negative enrichment; High yield (97%) and purity (3.8-log depletion of WBC)	27
	IsoFlux™ (Fluxion)	Immunomagnetic sorting	Complete kits for enumeration and NGS are available.	28
	Modular Sinusoidal Microsystems (BioFluidica)	Plastic chip with sinusoidally-shaped-channels coated with Abs[a]	Can release captured CTCs; electrical sensor for single cell counting	29
	OncoCEE (Biocept)	Irregular arrangement of multiple sized posts coated with Abs[a]	Combinational analysis of CTC and ctDNA	30, 31
	CytoTrapNano™ (CytoLumina)	Nanostructured substrates coated with Abs[a] (NanoVelcro chip)	High efficiency CTC enumeration when coupled with chaotic mixer; can release captured CTCs	32
In-vivo	GILUPI CellCollector.	Medical stainless steel wire with Abs[a]-coated polymer tip	Target cell capture in vivo directly from the bloodstream	33
Label-free CTC isolation technologies				
Size-based membrane filtration	ISET® (Rarecells Diagnostics)	Micropore membrane filtration	Immuno-theranostics; Incubation with dilution buffer followed by filtration; High detection sensitivity (1 CTC / 10 mL of blood)	34
	ScreenCell® Cyto	Micropore membrane filtration	Incubation with filtration buffer followed by filtration; filter can be released onto a glass slide	35
	CellSieve (Creatv MicroTech)	Micropore membrane filtration	Precise pore size and distribution for high capture efficiency and lower background contamination	36
Size-based microfluidic separation	ClearCell® FX (Clearbridge BioMedics)	Dean flow fractionation in a spiral microfluidics system	Isolation of intact CTCs; After RBC lysis step, nucleated cells are loaded on the chip for size-based separation	37
	Parsortix® technology (Angle plc)	Trapping cells in steps with 10 µm height	Size and compressibility-based separation	38
	CD-CTC™ (Clinomics)	Tangential flow filtration on a disc with fluid-assisted separation technology (FAST)	Whole blood, ultrafast (> 3 mL/min), clog-free, low pressure drop	39
	VTX-1 (Vortex Biosciences)	Inertial microfluidics using laminar microscale vortices to isolate CTCs	Fully automated system for CTCs isolation directly from whole blood	40
Density gradient separation	AccuCyte–CyteFinder (RareCyte)	Density-based collection of nucleated cells to slides	No-wash; no-lysis collection of blood cells to slides	41
DEP[3]	DEPArray™ system. (Menarini-Silicon Biosystems)	DEP[c] cages for trapping, manipulation, and recovery of individual cells	Individual cells of interest among heterogeneous sample can be retrieved	42
	ApoStream® (Precision medicine group)	DEP[c] field-flow-fractionation (DEP[c]-FFF) in a continuous flow	Cell levitation is controlled by balancing DEP[c], hydrodynamic, and sedimentation forces	43
Direct imaging	Epic (Epic Sciences)	No isolation, all nucleated cells are analysed	After RBC lysis step, all nucleated cells are deposited on glass slides for immunostaining and scanning	44

[a]Antibody (Ab), [b]Deterministic lateral displacement (DLD), [c]Dielectrophoresis (DEP).

Table. 2. Examples of EVs isolation technologies

Isolation principles	Technology	Requirements (time)	Advantages	Disadvantages	Ref.
Density	D-UC[h]	> 100,000 × g (> 4 hr)	Most commonly used method	Time-consuming, labour-intensive, requires expensive equipment, low yield, protein contaminants, low reproducibility	45
	DG-UC[e]	> 100,000 × g (> 6 hr)	EV fractionization with fewer impurities and aggregation	Time-consuming, labour-intensive and difficult handling processes, requires expensive equipment, low yield, low reproducibility	46
Size	SEC[n]	SEC[n] column (0.5 hr)	High yield and purity, EV fractionization	Multiple handling processes, samples are often diluted and requires additional concentration step, cannot discriminate similar sized other particles	47
	UF[p]	Nanoporous membrane (volume dependent)	Less hands-on process	Membrane clogging, low purity, EV deformation, cannot discriminate similar sized other particles	48
	TFF[o]	Nanoporous membrane (volume dependent)	Large sample volume processing	Not good for small sample volume processing, cannot discriminate similar sized other particles	49
	Exodisc	Exodisc equipped with AAO[b] membrane and operating system (< 0.5 hr)	High yield, semi-automated, high reproducibility, gentle (< 500 × g) and rapid, on-chip detection or EV staining	For small sample volume (< 200 µL plasma, < 4 mL urine, < 10 mL CCM[d]), require specialized operation machine, cannot discriminate similar sized other particles	50, 51
	ExoTic[j]	A device equipped with nanoporous membrane and syringe pumps (~ 5 ml/hr)	High yield and purity, small sample volume, size fractionization	Not commercialized, multiple handling processes, cannot discriminate other particles with similar size	55
	EXODUS[i]	A catridge with dual-membrane (AAO[b]) filter coupled with harmonic oscillations and operating workstation (urine 28 ml/hr)	High speed, high purity, high yield	For small sample volume (1/50 diluted plasma (20 µL), < 10 mL urine), require specialized operation machine, cannot discriminate other particles with similar size	56
	DLD[f]	DLD[f] pillar array, syringe pump (NA[l])	High precision size fraction (20 – 110 nm)	Slow operation	57
	Acoustofluidic device	Acoustofluidic chip, syringe pump (4 hr/ml)	Full automation from whole blood, high purity, and yield.	Long process time, samples are often diluted and requires additional concentration step, cannot discriminate similar sized other particles	58
Charge	IEC[k]	IEC[k] column (2 ml/min)	Extremely fast, large sample volume	Low yield, requires further purification	59
	Electrophoresis	Electrophoresis apparatus (3 hrs)	Available to separate EV from lipoprotein, high recovery	Not commercialized, long time, sample to sample variation	60
	DEP[g]	ACE[a] microarray (< 0.25 hr)	Fast, small sample volume	Low purity, scale-up is difficult	61
	Precipitation	NaAc	Simple, low cost	Low yield, low purity, long process time	62
Water solubility	Precipitation	Hydrophilic polymers (PEG[m])	Simple, low cost, high yield	Low purity, contamination with precipitation reagents	63
	Phase-separation	ATPS[c] (PEG[m]/Dextran)	Simple, low cost, gentle condition, high yield	Low purity, contamination with precipitation reagents	64
Immuno-affinity	Magnetic beads	Antibody-coated beads	Simple, high purity	Antigen-dependence, requires pre-concentration for large volume sample, binding is not reversible	65-67
	Click Chip	Click chip, syringe pump (1 ml/hr)	High selectivity, retrieval, on-chip detection	Long process time, antigen-dependence	68
	ExoTENPO	ExoTENPO chip, antibody-coated magnetic beads, syringe pump (10 ml/hr)	High selectivity, high throughput	Antigen-dependence, binding is not reversible	69
	OncoBean Chip	OncoBean chip, syringe pump (0.05 ml/min)	High throughput, retrieval	Antigen-dependence, For small sample volume (< 1.2 ml for plasma, <10 ml for CCM)	70
	[EV]HB-Chip	[EV]HB-Chip, syringe pump (3 hrs)	High yield and specificity,	Long process time, antigen-dependence	71

[a]Alternating current electrokinetic (ACE), [b]Anodic aluminum oxide (AAO), [c]Aquous two phase system (ATPS), [d]Cell culture media (CCM), [e]Density gradient ultracentrifugation (DG-UC), [f]Deterministic lateral displacement (DLD), [g]Dielectrophoresis (DEP), [h]Differential-Ultracentrifugation (D-UC), [i]Exosome detection method via the ultrafast-isolation system (EXODUS), [j]Exosome total isolation chip (ExoTic), [k]Ion-exchange chromatography (IEC), [l]Not available (NA), [m]Poly ethylene glycol (PEG), [n]Size exclusion chromatography (SEC), [o]Tangential-flow-filtration (TFF), [p]Ultrafiltration (UF).

Table. 3. ***Comparison of various circulating biomarkers in liquid biopsy applications***

Circulating biomarkers	Pros	Cons
CTCs	• Cell phenotype studies (morphology, immunostaining, FISH[a]) • Comprehensive downstream analysis (DNA, RNA, protein) • Single cell analysis • Functional studies (PDX[b])	• Extremely rare and fragile • Heterogeneity of CTCs • No standard isolation method • Enumeration method dependent results • Not for biobanked samples
EVs	• Relatively stable and abundant • Comprehensive downstream analysis (DNA, RNA, protein) • Biobanked samples with minimum freeze and thaw • Potential for therapeutics	• No standard isolation and detection method • Difficult to isolate tumour-specific EVs • Contamination with EVs from normal cells and other proteins • Heterogeneity of EVs (size, density, surface protein) • Lack of cancer-specific EVs marker
cfDNA	• Relatively simple isolation method available • High sensitivity detection method available • Overcome spatial and temporal tumour heterogeneity • Sensitive to disease status (diagnosis, treatment monitoring, and drug resistance detection)	• Rare and fragile • Limited downstream analysis (DNA only) • Difficult to isolate tumour-specific DNA • Contamination with DNA from normal cells. • No functional assays

[a]Fluorescent in situ hybridization (FISH), [b]Patient-derived xenograft (PDX).

Table. 4. Diagnosis of prostate cancer using liquid biopsy

Circulating biomarkers	Patient cohort	Sample type/ Volume	Detection marker	Readout to diagnosis	Isolation method	Detection method	Year[Ref.]
CTC	9 PCa[o], 1 KCa[h], 17 HD[e]	WB[u]/ NA[l]	CTCs counts per 1 mL	Present	ISET®	Immunofluorescent imaging	2020[116]
	41 CRPC[b], 13 HD[e]	WB[u]/ 6 – 12 mL	CTC counts per 7.5 mL	Present	CTC-iChip, CellSearch	Immunofluorescent imaging	2013[115]
	19 PCa[o], 20 HD[e]	WB[u]/ 0.9 – 5.1 mL	CTC counts per 1 mL	Present	CTC-Chip	Immunofluorescent imaging	2007[114]
EV	269 PCa[o], 234 Benign	U[s]/ 15-20 mL	EPI (*EGR[t], SPDEF[q], PCA3*)	Increased	EXOPRO Urine Clinical Sample Concentrator Kit	RT-qPCR	2016[118], 2018[120]
	44 PCa[o], 4 BPH[a], 4 HD[e]	S[r]/ 0.25 mL	miRNA (*miR-1246*)	Increased	Total Exosome Isolation Reagent	NanoString	2018[122]
	20 PCa[o], 9 HD[e] for NGS; 28 PCa[o], 19 HD[e] for RT-qPCR	U[s]/ 50-150 mL	miRNA (*miR-196a-5p, miR-501-3p*)	Decreased	UC	NGS/ RT-qPCR	2017[123]
	18 PCa[o], 7 BPH[a]	P[n]/ 1 mL	miRNA (*miR-10a-5p, miR-29b-3p*)	Increased	ExoRNeasy midi kit	RT-qPCR	2019[124]
	117 PCa[o], 48 HD[e]	U[s]/ 15-40 mL	mRNA (*GATA2, PCA3, TMPRSS2-ERG*)	Increased	UC	RT-qPCR	2020[125]
	43 PCa[o], 30 HD[e]	WB[u]/ 0.03 – 0.6 mL	Proteins (*PSA, PSMA, HSP90, EpCAM, EGFR1*)	Increased	ExoDisc-P	ELISA	2019[51]
	15 PCa[o], 13 HD[e]	U[s]/ 50 - 150 mL	Lipids: *Phosphatidylserine (PS) (18:1/18:1), Lactosylceramide (LacCer) (d18:1/16:0)*	*PS*: descreased *LacCer*: increased	UC	MS[i]	2017[126]
cfDNA/ cfRNA	121 PCa[o], 136 HD[e]	P[n]/ 2-3 mL	DNA methylations (*FOXA1, GSTP1, HOXD3, RARβ2, RASSF1A, SEPT9, SOX17*)	Increased	QIAmp MinElute ccfDNA	Met-PCR (RT-qPCR)[j]	2019[127]
	84 PCa[o], 7 HGPIN[f], 30 HD[e]	S[r]/ 1 mL	Promoter DNA methylations (*MCAM, ERα and ERβ*)	Increased	Phe/chl Ext.[m], EtOH Precip.[c]	QMet-PCR[p] (RT-qPCR)	2017[128]
	42 PCa[o], 52 HD[e]	U[s]/ 20 – 90 mL	DNA methylations (32 markers)	Increased	Chl. Ext., IPA precip.[g]	RT-qPCR	2018[129], 2019[130]
	809 PCa[o], 241 NPBx[k], 41 HD[e]	S[r]/ 0.3 mL	Circulating miRNA (*miR-17-3p, miR-1185-2-3p*)	Increased	3D Gene RNA Extraction Reagent	miRNA microarray	2019[131]

[a]Benign prostatic hyperplasia (BPH), [b]Castration resistant prostate cancer (CRPC), [c]Ethanol precipitation (EtOH Precip.), [d]ExoDx Prostate (IntelliScore) (EPI), [e]Healthy donor (HD), [f]High grade prostate intraepithelial neoplasia (HGPIN), [g]Isopropanol precipitation (IPA precip.), [h]Kidney cancer (KCa), [i]Mass spectrometry (MS), [j]Methylation specific-PCR (Met-PCR), [k]Negative prostate biopsy (NPBx), [l]Not available (NA), [m]Phenol/chloroform extraction (Phe/Chl Ext.), [n]Plasma (P), [o]Prostate cancer (PCa), [p]Quantitative methylation specific-PCR (QMet-PCR), [q]SAM pointed domain-containing Ets transcription factor (SPDEF), [r]Serum (S), [s]Urine (U), [t]V-ets erythroblastosis virus E26 oncogene homologs (EGR), [u]Whole blood (WB)

Table 5. Prognosis of prostate cancer using liquid biopsy

Circulating biomarkers	Patient cohort	Sample type/ Volume	Prognostic marker	Outcome	Isolation method	Detection method	Year[ref]
CTC	120 mCRPC[l] patients treated with carbazitaxal	WB[s]/7.5 mL	Baseline CTC number	PFS[p] and OS[o]	CellSearch	IF imaging[k]	2019[138]
	949 mCRPC[l] patients treated with AA[a] plus prednisone or prednisone alone	WB[s] /7.5 mL	Baseline and relative change in CTC number, together with ALPHA model	OS[o]	CellSearch	IF imaging[k]	2017[139]
	41 mCRPC[l] patients	WB[s] /5 mL	*WNT5a, AURKA, BMP7* expression on CTCs	OS[o]	Anti-EpCAM antibody-conjugated microbeads	RT-qPCR	2018[142]
	79 CRPC[f] patients	WB[s] /5 mL	*PSMA* expression on CTCs	PFS[p] and OS[o]	AdnaTest	RT-qPCR	2020[144]
	44 CRPC[f] patients	WB[s] /7.5 mL	Baseline CTC number and synaptophysin expression on CTCs	PFS[p]	CellSearch	IF imaging[k]	2018[141]
	108 mCSPC[m] patients	WB[s] /5 mL	Mesenchymal markers (*Vimentin, Twist*) expression on CTCs	Castration-sensitive prostate cancer to CRPC[f]	CanPatrol	Multiplex *in situ* hybridization	2019[143]
	40 mCRPC[l] patients treated with docetaxel	WB[s] /7.5 mL	Baseline CTC number and EGFR expression on CTCs	OS[o]	CellSearch	IF imaging[k]	2016[140]
	155 treatment-naïve patients with localized PCa[q]	WB[s] /7.5 mL	The presence of CTCs	Higher GS[i], risk group and clinically significant PCa[q]	Parsortix	Multiplex *in situ* hybridization	2020[145]
	34 patients with localized PCa[q]	WB[s] /20 mL	Preoperative CTCL score	Microscopic cancer dissemination to seminal vesicles and/or lymph nodes	Microfluidic CTC-iChip	ddPCR[g]	2018[146]
	203 post-RP patients with undetectable serum PSA	WB[s] /5 mL	The presence of CTCs after RP	BCR[e]	Ad5/35E1aPSESE4 infection	IF imaging[k]	2020[147]
	37 high-risk localized PCa[q]	WB[s] /8 mL	The presence of CTCs after RP	BCR[e]	Epic Sciences	IF imaging[k]	2019[148]
EV	535 PCa[q] patients for development, 264 patients for validation	Post-DRE[h] urine/-	Four prostate urine risk (PUR) signatures based on urine EV RNAs	Intermediate- or high-risk disease and time to progression in AS[b]	Microfiltration	NanoString	2019[156, 158]
	235 PCa[q] patients for development, 1436 patients for validation	None-DRE[h] urine/-	The miR Sentinel CS Test and the miR Sentinel HG Test based on urine EV small non-coding RNAs	Grade Group 2 or greater cancer (CS Test); Grade Group 3 or greater cancer (HG test)	Exosome RNA Isolation Kits	Microarray	2020[159]
	35 CRPC[f] patients	Plasma/-	*AR-V7* mRNA level in plasma EVs	Time to progression	ExoQuick	ddPCR[g]	2019[160]
cfDNA/ cfRNA	140 CRPC[f] patients	Plasma from 10 mL WB	Estimated tumor fractions in cfDNA	Metastasis location, high ALP[c] and low Hb[j]	Qiagen Circulating DNA kit	WGS[u]	2018[162]
	250 mPCa[n] patients	Plasma/1-5 mL	*TP53* mutations, *RB1* loss, *AR* amplifications and mutations in multiple DNA repair genes (*ATM, BRCA1, BRCA2, CHEK2*)	OS[o] and time to ADT[d] failure	QIAamp Circulating Nucleic Acid Kit	NGS	2020[163]
	514 mCRPC[l] patients	Plasma/5-30 ng cfDNA	Higher number of ctDNA alterations, *AR* alterations and amplifications of *MYC* and *BRAF*	Treatment failure-free survival and OS[o]	Guardant360 platform	NGS	2019[164]
	50 CRPC[l] patients	Plasma/1.5 mL	Pre-treatment hypermethylation patterns of *GSTP1* and *APC* in cfDNA	OS[o]	Quick-cfDNA Serum&Plasma kit	Methylation-specific PCR	2018[165]
	62 mPCa[n] patients	Plasma/2 mL	ctDNA methylation	CRPC[f] neuroendocrine differentiation	NeoGeneStar Cell-Free DNA Purification kit	WES[t]	2020[166]
	60 patients with localized PCa[q]	Plasma/1 mL	Preoperative cfRNA expression (*GOLM1, NKX3-1 and TRPM8*)	High risk disease	miRNeasy Mini kit	RT-qPCR	2020[168]

[a]Abiraterone acetate (AA), [b]Active surveillance (AS), [c]Alkaline phosphatase (ALP), [d]Androgen deprivation therapy (ADT), [e]Biochemical recurrence (BCR), [f]Castration-resistant prostate cancer (CRPC), [g]Digital droplet PCR (ddPCR), [h]Digital rectal examination (DRE), [i]Gleason score (GS), [j]Haemoglobin (Hb), [k]Immunofluorescent imaging (IF imaging), [l]Metastatic castration-resistant prostate cancer (mCRPC), [m]Metastatic castration-sensitive prostate cancer (mCSPC), [n]Metastatic prostate cancer (mPCa), [o]Overall survival (OS), [p]Progression-free survival (PFS), [q]Prostate cancer (PCa), [r]Radical prostatectomy (RP), [s]Whole blood (WB), [t]Whole exon sequencing (WES), [u]Whole genome sequencing (WGS).

Table 6. Precision medicine and treatment response monitoring using liquid biopsy

Circulating biomarkers	Patient cohort	Sample type/ Volume	Predictive marker	Outcome	Isolation method	Detection method	Year[ref]
CTC	511 mCRPC[g] patients with baseline CTC <5/7.5 mL	WB[i]/7.5 mL	The increase of CTC number during the first 12 weeks of treatment	Response to AA[a] or chemotherapy	CellSearch	IF imaging[f]	2018[177]
	6081 mCRPC[g] patients	WB[i]/7.5 mL	CTC0 (≥1 CTC at baseline, 0 at week 13) and CTC conversion (≥5 CTCs at baseline, ≤4 at week 13)	Response to AA[a] or ENZ[e] or orteronel or carbozantinib	CellSearch	IF imaging[f]	2018[178]
	161 mCRPC[g] patients	WB[i]/7.5 mL	Nuclear-localized *AR-V7* in CTCs	Response to taxane-based chemotherapy	Epic Sciences	IF imaging[f]	2017[185]
	142 mCRPC[g] patients	WB[i]/10 mL	Nuclear-localized *AR-V7* in CTCs	Treatment decision between ARS[b] inhibitor and taxane-based chemotherapy	Epic Sciences	IF imaging[f]	2018[186]
	118 mCRPC[g] patients	WB[i]/5 mL WB[i]/7.5 mL	*AR-V7* positive CTCs (Epic Sciences or AdnaTest)	Response to AA[a] or ENZ[e]	Epic Sciences, AdnaTest	IF imaging[f], RT-qPCR	2019[187]
EV	36 CRPC[d] patients	Plasma from 3 mL WB[i]	*AR-V7* mRNA level in plasma EVs	Response to AA[a] or ENZ[e]	ExoRNeasy kit	ddPCR[c]	2017[188]
cfDNA/ cfRNA	62 mCRPC[g] patients	Plasma/3 mL	cfDNA concentration, TP53 loss and PI3K pathway defects	Response to ENZ[e] and/or AA[a]	QIAamp Circulating Nucleic Acid Kit	NGS	2019[193]
	49 mCRPC[g] patients	Plasma/4-8 mL	cfDNA concentration and DNA repair mutations	Response to PARP[h] inhibitor	QIAsymphony and the Circulating DNA Kit	Targeted and WES[j]	2017[192]
	571 mCRPC[g] patients	Plasma/1 mL	cfDNA concentration	Response to taxane-based chemotherapy	QIAamp Circulating Nucleic Acid Kit	Quant-IT Picogreen HS DNA kit	2018[191]
	88 mCRPC[g] patients	Plasma/0.5 mL	*AR* and *AR* enhancer amplification, *ZFHX3* deletion and *PIK3CA* amplification	Response to AA[a]	QIAmp DNA Blood Mini Kits	WGS[k]	2020[194]
	202 mCRPC[g] patients	Plasma/-	Defects in *BRCA2* and *ATM*, and somatic alterations in *TP53*	Response to AA[a] or ENZ[e]	NimbleGen SeqCap EZ Choice target capture panel	Targeted and WES[j]	2018[195]
	2 mCRPC[g] patients	Plasma/6 mL	*BRCA2* reversion mutations	Response to PARP[h] inhibitor	Circulating Nucleic Acids Kit	NGS	2017[198]
	14 mCRPC[g] patients	Plasma/-	Microsatellite instability high in ctDNA	Response to pembrolizumab	Guardant360	NGS	2020[203]

[a]Abiraterone acetate (AA), [b]Androgen receptor signalling (ARS), [c]Digital droplet PCR (ddPCR), [d]Castration-resistant prostate cancer (CRPC), [e]Enzalutamide (ENZ), [f]Immunofluorescent imaging (IF imaging), [g]Metastatic castration-resistant prostate cancer (mCRPC), [h]Poly (adenosine diphosphate ribose) polymerase (PARP), [i]Whole blood (WB), [j]Whole exon sequencing (WES), [k]Whole genome sequencing (WGS).