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Review Article

Research landscape of liquid biopsies in prostate cancer

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Abstract: Studies show that liquid biopsies are efficient in the detection of circulating cancer products. However, scientific community has not yet implemented this technology in routine clinical practice. Liquid biopsies are less invasive than traditional surgical ones because they rely on the detection of specific biomarkers in readily accessible body fluid samples. The clinical management of prostate cancer depends on the controversial blood serum biomarker PSA (prostate specific antigen). PSA tests have a low accuracy. In addition, a positive PSA result for prostate cancer needs a confirmation through a tissue biopsy. Thus, liquid biopsies are considered tools to find a surrogate biomarker. This review aimed to show the landscape of liquid biopsies in prostate cancer research to understand its challenges and foresee the trends in this area. We performed an exhaustive Pubmed search of articles reporting the study of liquid biopsies in prostate cancer with circulating tumor cells, cell-free nucleic acids and extracellular vesicles as targets. After a thorough analysis, we retrieved sixty-two relevant articles. Among the identified articles, the most used target and body fluid were circulating tumor cells and blood, respectively. Enumeration of circulating tumor cells was the most reported parameter, but it was often combined with other biomarkers. The most used methods for biomarker detection were those based on transcriptome analysis. Despite the vast literature about liquid biopsy in prostate cancer, most studies seem to be stuck on improving the yield of technologies. Consequently, they seem to test a limited number of samples. Larger cohorts could provide robust evidence to translate liquid biopsies of prostate cancer to the clinics.

Keywords: Liquid biopsy, prostate cancer, circulating tumor cells, extracellular vesicles, exosomes, cell-free nucleic acids, circulating nucleic acids, cell-free DNA, cell-free RNA

Introduction

The liquid biopsy was first applied in 1869 by the pathologist Thomas Ashworth, who showed the existence of circulating tumor cells (CTCs) in the blood of a patient with metastatic cancer [1]. Tumor cell masses, as well as normal tissues, shed components such as whole cells known as CTCs, cell-free nucleic acids (cf-nucleic acids) and extracellular vesicles (EVs), which end up in the bloodstream or other body fluids depending on their place of origin. These tumor components are targets of biomarker search that may enable to monitor tumor development. Thus, any sample from a body fluid containing these targets is called liquid biopsy.

The prostate specific antigen (PSA) is the gold standard biomarker for the clinical management of prostate cancer (PCa). However, the PSA shows a low accuracy, with a high rate of false-negative and false-positive results [2]. Although PSA is a circulating glycoprotein detected in blood serum samples and can be considered as a liquid biopsy itself, a positive result in PSA (high serum levels) needs a confirmation through histological examination of a surgical prostate biopsy from the patient. In an attempt to find a more effective and less invasive clinical tool for PCa, researchers from all over the world are developing new strategies based on liquid biopsies from blood, urine, seminal fluid and stool (**Figure 1**).

CTCs are rare cells from primary and metastatic tumors that circulate throughout the body to form metastatic niches in other tissues. These cells are detectable in cancer patients while

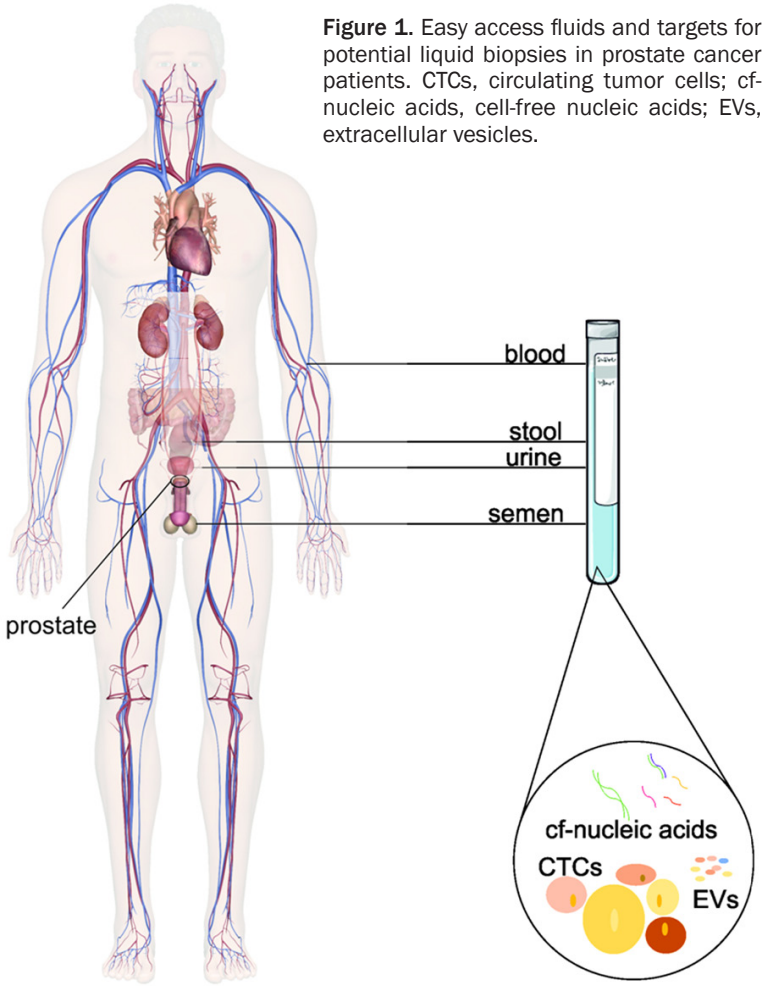

Figure 1. Easy access fluids and targets for potential liquid biopsies in prostate cancer patients. CTCs, circulating tumor cells; cf-nucleic acids, cell-free nucleic acids; EVs, extracellular vesicles.

they are undetectable in healthy individuals [3]. Since CTCs may derive from different tissues, they constitute a heterogeneous population of cells that represents a comprehensive view of cancer progression in a patient [4]. CTCs are released into blood, but CTCs from PCa can be also found with a high probability in urine, seminal fluid or stool because of their organ of origin. Nucleic acids contained in CTCs, which allow monitoring epigenetic and genetic alterations, proteins expressed on the surface or inside CTCs, as well as the number of these CTCs are potential cancer biomarkers [5].

Cf-nucleic acids are cell-free circulating DNA or RNA fragments released after the lysis of apoptotic or necrotic cells [6, 7]. In patients with PCa, cell-free DNA (cf-DNA) in blood is detectable in higher levels when compared to control individuals; with a sensitivity and specificity of 80% and 82%, respectively [8]. Furthermore, more than 50% of blood samples and over 70% of urine samples from PCa patients showed alterations on cell-free DNA that may be used as PCa biomarkers [9].

EVs are membrane-covered cellular components and are involved in intercellular communication [10]. EVs from tumor cells were also described as tumor-derived microvesicles [11], and observed in PCa among other types of cancer [12]. These targets have already been successfully isolated from diferent body fluids [13].

The numerous studies about liquid biopsy in PCa indicate the relevance of this technology. However, there is no evidence of a wide clinical implementation of liquid biopsy in PCa. Therefore, we wondered why liquid biopsy in PCa fails to translate to the medical practice and what the level of development achieved by this technology in PCa is. The goal of this review was to gather information about the different approaches used for liquid biopsy in PCa, analyze them and discuss their characteristics, challenges and potentials for their use in the clinical oncology.

Materials and methods

Literature search

A PubMed literature search was conducted with the key terms (prostate cancer) AND (biomarker) AND (patients) AND (diagnosis OR prognosis OR treatment) AND ((extracellular vesicles OR exosomes) OR (cell-free DNA OR circulating tumor DNA) OR circulating tumor cells) to identify studies that reported biomarkers for PCa in liquid biopsies. Following this step, the filters for publication dates (from 2010/01/01 to 2017/12/31) and languages (English) were applied to the literature search.

Titles and abstracts from this PubMed literature search were retrieved and considered for

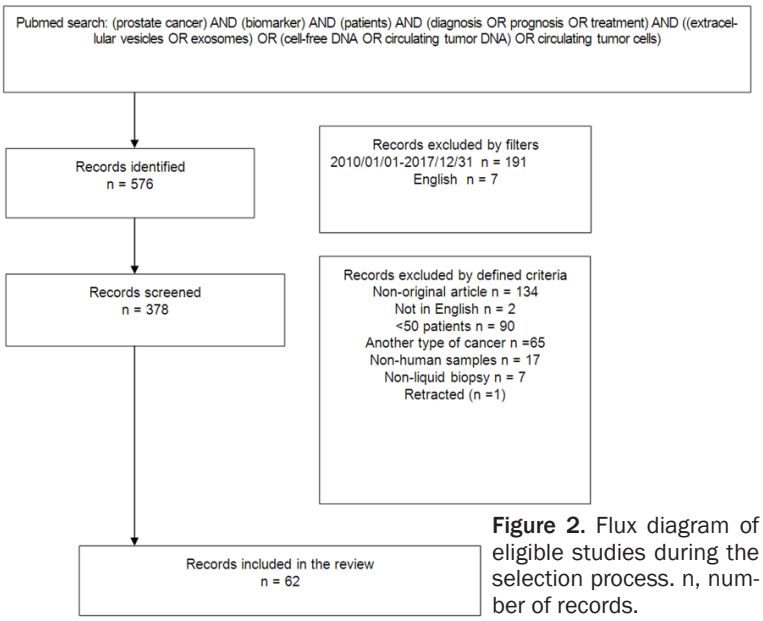

Figure 2. Flux diagram of eligible studies during the selection process. n, number of records.

our eligibility analysis. Those studies such as reviews, case studies and letters to the editor were excluded as non-original articles. Studies in animal models or cell lines, with traditional biopsies, or evaluating other type of cancer were also excluded. The remaining articles were further assessed from their full-texts to ensure that our pre-established criteria were met. Furthermore, studies with less than 50 patient samples were excluded from this search. Then, the selected articles were scanned and relevant data were gathered together onto a Microsoft Excel spreadsheet. The extracted data were digital object identifier (DOI), types of target, body fluids for target extraction, biomarkers, isolation and detection methods, number of samples, and clinical values such as diagnosis, prognosis, recurrence and treatment (including treatment monitoring and/or treatment choice). Two authors reviewed each full-length article and discrepancies between them were solved by discussion with a third author.

Quality of the selected articles

The following questions constituted the tool used to assess the quality and bias of the selected studies: (1) Was the spectrum of patients representative of the patients who will receive the test in practice? (2) Were selection criteria clearly described? (3) Was the execution of the index test described in sufficient detail to permit replication of the test? (4) Were the same clinical data available when test results were interpreted as would be available when the test is used in practice? (5) Were uninterpretable/intermediate test results reported? (6) Were withdrawals from the study explained? (7) Did the study provide a clear definition of what was considered to be a positive result? (8) Were objectives pre-specified? (9) Was the study free of commercial funding?

The first six questions were obtained from the QUADAS (Quality Assessment of Diagnostic Accuracy Studies) checklist [14] and the last four questions are potential additional quality items that were extracted from the Cochrane Handbook for Reviews of Diagnostic Test Accuracy [15]. Other questions from these sources were omitted because they were not applicable to the topic. Some of these missing questions compared the index test and the reference standard test; and as we mentioned before, PCa tests lack a proper reference standard. Others were exclusive for diagnostic tests.

Results

General findings

In total, 576 studies were found in the Pubmed search and the filters excluded 198 studies. The 378 articles left were screened and only 62 articles fulfilled the inclusion criteria for this review (**Figure 2**). After non-original article criterion, the most common exclusion criterion within our selected articles was a cohort with less than 50 patients.

Regarding the quality of the selected articles, the analysis showed that studies seemed to be poorly described to answer adequately to the quality questions. The section of material and methods was insufficiently described in the articles. Nearly half of the studies showed a representative spectrum of the patients, clear selection criteria, an index test described in

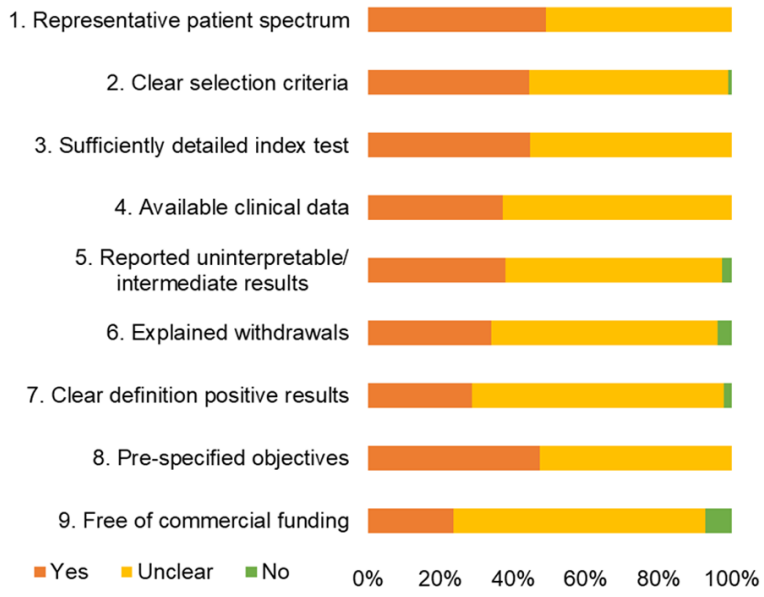

Figure 3. Quality of selected studies according to the nine pre-defined assessment questions.

sufficient detail for replication and pre-specified objectives. Less than 30% of the studies provided a clear definition for a positive result. Moreover, less than 25% was unquestionably free of commercial funding, which shows a potential conflict of interests (**Figure 3**).

All the selected studies but two [16, 17] reported positive results, with a strong association between the biomarkers detected form liquid biopsies and their clinical values. Patient samples used in the eligible articles included whole blood, blood plasma or blood serum (80%) and urine (20%) with no mention of other alternative samples.

Regarding the type of target used for biomarker identification, most studies reported CTCs (55.8%, n = 34) (**Table 1**), followed by cf-nucleic acids (24.2%, n = 15) (**Table 2**) and EVs (21%, n = 13) (**Table 3**). In the next section, we further assess the targets in liquid biopsies of PCa according to the body fluids where they were isolated, the biomarkers detected on them, the detection methods used and the clinical values of the biomarkers.

CTCs in liquid biopsies of PCa

Figure 4 summarizes the biomarkers, the body fluids, the detection methods and the clinical values in the retrieved studies about CTCs in PCa (**Table 1**).

All CTCs from these studies were isolated from patient samples of peripheral blood collected in tubes containing ethylenediaminetetraacetic acid (EDTA) (**Figure 4A**).

The analysis of our selected articles showed that CTC enumeration alone was the least reported biomarker. Most of the selected articles reported CTC enumerations combined with specifics biomarkers on their surface or within the CTCs, such as intracellular proteins and gene expression profiles (**Figure 4B**).

Our research yielded three commercial approaches for CTC detection that use fluorescent dye-conjugated antibodies. More than fifty percent of our retrieved studies applied the FDA (Food and Drug Administration)-approved system CellSearch (Veridex, Raritan, NJ, USA). Four of the selected articles reported the use of the Epic CTC platform (Epic Sciences, San Diego, CA, USA) and two reported the AdnaTest (Quiagen, Hilden, Germany). Similar to the AdnaTest, 15% of the articles used an enrichment technique coupled to gene expression analysis by different types of PCR. Immunocytochemistry and flow cytometry were the least used methods for CTC detection in PCa. Techniques based on microfluidics for CTC purification and detection were not represented in our selected articles (**Figure 4C**).

According to the clinical values reported in the list of studies about CTCs, almost half of them were prognosis; followed by treatment, diagnosis and recurrence (**Figure 4D**).

Cf-nucleic acids in liquid biopsies of PCa

The biomarkers, the body fluids, the detection methods and the clinical values in the retrieved studies about cf-nucleic acids in PCa (**Table 2**) are summarized in **Figure 5**.

The screening of cf-nucleic acids from blood samples was the most used approach, with plasma screening comprising three-quarters of the retrieved studies and serum screening

Table 1. List of selected studies with circulating tumor cells as main targets for liquid biopsy in prostate cancer including the specific biomarkers, the body fluids where the biomarkers were detected, the methods used for their detection and their clinical values

Study	Biomarkers	Body fluids	Detection methods	Clinical values
[50]	CTC enumeration	Blood	CellSearch (Veridex)	Prognosis Treatment
[51]	AR-V7 in CTCs	Blood	Epic CTC platform (Epic Sciences)	Prognosis Treatment
[52]	PSMA+CTC enumeration and PSMA in plasma	Blood	ICPMS	Diagnosis
[53]	Apo10 and TKTL1 in $CD14^{+}CD16^{+}$ macrophages	Blood	Flow cytometry	Treatment
[54]	CTC enumeration and *AR-V7* mRNA in CTCs	Blood	Adnatest platform (AdnaGen)	Prognosis Treatment
[55]	mRNA of *PSA*, *PSMA* and *EGFR* in CTCs	Blood	Adnatest platform (AdnaGen)	Prognosis Treatment
[56]	Nuclear localization of AR-V7 in CTCs	Blood	Epic CTC platform (Epic Sciences)	Prognosis Treatment
[57]	CTC enumeration and EGFR in CTCs	Blood	CellSearch (Veridex)	Prognosis Treatment
[23]	Albumin, LDH, PSA, hemoglobin, and ALK (ALPHA) in serum and CTC enumeration	Blood	Immunoassays and Epic CTC platform (Epic Sciences)	Prognosis
[58]	RNA levels of AR-V7 and PSA in CTCs	Blood	Centrifugation and RT-PCR and ddPCR	Prognosis
[59]	CTC enumeration	Blood	CellSearch (Veridex)	Prognosis
[60]	CTC enumeration	Blood	CellSearch (Veridex)	Prognosis Treatment
[61]	CTC enumeration and AR-V7 in CTCs	Blood	Epic CTC platform (Epic Sciences)	Prognosis Treatment
[22]	CTC enumeration and Ki67 and vimentin in CTCs	Blood	CellSearch (Veridex)	Prognosis Treatment
[16]	CTC enumeration	Blood	CellSearch (Veridex)	Prognosis
[21]	CTC enumeration, stem cell-related genes (ABCG2, *PROM1* and *PSCA*) and EMT-related genes (*TWIST1* and vimentin) in PBMCs	Blood	CellSearch (Veridex) and RT-qPCR	Prognosis Recurrence
[62]	CTC enumeration	Blood	CellSearch (Veridex)	Prognosis Treatment
[63]	CD117/c-kit, CD133/prominin-1, CD34, CD184/CXCR4 and EpCAM/CD326 in lymphocytes	Blood	Flow cytometry	Prognosis Treatment Recurrence
[64]	Telomerase activity in CTCs and CTC enumeration	Blood	qPCR-TRAP and CellSearch (Veridex)	Diagnosis Prognosis Treatment

[65]	CTC enumeration	Blood	CellSearch (Veridex)	Prognosis
[66]	CTC enumeration	Blood	CellSearch (Veridex)	Prognosis Treatment
[67]	PSA and P504S in CTCs	Blood	Immunocytochemistry	Treatment
[68]	CTC enumeration	Blood	CellSearch (Veridex)	Diagnosis
[69]	CTC enumeration	Blood	CellSearch (Veridex)	Prognosis Treatment
[70]	mRNA of *KLK3, KLK2, HOXB13, GRHL2* and *FOXA1* in whole blood and CTC enumeration	Blood	RT-PCR and CellSearch (Veridex)	Treatment
[71]	PSA in CTCs	Blood	Immunocytochemistry	Prognosis
[72]	CTC enumeration	Blood	CellSearch (Veridex)	Recurrence Prognosis
[73]	mRNA of antioxidant genes (*GPX1* and *SOD2*) and prostate genes (*AR, cyclin B* and *bFGF*) in CTCs	Blood	Anti-EpCAM or a retention mesh of 20 microm width; and RT-qPCR	Prognosis
[74]	PSA, CD82, MMP-2 and HER-2 in CTCs and DTCs from bone marrow aspirates	Blood	Immunocytochemistry	Diagnosis Prognosis Treatment
[75]	mRNA of *PSA* and *PSMA* in CTCs	Blood	Ficoll density gradient centrifugation and RT-PCR	Diagnosis Prognosis
[76]	CTC enumeration, ($DAPI^+CD146^+CD105^+CD45^-$) CEC enumeration and ET-1 and TF in serum	Blood	CellSearch (Veridex) and ELISA	Prognosis Treatment Recurrence
[77]	CTC enumeration and methylation in regulatory regions of genes (*GSTP1, APC, PTGS2, MDR1* and *RASSF1*) in serum (cf-DNA)	Blood	CellSearch (Veridex); and sodium bisulfite modification and methylation-specific qPCR	Prognosis Treatment
[78]	EpCAM+CK+CD45- object and CTC enumerations	Blood	CellSearch (Veridex)	Prognosis Treatment
[79]	mRNA of antioxidant genes (*GPX1, SOD2* and *TXNRD1*), epithelial gene (*CK20*) and organ genes (*AR, PSA* and *PSMA*) in CTCs	Blood	Anti-EpCAM or a retention mesh of 20 microm width; and RT-qPCR	Prognosis

CTC, circulating tumor cell; AR-V7, androgen receptor splice variant 7; PSMA, prostate specific membrane antigen; ICPMS, inductively coupled plasma mass spectrometry; Apo10, apoptosis related protein DNaseX; TKTL1, transketolase-like 1; CD, cluster of differentiation; mRNA, messenger RNA, PSA, prostate specific antigen; EGFR, epithelial growth factor receptor; LDH, lactate dehydrogenase; ALK, alkaline phosphatase; ((RT)-(q))PCR, ((reverse transcription) (quantitative)) polymerase chain reaction; ddPCR, droplet digital polymerase chain reaction; ABCG2, ATP-binding cassette super-family G member 2; PROM1, pominin 1; PSCA, prostate stem cell antigen; EMT, epithelial-mesenchymal transition; TWIST1, TWIST family BHLH transcription factor 1; PBMC, peripheral blood mononuclear cell; c-kit, cytokine-tyrosine kinase receptor; CXCR4, C-X-C motif chemokine receptor type 4; EpCAM, epithelial cell adhesion molecule; TRAP, telomeric repeat amplification protocol; KLK, kallikrein-related peptidase; HOXB13, homeobox B13; GRHL2, grainyhead-like 2; FOXA1, forkhead box A1; GPX1, glutathione peroxidase 1; SOD2, superoxide dismutase 2; AR, androgen receptor; bFGF, basic fibroblast growth factor; MMP2, matrix metalloproteinase-2; HER-2, human epidermal growth factor receptor-type 2; DTC, disseminated tumor cell; DAPI, 4',6-diamidino-2-phenylindole; CEC, circulating endothelial cell; ET-1, endothelin-1; TF, tissue factor; ELISA, enzyme linked immunosorbent assay; GSTP1, glutathione S-transferase P1; APC, adenomatous polyposis coli; PTGS2, prostaglandin-endoperoxide synthase 2; MDR1, multidrug resistance protein 1; RASSF1, Ras association domain family member 1; cf-DNA, cell-free DNA; CK, cytokeratin; TXNRD1, thioredoxin reductase 1.

Table 2. List of studies with cell-free nucleic acids used as main targets for liquid biopsy in prostate cancer including the specific biomarkers, the body fluids where the biomarkers were detected, the methods used for their detection and their clinical values

Study	Biomarkers	Body fluids	Detection methods	Clinical values
[36]	AR copy number and choline uptake	Blood plasma	qPCR, dPCR and FCH-PET/CT	Prognosis Treatment
[29]	miR-21-5p, miR-200c-3p, miR-210-3p, miR-375, miR-30c-5p, miR-223-3p,r Let-7a-5p, miR-141-3p and miR-106a-5p	Blood, plasma	RT-qPCR	Diagnosis Prognosis
[80]	CTC enumeration, miR-141, miR-200a, miR-200b, miR-210, miR-375	Blood plasma	CellSearch (Veridex) and RT-qPCR	Prognosis
[81]	AR copy number	Blood plasma	qPCR and dPCR	Prognosis Treatment
[34]	ALU sequence quantification and integrity	Blood plasma	qPCR	Prognosis
[82]	AR copy number, and 19 cancer associated genes	Blood plasma	Deep (Illumina) and targeted (Ion Torrent) NGS	Prognosis
[83]	c-MYC, HER-2 and AR integrity	Urine	RT-qPCR	Treatment response
[84]	cf-DNA concentration	Blood serum	Spectrophotometry	Diagnosis
[85]	Copy number of cancer-related genes, AR and CTC enumeration	Blood plasma	Comparative genomic hybridization, deep sequencing (Illumina) and CellSearch (Veridex)	Prognosis
[86]	let-7c, miR-30c, miR-141, and miR-375	Blood plasma	RT-qPCR	Treatment
[33]	Copy number variations of cf-DNA (chromosomal instability)	Blood serum and plasma	WGA and massive parallel sequencing by SOLiD (Life Technologies)	Diagnosis Prognosis
[87]	ALU sequence quantification and integrity	Blood plasma	qPCR	Prognosis
[88]	cfDNA quantification	Blood plasma	PicoGreen dsDNA Quantification Reagent Kit (Molecular Probes) and fluorometry	Prognosis
[89]	cfDNA quantification	Blood serum	qPCR for the GSTP1 gene	Recurrence
[17]	cBMP6 mRNA, cf-DNA, apoptotic nucleosomes and H3K27me3	Blood plasma	RT-qPCR, qPCR for the Sat2 sequence, Cell Death Detection ELISA kit (Roche Diagnostics) and ELISA-based EpiQuik Global Histone H3K27me3 Assay Kit (Epigentek)	Diagnosis

AR, androgen receptor; ((RT)-(q))PCR, reverse tanscription quantitative polymerase chain reaction; dPCR, digital polymerase chain reaction; FCH-PET/CT, 18F-fuorocholine positron emission tomography/computed tomography; miR(NA), microRNA; CTC, circulating tumor cell; ALU, Arthrobacter Luteus; NGS, next generation sequencing; cMYC, cellular MYC; HER-2, human epidermal growth factor receptor-type 2; cf-DNA, cell-free DNA; WGA, whole genome amplification; SOLiD, sequencing by oligonucleotide ligation and detection; dsDNA, double-stranded DNA; GSTP1, glutathione S-transferase P1; ELISA, enzyme linked immunosorbent assay; cBMP6, circulating mone-morphogenetic protein 6; mRNA, messenger RNA; H3K27me3, trimethylation at lysine (K) 27 of histone 3.

Table 3. List of studies with extracellular vesicles used as main targets for liquid biopsy in prostate cancer including the specific biomarkers, the body fluids where the biomarkers were detected, the methods used for their detection and their clinical values

Study	Biomarkers	Body fluids	Detection methods	Clinical values
[90]	IAP proteins (Survivin, XIAP, cIAP-1, cIAP-2)	Urine	Western blot	Prognosis
[91]	miR-572, miR-1290, miR-141, and miR-145	Urine	All-in-One miRNA qRT-PCR Detection Kit (GeneCopoeia)	Diagnosis
[41]	Prostate-specific transcripts such as KLK3, PCA3, and ERG; kidney- and bladder-specific transcripts in EVs	Urine	TaqMan qPCR and targeted sequencing using multiplex Precise Assay (Cellular Research)	Diagnosis
[92]	miR-21, miR-141, miR-214, miR-375, and let-7c	Urine	miRNeasy serum/plasma kit (Qiagen) and RT-qPCR	Diagnosis
[93]	ADSV-TGM4 and CD63-GLPK5-SPHM-PSA-PAPP	Urine	Mass spectrometry, Western blot and immunohistochemistry	Diagnosis Prognosis
[94]	Protein N-glycosylation, EV concentration and PSA	Urine	DNA-sequencer-assisted fluorophore-assisted carbohydrate electrophoresis, and centrifugation with *n*-butanol	Diagnosis
[43]	ERG+PCA3 transcripts (ExoDx Prostate Intelliscore)	Urine	RT-qPCR	Diagnosis
[95]	PSMA in prostate microparticles and CTC enumeration	Blood plasma	Nanoscale flow cytometry and CellSearch	Prognosis
[96]	miRNA-141	Blood serum	miRNeasy Serum/Plasma kit (Qiagen) and RT-qPCR using the PrimeScript RT Reagent kit and SYBR Premix Ex Taq kit (Takara Bio)	Diagnosis Prognosis
[97]	RNA copy numbers of ERG and PCA3 (EXO106 score)	Urine	RT-qPCR	Diagnosis
[45]	CD63, CD9	Urine	TR-FIA (a sandwich ELISA)	Diagnosis Prognosis
[48]	miRNAs: miR-1290 and miR-375	Blood plasma	RNAseq by Illumina HiSeq2000 DNA sequence analyzer and RNA quantification by TaqMan MicroRNA Assays (Life Technologies) and miScript SYBR Green PCR Kit (Qiagen)	Prognosis
[98]	Serum miR-141 and miR-37; and urine miR-107 and miR-574-3p	Blood plasma and serum, and urine	RT-qPCR by miRCURY LNA Universal RT miR PCR kit (Exiqon) and miR ready-to-use PCR Human Panel I+II (Exiqon)	Prognosis Diagnosis

IAP, inhibitor of apoptosis protein; XIAP, X-linked inhibitor of apoptosis protein; cIAP, cellular inhibitor of apoptosis protein; miR(NA), microRNA; ((RT)-(q))PCR, reverse transcription quantitative polymerase chain reaction; KLK3, kallikrein-related peptidase 3; PCA3, prostate cancer antigen 3; ERG, erythroblast transformation-specific related gene; ADSV, adseverin; TGM4, transglutaminase 4; CD, cluster of differentiation, GLPK5, glycerol-3-phosphate kinase 5; SPHM, N-sulphoglucosamine sulphohydrolase; PSA, prostate specific antigen; PPAP, prostatic acid phosphatase; PSMA, prostate specific membrane antigen; TR-FIA, time-resolved fluorescence immunoassay; ELISA, enzyme linked immunosorbent assay; RNA-seq, RNA sequencing.

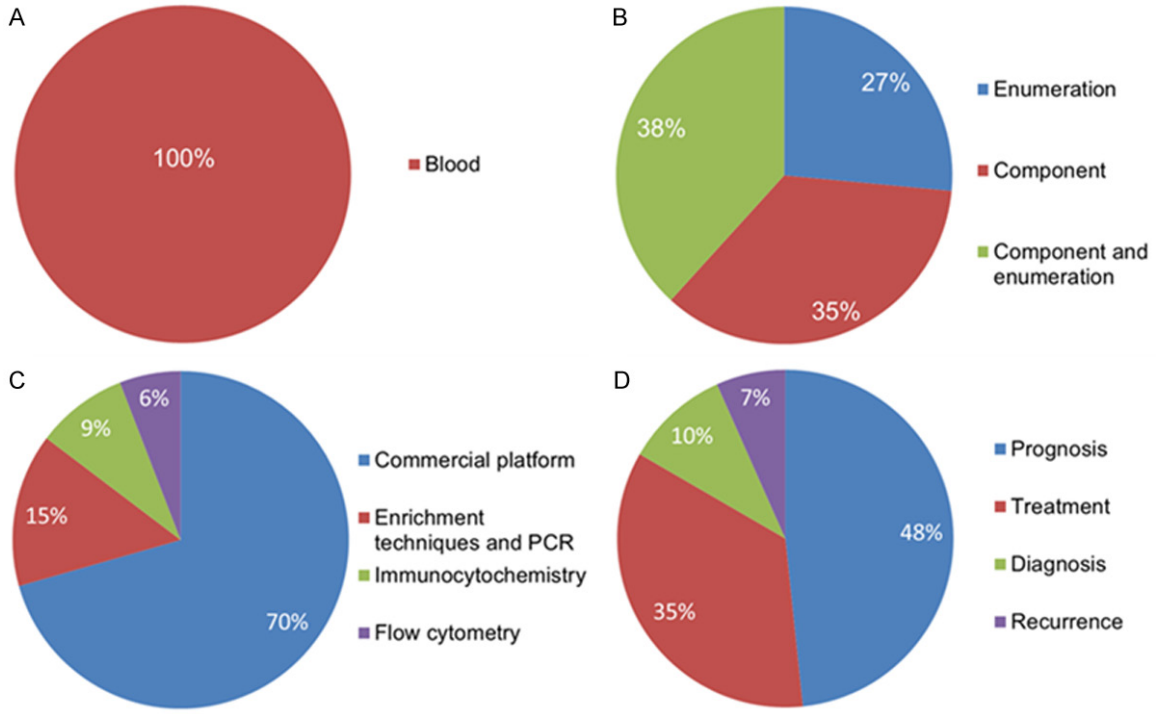

Figure 4. Characteristics of liquid biopsies from CTCs of prostate cancer patients evaluated in the selected studies. A. Body fluids as source of CTCs. B. Biomarkers assessed in CTCs. C. Detection methods of biomarkers. D. Clinical values of biomarkers. CTCs, circulating tumor cells; PCR, polymerase chain reaction.

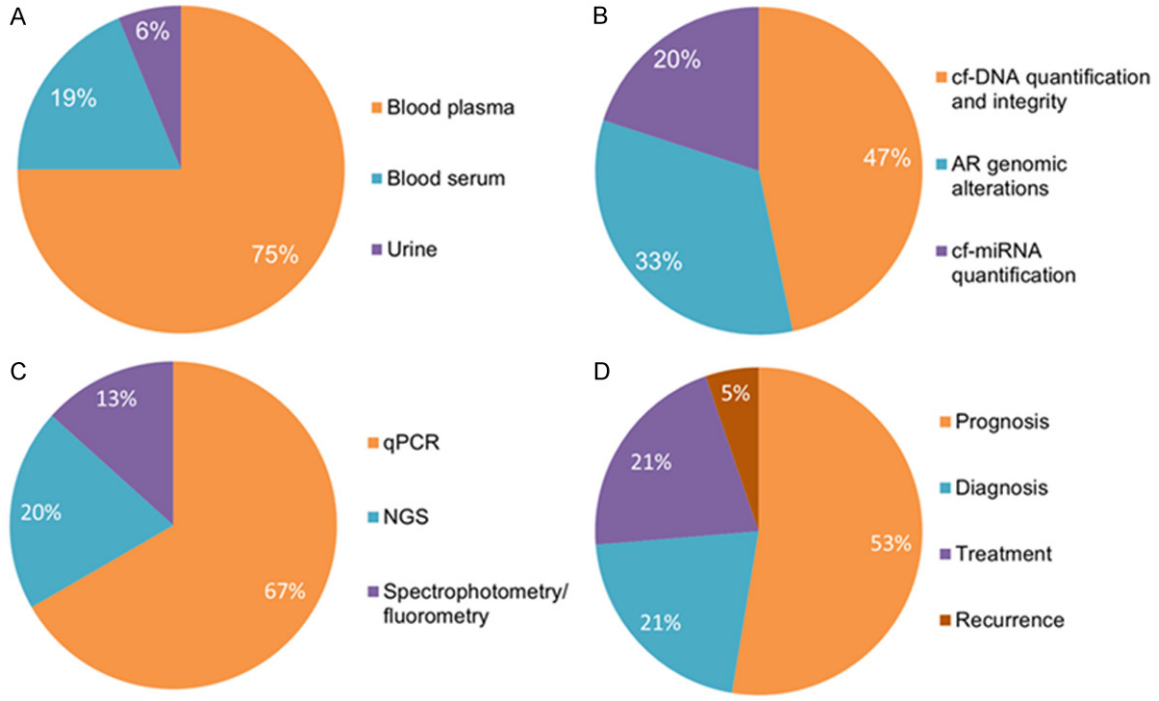

Figure 5. Characteristics of liquid biopsies from cf-nucleic acids of prostate cancer patients evaluated in the selected studies. A. Body fluids as source of cf-nucleic acids. B. Biomarkers assessed in cf-nucleic acids. C. Detection methods of biomarkers. D. Clinical values of biomarkers. cf-nucleic acids, cell-free nucleic acids; cf-DNA, cell-free DNA; AR, androgen receptor; cf-miRNA, cell-free microRNA; qPCR quantitative polymerase chain reaction; NGS, next generation sequencing.

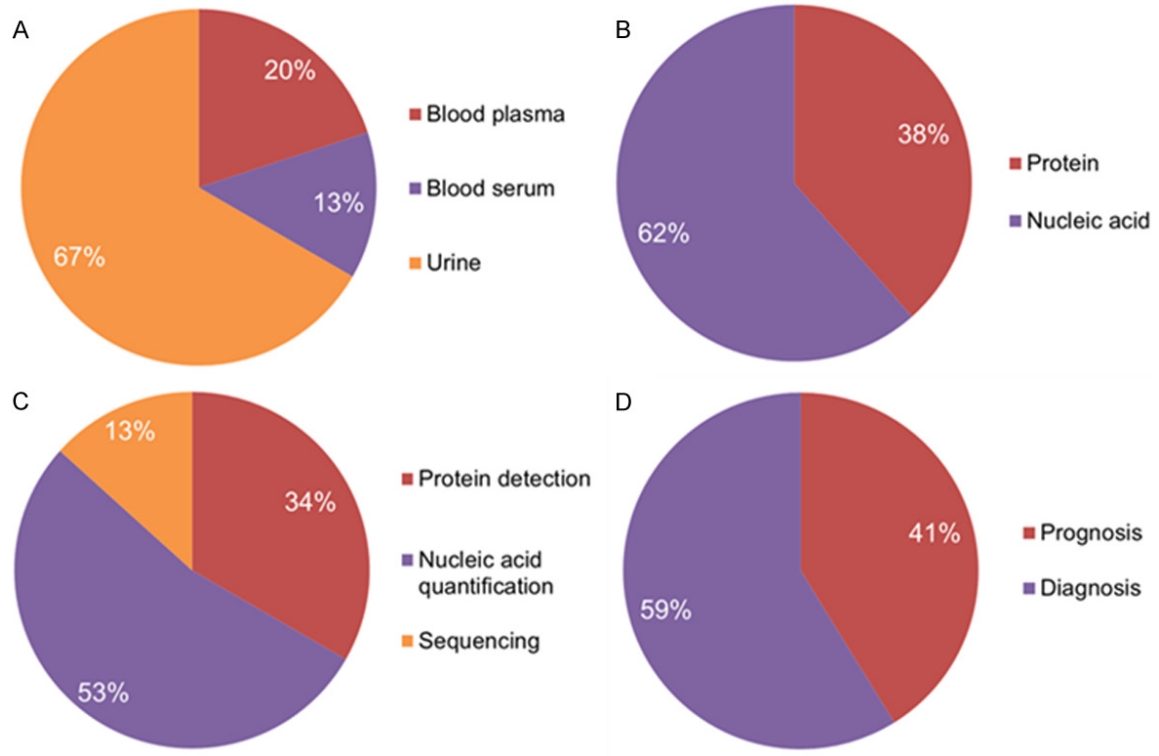

Figure 6. Characteristics of liquid biopsies from EVs of prostate cancer patients evaluated in the selected studies. A. Body fluids as source of EVs. B. Biomarkers assessed in EVs. C. Detection methods of biomarkers. D. Clinical values of biomarkers. EVs, extracellular vesicles.

reaching almost 20% of the studies. Only one study reported urine samples (**Figure 5A**).

Our findings showed that most of the articles (seven) analyzed the quantity and integrity of cf-DNA as a molecular marker of PCa (**Figure 5B**). Five out of our 15 selected articles on cf-nucleic acids reported the androgen receptor (AR) as a PCa biomarker. In addition, three studies associated the quantification of a pool of several cell-free microRNAs (cf-miRNAs) [29, 80, 86] (**Table 2**).

Around 70% of the retrieved articles performed PCR assays to quantify and/or detect specific genes or non-coding sequences. Twenty percent of them used next generation sequencing (NGS) technologies. Just two articles [84, 88] quantified and assessed the quality of cf-nucleic acids by spectrophotometry or fluorometry (**Figure 5C**).

More than half of the clinical values reported were prognosis, whereas both diagnosis and treatment accounted for more than 20% of them (**Figure 5D**). Recurrence was reported once [89] (**Table 2**).

EVs in liquid biopsies of PCa

Figure 6 shows the biomarkers, the body fluids, the detection methods and the clinical values of the retrieved studies about EVs in PCa (**Table 3**).

Of the 13 articles selected on EVs as targets in liquid biopsies of PCa, almost 70% of the analyses were performed with urine and the remaining ones with blood (**Figure 6A**).

Among the biomarkers studied, more than 60% of the articles reported about nucleic acids and less than 40%, about proteins (**Figure 6B**). The quantification of cell-free RNA (cf-RNA), namely cell-free messenger RNAs (cf-mRNAs) and cf-miRNAs, was performed in eight of the selected papers. The biomarkers erythroblast transformation-specific related gene (ERG) and prostate cancer antigen 3 (PCA3) were analyzed in the three selected papers about cf-mRNA in

EVs [41, 43, 97]. Regarding the explored proteins, they were either PCa-related such as PSMA, or EV-related proteins such as the tetraspanin (TSPAN) CD63 (**Table 3**).

From the selected articles about EVs, more than half of them has a method based on nucleic acid quantification. In these articles, authors validated total yield of DNA or RNA extractions by spectrophotometry and detected specific sequences by additional PCR. Thirteen percent of the articles went further with sequencing technologies to detect mutations in specific biomarkers such as oncogenes or tumor suppressor genes. Alternatively, more than 30% of the articles identified biomarkers based on methods for protein detection such as Western blot, enzyme linked immunosorbent assay (ELISA), mass spectrometry, flow cytometry and electrophoresis (**Figure 6C**).

Among our selected papers about EVs in PCa, diagnosis was the main clinical value, followed by prognosis value (**Figure 6D**).

Discussion

Body fluids in liquid biopsies of PCa with CTCs as targets

The prostate gland produces the prostatic fluid that contributes up to 30% of the seminal fluid mixture [18]. Consequently, the seminal fluid is a direct source of PCa components for early-detection biomarkers. In comparison with other body fluids, PCa components in the seminal fluid do not need to get through other tissues to be detected. Regarding the urine, PCa components may remain in the urethra and can be incorporated to the flux of urine. As the prostate gland surrounds the urethra, another way to find PCa components in the urine can be the migration of them from the prostate to the urinary tract tissues. This can be also the case of PCa components in stool samples. The prostate gland is situated in front of the rectum. Therefore, stool may load some PCa components. Blood delivers nutrients to PCa cells and can transport PCa components away from the prostate gland to other tissues. Therefore, blood, seminal fluid, urine, and stool would be the body fluids with higher chances of carrying PCa components.

In our selection process, we did not find articles reporting seminal fluid, stool or other body fluids more distal from the prostate gland such as saliva, tears or sweat as sources for PCa biomarkers. This may indicate that articles reporting these body fluids for PCa monitoring are emerging and only test small groups of patients. All body fluids previously mentioned can be consider as sources of liquid biopsies, since they are susceptible of being involved in non-invasive procedures. However, it should be noted that blood extraction requires a puncture that it may cause some discomfort to the patients. Therefore, blood extraction is sometimes referred to as a source of liquid biopsy with a minimal-invasive technique.

With the discovery of Ashworth in 1869 [1], CTCs were the first targets of liquid biopsies. In addition, blood was the first body fluid to be investigated for liquid biopsies, probably because of this historical link between CTCs and blood. In fact, our selected articles evidenced a higher proportion of research on liquid biopsies of PCa with CTCs and blood samples.

The research on CTCs developed a broad range of approaches and improvements for their clinical utility, reflected in some advances in oncology related to the fields of scientific knowledge and technology. One example of these advances is the FDA approval of the CellSearch method (Veridex, Raritan, NJ, USA) for CTC enumeration as a prognostic biomarker in metastatic castration-resistant prostate cancer (mCRPC). Thus, this important breakthrough has further increased the research of CTCs in this type of cancer.

The crossing of tissue barriers by CTCs until reaching the bloodstream may yield a low CTC detection rate in blood. Thus, CTCs could be detected earlier and in larger quantities in seminal fluid [19] and urine than in blood. Furthermore, localized PCa shows lower levels of CTCs and other tumor components in blood than metastatic PCa. These facts mean a technological challenge for researchers. Therefore, enrichment techniques are necessary for CTC detection in blood of patients with PCa, particularly in those with non-metastatic PCa [20].

Biomarkers in liquid biopsies of PCa with CTCs as targets

Although the use of CTC enumeration in peripheral blood has been considered a biomarker for

reliable prognosis of mCRPC, researchers try to improve its performance with the combination of other biomarkers. For example, Chang et al. designed a reverse transcription quantitative polymerase chain reaction (RT-qPCR) assay to determine the expression profiles of epithelial-mesenchymal transition (EMT) and stem cell genes of peripheral blood mononuclear cells to verify whether they could complement CTC enumeration [21]. Studies of CTC enumeration associated with specific biomarkers showed that additional biomarkers increase the predictive accuracy of CTC enumeration. Lindsay *et al.* showed that CTC enumeration associated with the analysis of vimentin and Ki67 protein expression can straightforwardly be assessed in CTCs from patients with mCRPC [22]. Addition of CTC enumeration to the "ALPHA model" that contains the standard markers albumin, lactate dehydrogenase, PSA, hemoglobin and alkaline phosphatase improved the prognostic power of this model in mCRPC patients [23]. Thus, CTC enumeration would provide complementary information for clinical practice. On this account, molecular analysis of CTCs rather than CTC enumeration may yield more valuable information for the clinical management of PCa patients [20].

Detection methods in liquid biopsies of PCa with CTCs as targets

CTC detection in blood requires a first step of enrichment to isolate these rare cells from other more abundant circulating components. Consequently, the subsequent analysis for the detection of molecular biomarkers in CTCs will depend on the enrichment technique [24]. Centrifugation, sometimes combined with density-gradient solutions, allows the physical isolation of any type of CTCs from the peripheral blood mononuclear cell layer. Microfiltration is an alternative for physical isolation that is able to isolate even CTC clusters. According to Sackmann *et al*., microfluidics are being widely used in experimental research; but despite their advantages over other approaches, they are not a prevailing methodology because other well-established technologies have been improving over the last years [25]. Therefore, the handful of articles about microfluidics in liquid biopsy of PCa that we may find in the literature is probably testing their potential on a small scale and it is not among our selected articles.

Isolation techniques can be combined with immunofluorescence techniques to increase their sensitivity. As most CTCs in PCa derive from epithelial cancer cells, they usually express cytokeratins and other epithelial markers such as the epithelial cell adhesion molecule (EpCAM) and lack the leukocyte antigen cluster of differentiation 45 (CD45). Thus, our retrieved articles showed that antibodies against these markers could label PCa CTCs for their selection. The commercial method CellSearch use these antibodies for immunomagnetic capture of epithelial CTCs from centrifuged peripheral blood to enumerate CTCs. However, CTCs from prostate also express tissue-specific markers. Besides immunomagnetic capture of CTCs from peripheral blood by epithelial and tumor antigens, the AdnaTest method lyses CTCs to isolate their mRNA. In this way, this commercial test analyzes the expression profiles of tissue-specific antigens such as the prostate specific membrane antigen (PSMA), the PSA, the epidermal growth factor receptor (EGFR) and the AR through RT-PCR. In addition, the Epic CTC platform found in our search combines immunofluorescence with size and shape measurements to detect a wide variety of CTCs from images of lysed blood on glass slides. Interestingly, the Epic CTC platform is not limited to the analysis of CTCs that express epithelial markers. This commercial method allows the analysis of non-epithelial CTCs, such as those that undergo the EMT, and even clusters of CTCs.

Clinical values in liquid biopsies of PCa with CTCs as targets

The development of tools aimed at deciphering the clinical outcome is becoming a significant area in liquid biopsies of PCa [26]. However, researchers still find some troubles. In line with our results from studies on CTCs, most studies with biomarkers in PCa remain unsatisfactory to predict recurrence of the disease after treatment of patients [16].

Body fluids in liquid biopsies of PCa with cf-nucleic acids as targets

Cf-nucleic acids are of diverse nature, RNA or DNA, and appear in a wide variety of body fluids. Although we mostly found them in blood plasma, the detection of cf-nucleic acids from urine samples for PCa diagnosis seems a prom-

ising technology due to its high adaptability to the clinical settings [27].

Biomarkers and clinical values in liquid biopsies of PCa with cf-nucleic acids as targets

Cf-nucleic acid signatures have potential uses in PCa clinical practice because they are able of updating the status of cancer. During cancer progression, cf-nucleic acids have been mainly explored in terms of levels of content or quantity, rather than levels of genetic expression. Among cf-RNAs in the scientific literature, we find chronologically cf-mRNAs, cf-miRNAs and cell-free long non-coding RNAs (cf-lncRNAs). Remarkably, PCA3 is a cf-lncRNA isolated from urine and the only marker of the cell-free nucleic acid type that is approved by the FDA, specifically for diagnosis of patients with a first negative tissue biopsy [28]. Our findings showed that combinations of cf-miRNAs are promising biomarkers but they are still validating their clinical value. Interestingly, one selected article compared the diagnostic application of cf-miRNAs and miRNAs in blood plasma [29]. As the first time that cf-miRNAs were associated to cancer was in 2008 [30], our results provide evidence of the growing interest of this target in liquid biopsies of PCa during the last years. Regarding cf-mRNAs, they have been studied for long, as they represent the DNA that is being translated at that moment. However, there are some challenges in relation to their clinical applications. Since cf-mRNAs are expressed under certain conditions of the cancer development, they display a high variability among patients [31]. At the same time, this characteristic makes cf-mRNAs promising candidates for personalized medicine.

The integrity, copy number variation, mutations and methylation of cf-DNAs have also been extensively studied as cancer biomarkers. In fact, cf-DNA was the first cf-nucleic acid that was related to cancer and it was found in blood [32]. The term cf-DNA is commonly associated with cancer, but other physiological processes and conditions can release cf-DNA to the bloodstream and other body fluids. The copy number variation of several random chromosomal regions on cf-DNA is a way to analyze the quantity and integrity of cf-DNA [33]. Another way is the use of a short interspersed element (SINE) such as the *Arthrobacter luteus* (ALU) sequence. The ALU sequence is the most frequent repetitive DNA sequence of the human genome. The ratio of their long (247 base pairs) and short (115 base pairs) amplicons is used to estimate cf-DNA integrity by qPCR, considering that the small amplicons are derived from tumor cells [34]. For example, the ALU biomarker in cfDNA from blood plasma samples demonstrated to determine prognosis of metastatic PCa patients [34]. Another research by Deligezer *et al*. cunningly combined the quantifications of cf-DNA and a tumor-related mRNA with the detections of histone-complexed DNA fragments and a post-translational histone modification [17]. Moreover, the high proportion of selected studies about cf-nucleic acids reporting the AR as a PCa biomarker may be explained because its signaling axis is the most important in the pathogenesis of this tumor. The AR signaling axis promotes mechanisms of somatic mutations, splice variants and, in some cases, therapeutic resistance [35]. For instance, the AR gene aberrations in cf-DNA are associated with therapeutic resistance in mCRPC [36].

Detection methods in liquid biopsies of PCa with cf-nucleic acids as targets

Although the presence of endogenous nucleases may jeopardize the stability of cf-nucleic acids during manipulation and researchers have stated some concerns about it, cf-nucleic acids seemed to have enough resistance to nuclease action. In fact, cf-nucleic acids are naturally protected in EVs and protein complexes such as nucleosomes, and even attached to cell surfaces [37].

Cf-nucleic acid research requires PCR and sequencing techniques for their analysis. Concentration and purification processes of cf-nucleic acids are usually performed with commercial kits for their maximum recovery, using simple and fast methods to obtain great quality results for the early screening and staging of PCa [38]. Both urine and blood (serum and plasma) samples undergo several centrifugation steps and resulting supernatants are stored at -80°C until their use. Purified DNA and RNA are quantified by spectrophotometry or fluorometry techniques in order to verify not only their amount but also their quality. Of the five types of applications of NGS technologies

[39], all were reported except for the interactome-based sequencing, also known as ChiP sequencing (chromatin immunoprecipitation combined with DNA sequencing). However, one of our retrieved studies used ELISA-based techniques instead [17]. These reported NGS technologies included genome-based or DNA sequencing, transcriptome-based or RNA sequencing, methylome-based sequencing and sequencing for the detection of chromosomal rearrangements and copy number variations derived from insertions and/or deletions. These technologies applied in the analysis of cf-nucleic acids allow the comparison of different parameters and genes at the same time, becoming an attractive approach for the search of PCa biomarkers.

Body fluids and biomarkers in liquid biopsies of PCa with EVs as targets

EVs can be isolated from body fluids by diverse types of centrifugation, mainly gradient-density centrifugation or ultracentrifugation. The detection of the family of membrane proteins TSPANs is another common method for EV purification, which we reported in our selected articles. Cells exchange proteins, nucleic acids, sugars and lipids through EVs to induce changes in the recipient cells. Therefore, EVs are potential carriers of cancer biomarkers from tumor cells to other tumor or non-tumor cells. Since biomolecules are less susceptible to degradation when they are protected by the lipid layer of the EVs, the stability of cf-nucleic acids in body fluids is lower compared to that of the nucleic acids contained in EVs from the same body fluid [40]. In this case, the concentration of PCa biomarkers, such as the serum PSA concentration, would be higher in EVs. Therefore, the study of the EV content is interesting because it may improve the sensitivity of new and well-established PCa biomarkers.

It was found that concentration of RNA-based biomarkers was higher in EVs than in CTCs from urine samples [41]. Remarkably, in our search, urine was the most used body fluid for detection of biomarkers in EVs from liquid biopsies of PCa. Despite sometimes urine samples are collected after a digital rectal examination, which is considered as an embarrassing procedure, the use of urine as a body fluid for clinical testing seems to be a popular choice. Comparing to blood, EVs from urine have larger size, but yet lower concentration [42]. However, the relevance of EVs from urine samples in the PCa clinical context is supported by the use of the biomarkers ERG and PCA3 in EVs that were combined with the serum PSA in a diagnostic test that was reported in our search [43].

Detection methods in liquid biopsies of PCa with EVs as targets

Isolation methods for EVs still challenge authors [44], and this may be reflected in the later publication of our selected papers about EVs in PCa from 2012. Authors are mostly constrained by the purity and efficiency achieved by the laboratory procedures. In this perspective, our results showed a wide use of extraction kits for the isolation and purification of EVs. Detection of EVs requires previous purification and concentration steps with filtration, ultracentrifugation, precipitation or immunological techniques [45]. Following these steps, EV enrichment is usually verified by visualization through transmission electron microscopy or nanoparticle tracking analysis. Additional verification is performed by indirect quantification through the measurement of their protein content, or the detection of specific proteins or their corresponding activity. One thing to bear in mind when discussing about the EVs is that those of endosomal origin and size from 40 to 100 nanometers are called exosomes. However, current detection methods fail to completely purify one type of the EVs [46].

Clinical values in liquid biopsies of PCa with EVs as targets

Although some studies have reported the ability of EVs to monitor PCa treatments [47], we did not find this clinical value in liquid biopsies of PCa with EVs. The miRNAs in EVs are promising prognostic biomarkers for mCRPC patients, but prospective validation is needed for the development of these candidates [48]. Further research in this area will improve their application in diagnostic and prognostic tests [49]. Therefore, the incipient use of EVs for treatment monitoring could also open the way to use EVs to detect recurrence of previously diagnosed PCa patients.

Conclusions

In this literature review, we examined the different targets for liquid biopsy in PCa from a selection of reliable articles published in the decade of 2010s. The retrieved articles, with cohorts of at least 50 patients, aimed to contribute with their findings to a critical foundation for the analysis of the clinical application of liquid biopsies in PCa. Accordingly, evidence-based changes detected by patient-friendly techniques during PCa progression could promote a better orientation in patient care.

Despite commercial approaches exist, the fact that 90 articles were excluded due to a small patient cohort, together with the moderate quality of the selected articles, may reflect that the implementation of liquid biopsies in PCa is in its infancy and preliminary studies are still taking place to test better approaches. Although there are hundreds of studies using different methods for liquid biopsies of PCa, validation of data from small cohorts is a great challenge. Larger cohorts of PCa patients and multicenter studies will yield robust results that will translate these technologies to medical practice to develop the definitive liquid biopsy.

We found a wider use of CTCs as targets for biomarker discovery. Researchers preferred blood as body fluid to be tested. However, they show a trend towards the use of urine for PCa testing that it will probably continue in coming years. The main methods for biomarker prospection in PCa were related to gene expression, RT-PCR and NGS, demonstrating the growing interest in transcriptome analysis.

The main problem of researchers found in liquid biopsies of PCa was the isolation technologies involving enrichment techniques of low efficiency. Moreover, specific guidelines should be implemented to tackle the high variability of isolation and detection techniques for CTCs, cf-nucleic acids and EVs. These guidelines will standardize the laboratorial procedures in order to allow better comparisons between different studies. Consequently, we could establish the best line of research to better invest our resources bringing high-throughput techniques to detect biomarkers with high accuracy. Thus, advances in liquid biopsies will result in theranostic tools for PCa management, with more personalized therapies and new drugs.

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Disclosure of conflict of interest

None.

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