Liquid biopsies, comprising the noninvasive analysis of circulating tumor-derived material (the ‘tumor circulome’), represent an innovative tool in precision oncology to overcome current limitations associated with tissue biopsies. Within the tumor circulome, circulating tumor DNA (ctDNA) and circulating tumor cells (CTCs) are the only components the clinical application of which is approved by the US Food and Drug Administration (FDA). Extracellular vesicles (EVs), circulating tumor RNA (ctRNA), and tumor-educated platelets (TEPs) are relatively new tumor circulome constituents with promising potential at each stage of cancer management. Here, we discuss the clinical applications of each element of the tumor circulome and the prevailing factors that currently limit their implementation in clinical practice. We also detail the most recent technological developments in the field, which demonstrate potential in improving the clinical value of liquid biopsies.

**Liquid Biopsies: Investigating the ‘Tumor Circulome’**

Cancer is one of the leading causes of death worldwide, with 9.6 million cancer deaths estimated in 2018. In the USA, more than 1.735.350 diagnoses of cancer were estimated in 2018, causing more than 609,640 deaths. The development of omics technologies has led to the field of precision oncology, which comprises tailoring treatment regimens to the molecular characteristics of each patient’s tumor [1]. The current gold standard for genetic profiling of tumors typically involves the use of tissue biopsies. Given their invasive nature, tissue biopsies are associated with many limitations, including patient risk, sample preparation, sensitivity (see Glossary) and accuracy, procedural costs, and invasive testing. This makes the procedure incompatible for clinical longitudinal monitoring [2]. Furthermore, a significant limitation of tissue biopsies is that they fail to capture intratumoral and intermetastatic genetic heterogeneity, impacting the accuracy of the test [3].

Liquid biopsies have a great potential to overcome these existing sampling limitations. Such biopsies comprise the sampling and analysis of liquid biological sources, typically blood, for cancer diagnosis, screening, and prognosis. The ‘tumor circulome’, defined as the subset of circulating components, is derived from cancer tissue and can be directly or indirectly used as a source of cancer biomarkers in liquid biopsies [4]. These components include circulating tumor proteins, circulating tumor nucleic acids (ctDNA and ctRNA), CTCs, EVs, and TEPs (Figure 1, Key Figure). Liquid biopsies present several advantages over conventional tissue biopsies (Table 1), and technological advancements in sample isolation (e.g., the development of nucleic acid extraction chips to minimize the manipulation of samples [5]) and detection platforms (e.g., the development of high-resolution flow cytometers [6] or single-cell western blot (scWB) platforms [7]) are evolving to support this approach. The first important milestone in this field was reached in 2016 with the FDA approval of the first companion diagnostic test for lung cancer based on the ctDNA content of a liquid biopsy [8].

---

**Highlights**

Liquid biopsies are emerging as a favorable alternative to conventional tissue biopsies, providing a noninvasive approach for the detection and monitoring of cancer biomarkers.

- The FDA approval of a companion diagnostic test for lung cancer and a screening test for colorectal cancer based on the analysis of ctDNA were important milestones in the clinical implementation of liquid biopsies.

- Circulating EVs, RNAs, and TEPs are promising new constituents of liquid biopsies.

- Recent technological developments in the field have the potential to address current limitations of liquid biopsies and enhance the sensitivity of cancer biomarker detection.

---

1 Discipline of Pharmacy, Graduate School of Health, University of Technology Sydney Australia, PO Box 123, Broadway, NSW 2007, Australia

Correspondence: mary.bebawy@uts.edu.au (M. Bebawy).
In this review, we detail the clinical significance and potential of liquid biopsies and provide an overview of recent reports supporting elements of the tumor circulome as biomarkers for the diagnosis and monitoring of cancer, with a particular focus on ctDNA, CTCs, tumor-derived EVs, and ctRNAs. We also discuss factors limiting their implementation in clinical practice and outline significant technological advances that may overcome these. Although our focus is on blood as the biological matrix for the liquid biopsy, other biological fluids also comprise a source of promising tumor-derived biomarkers (Box 1).

Circulating Tumor-Derived Proteins
The measurement of circulating protein markers has historically been the gold standard approach used for noninvasive diagnosis, screening, and postoperative follow-up in cancer management. Notable examples of circulating tumor-derived protein markers include the prostate-specific antigen (PSA) for prostate cancer screening [9] and cancer antigen (CA) 15-3 for postoperative follow-up of breast cancer recurrence [10]. These are compromised by high false positive rates, which can lead to overdiagnosis and, in some cases, unnecessary anticancer treatment [9]. In the case of CA 15-3, there are questions around its application in improvements in patient outcomes [10]. The use of panels or biosignatures comprising more than one protein is a more promising approach, because the combination of multiple biomarkers increases the diagnostic and/or prognostic capability of the assay by reducing the number of false positives and false negatives [11,12].

Circulating Tumor DNA
ctDNA comprises the fraction of circulating cell-free DNA (cfDNA) originating from cancer cells. This includes short nucleosome-associated fragments (80–200 bp) [13] and longer fragments (>10 kb) encapsulated within EVs [14]. The mechanisms of ctDNA release into circulation include apoptosis, necrosis, lysis of CTCs, and active secretion from the tumor [15]. The proof of the suitability of ctDNA as a cancer biomarker came with the identification of KRAS gene mutations in ctDNA from the blood of patients with pancreatic cancer [16].

Clinical Significance of ctDNA Analysis
Both qualitative and quantitative information can be obtained from ctDNA analysis [13]. Quantitative information can be obtained from the measurement of the mutant allele fraction (MAF, the percentage of mutant allele in a given locus) and is a reflection of tumor burden [13]. It finds application in the detection of minimal residual disease (MRD) and occult metastases [17] and in the monitoring of treatment response and therapeutic effectiveness [18]. ctDNA levels provide a ‘real-time’ snapshot of tumor bulk because of the short half-life of ctDNA (~2.5 h) [13]. The detection of ctDNA after treatment is a high-sensitivity and high-specificity predictor of relapse [19].

Qualitative information can be sourced through the profiling of mutations, amplifications, deletions, and translocations in ctDNA (Figure 1), allowing the identification of genetic alterations associated with response, hence supporting decision-making for personalized management [13]. For example, the first ctDNA-based companion diagnostic test (cobas® EGFR Mutation Test v2; Roche Diagnostics), recently approved by the FDA [8], is used to guide the use of epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors on the basis of specific EGFR-sensitizing mutations in patients with non-small cell lung cancer (NSCLC). Other qualitative information obtainable through ctDNA analysis includes assessment of methylation status. For example, a screening test for colorectal cancer (CRC), Epi proColon®, was recently approved by the FDA and analyzes the methylation pattern of the promoter of the SEPT9 gene, a region known to be hypermethylated in CRC compared with non-malignant samples [20].

Glossary
4,6-diamidino-2-phenylindole (DAPI): a fluorescent dye that binds to AT-rich regions on DNA and is used to stain nuclei.
Apoptosis: programmed cell death; can be induced by external stimuli and is a tightly regulated process.
Beads, emulsion, amplification, magnetics (BEAMing): highly sensitive dPCR method that combines emulsion PCR and flow cytometry to identify and quantify DNA mutations.
Cancer antigen 19-9 (CA 19-9): a portion of the Sialyl-Lewis A antigen. Its presence is highly correlated with advanced epithelial cancers.
Cancer personalized profiling by deep sequencing (CAPP-Seq): a sensitive method of analysis comprising the sequencing of cancer-specific (personalized) panels of genes to identify cancer-specific mutations.
Clonal hematopoiesis: the condition in which a substantial proportion of mature blood cells is derived from a single dominant hematopoietic stem cell lineage. Clonal hematopoiesis has been linked to a greater than tenfold increased risk of developing a hematological cancer.
Companion diagnostic: medical device, often an in vitro test, providing information that is essential and required for the safe and effective use of a corresponding drug. It is often developed simultaneously with the corresponding drug. The cobas® EGFR Mutation Test v2 described in the main text, for example, comprises a PCR-based analysis of a set of mutations, insertions, and deletions on the EGFR gene and is used to inform the use of erlotinib and osimertinib in NSCLC.
Digital PCR (dPCR): a quantitative PCR method that is used for the absolute quantification of DNA, without the need for a calibration curve with samples of known quantities. The initial sample mix (which is prepared as a common dPCR) is split into several individual wells before the amplification step. Following PCR amplification, the absolute quantification of the target is calculated using Poisson statistics, based on the number of positive and
Technological Approaches and Current Limitations

The currently available technologies for ctDNA analysis are based on PCR or next-generation sequencing (NGS). Allele-specific PCR was the first approach used in ctDNA detection [16] and a quantitative PCR (qPCR) variation of this technique is currently adopted by the cobas® EGFR test [8]. Given that the fraction of ctDNA in total ctDNA is usually very low, often <0.01% [21], more sensitive technologies have been developed and successfully used for ctDNA analysis, such as digital PCR (dPCR) [22], droplet digital PCR (ddPCR) [23], and beads, emulsion, amplification, magnetics (BEAMing) [24]. Although very sensitive, quick, and relatively inexpensive, PCR-based assays are limited by low multiplexing capacity, allowing for analysis of only a restricted number of loci in parallel [13].

The sensitivity of NGS-based technologies is lower than that of PCR-based technologies and inversely proportional to the number of loci analyzed, with whole-exome sequencing (WES) having the lowest sensitivity (>5% MAF) [13]. Approaches to enhance the sensitivity of NGS include considering patient- or cancer-specific gene panels, such as in the cancer personalized profiling by deep sequencing (CAPP-Seq) technology [25], or strategies to suppress the background noise generated by random errors occurring during library preparation. These strategies involve tagging each template molecule with unique molecular identifiers (UMIs). These are used by different NGS platforms, such as enhanced tagged amplicon sequencing (eTAm-Seq™) [26]. Another approach to enhance sensitivity includes the selective nuclease digestion of non-mutated DNA, which results in an increase in MAF and has enabled mutation detection down to 0.00003% MAF [27].

Despite its potential, the use of ctDNA as a liquid biopsy has many limitations. Detection sensitivity is a serious concern, especially in early cancer detection, where the low amount of ctDNA may result in a MAF lower than the limit of detection of existing techniques [13]. The sampling of other body fluids, proximal to the putative site of the tumor, can increase the detection rate, at least in individuals at risk due to, for example, hereditary predisposition. This is mainly because, especially at early stages, a proximal body fluid may have a higher concentration of tumor-derived DNA compared with blood [28]. Another concern in early detection is the predictive value of single or small sets of mutations, because cancer-associated mutations can be found in plasma of healthy individuals as a result of clonal hematopoiesis [13]. One approach to overcome this challenge is the CancerSEEK platform, which associates the analysis of eight tumor-derived proteins with ctDNA mutation profiling and has a specificity >99% [29].

Another limitation impeding the implementation of ctDNA analysis into clinical practice is the lack of standardized protocols for preanalytical sample preparation and ctDNA purification. Current procedures are complex and may cause ctDNA degradation and blood cell lysis [30]. A platform allowing the quick, single-step purification of ctDNA from blood is desirable, and lab-on-a-chip systems have the potential to address this need [5].

Circulating Tumor Cells

CTCs are a population of tumor cells that have detached from the primary tumor and can be found in the peripheral blood of patients. Their presence is thought to be fundamental to the development of metastasis [31]. CTCs present systemically through active invasion, with epithelial-to-mesenchymal transition (EMT) as a fundamental step [32], or through passive shedding from the primary tumor. This latter mechanism is supported by the presence of CTC aggregates or circulating tumor microemboli (CTMs) in the blood [33].
Clinical Significance of CTCs and Analytical Technologies

The information that can be obtained from CTCs is quantitative as well as phenotypic (qualitative) through single cell genomic, transcriptomic, and/or proteomic profiling (Figure 1). CTCs have great potential as tools for the diagnosis, monitoring, prognosis, and prediction of response to therapy, and also for the discovery of novel drug targets [34]. Furthermore, the ex vivo culture of CTCs has an important translational value, because it allows personalized drug sensitivity tests to be performed with the aim of basing treatment decision-making on evolving tumor mutational profiles and drug sensitivity patterns found in individual patients [35].

The simplest information obtainable from CTCs is their number, which is a prognostic predictor for many cancers, including metastatic breast, colon, and prostate cancers [36]. Currently the only FDA-approved clinical application of CTCs is the CellSearch™ platform, used for enumeration of epithelial CTCs [36]. The power of CTC counts as a criterion for the selection of the first-line treatment in metastatic breast cancer is currently being investigated in the METABREAST trial (clinicaltrials.gov identifier: NCT01710605).

With regards to the genetic and genomic information obtainable from CTCs, the technologies that can be used are similar to those of ctDNA analysis, and range from qPCR and dPCR-based mutational profiling to targeted NGS and whole-genome sequencing [37]. Additionally, CTCs can be analyzed by cytogenetic analyses, such as fluorescence in situ hybridization (FISH), for the identification of chromosomal rearrangements [38]. NGS technologies have fundamental importance for single-CTC genomic and transcriptomic characterization, in the study of tumor heterogeneity, and in comparative analysis with tissue biopsies [39]. Although the fields of single cell genomics and transcriptomics have experienced significant developments, single-CTC protein analysis is somewhat premature in comparison, with immunocytochemistry and flow cytometry being primarily used, both of which have poor multiplexing capacity. However, new technologies are emerging, and Sinkala and colleagues recently developed a microfluidic-based scWb that was used to assess the levels of eight proteins in single CTCs derived from three patients with metastatic cancer [7].

Despite numerous analytical platforms and technologies available for CTC analysis, their translation into clinical practice is limited by their isolation from blood. Challenges include their extreme rarity, fragility, and physical and phenotypic heterogeneity [34]. Currently available strategies for CTC enrichment and isolation exploit their biological and physical properties, while functionality assays allow CTCs identification (Box 2 and Table 2). Each of these alternatives has advantages and drawbacks, and only their combination can support a comprehensive characterization.

Extracellular Vesicles

EVs are membranous particles released from all cell types under physiological and pathological conditions, as well as following different types of stimuli, including proteases, ADP, thrombin, inflammatory cytokines, growth factors, biomechanical shear and stress inducers, and apoptotic signals [40]. They can be found in almost every bodily fluid, especially blood [41]. Once considered a simple means to eliminate unneeded cellular components from the cytoplasm of cells, over the past decade, EVs have been recognized as fundamental mediators of intercellular communication, regulating and participating in a plethora of physiological and pathological processes, including cancer [41]. Based on their biogenesis, content, and secretory pathways, EVs can be divided into two broad categories: exosomes and microvesicles [41].

that shares many similarities with genetic algorithms.
**Prostate-specific antigen (PSA):** an enzyme, the physiological function of which is to liquefy semen in the ejaculate. It is produced by the prostate gland and its blood concentration is elevated in prostate cancer.
**Sensitivity:** in a binary classification test (positive/negative; healthy/diseased) the sensitivity measures the proportion of actual positives that are correctly identified as positive by the test. It is also called the ‘true positive rate’ (TPR).
**Specificity:** in a binary classification test, the specificity measures the proportion of actual negatives that are correctly identified as negative by the test. It is also called the ‘true negative rate’ (TNR).
**Tyrosine kinase:** an enzyme that catalyzes the transfer of phosphate groups from ATP to tyrosine residues of proteins.
**Whole-exome sequencing (WES):** a genomic technique used for sequencing all the protein-coding genes in a genome (exome).
Clinical Significance of EVs as Cancer Biomarkers

The suitability of EVs as cancer biomarkers lies in the fact that the molecular cargoes they carry can be considered a molecular fingerprint of the cell of origin [42]. Compared with ctDNA and CTCs, the implementation of which in clinical cancer diagnostics is hampered by challenges in their isolation, analytical sensitivity, and stability concerns, the potential advantages of EVs are...
Table 1. Advantages of Liquid Biopsies over Conventional Tissue Biopsies

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Tissue biopsy</th>
<th>Liquid biopsy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Invasiveness</td>
<td>High</td>
<td>Minimal</td>
</tr>
<tr>
<td>Pain</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Risk of complications</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Time needed</td>
<td>Time-consuming</td>
<td>Quick</td>
</tr>
<tr>
<td>Tumor heterogeneity representation</td>
<td>Low/null</td>
<td>High/total</td>
</tr>
<tr>
<td>Tumor region selection bias</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Compatibility with longitudinal monitoring</td>
<td>No</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Box 1. Liquid Biopsy of Biological Specimens Other Than Blood

Although the “standard” concept of liquid biopsy comprises the sampling of blood, almost all bodily fluids are suitable for liquid biopsy. The anatomic localization of primary or metastatic tumors influences the presence of tumor-derived material in the corresponding bodily fluids. Common bodily fluids include urine, saliva, sputum, stool, cerebrospinal fluid (CSF), and pleural effusions. Many of these have demonstrated potential as a source of cancer biomarkers [96,97].

**Urine**

Urine is a valuable source of ctDNA for urogenital cancers, such as prostate, bladder, and cervical cancers, and non-urogenital malignancies, such as NSCLC, CRC, and gastric cancer [98], and its EV content is a source of several candidate biomarkers [99]. The first liquid biopsy test on urine, the Progensa® PCA3 Assay, was FDA approved in 2012 [98] to aid the decision-making of a repeat prostate biopsy in case of a first negative biopsy. This test measures the level of prostate cancer antigen 3 (PCA3) lncRNA, which is increased in >95% of primary prostate tumors [100]. Another promising urine-based test is the ExoDx® Prostate(IntelliScore)®, a laboratory-developed test (LDT) based on the analysis of the levels of three exosome-associated RNAs overexpressed in high-grade prostate cancer, which is used to “rule-out” potentially unnecessary prostate biopsies.

**Saliva**

Salivary biomarkers include ctDNA for head and neck squamous cell carcinoma [101], and miRNAs for detecting early malignancy in potentially malignant oral cancers [102]. Furthermore, EV-associated miRNAs have the potential to be used as biomarkers of oral squamous cell carcinoma [103].

**CSF**

CSF, as a result of its direct contact with the central nervous system (CNS), is set to become an important source of biomarkers for CNS-restricted cancers, potentially overcoming the relative scarcity of circulating biomarkers (especially ctDNA) in these diseases caused by the blood–brain barrier [104]. CSF-derived ctDNA has proven to represent genetic alterations of brain tumors better than plasma ctDNA [105], and a miRNA CSF signature for glioblastoma was recently reported [106].

**Other Bodily Fluids**

The analysis of stool-derived DNA was recently validated as a powerful diagnostic tool for CRC [107], while sputum DNA and protein content have potential in the context of lung cancer [108]. Pleural effusions are also sources of DNA biomarkers for lung cancer [109] and malignant pleural mesothelioma [110], and the presence of epithelial cell adhesion molecule (EpCAM) (see Box 2 in the main text) microparticles allows the distinction between malignant and non-malignant pleural effusions [111].

Many. EVs are typically produced and released in abundant quantities and in greater amounts compared with CTCs [43]. Likewise, the stability of the vesicular cargo is maintained through a protecting outer lipid membrane [44].

Similar to ctDNA and CTCs, EVs can be a source of quantitative and qualitative information. Quantitative information comprising EV numbers can inform the presence of malignant disease and tumor burden. For example, circulating exosome levels are increased in breast and pancreatic cancer [45] and the number of circulating microparticles (MPs) is higher in patients with multiple myeloma (MM) compared with healthy individuals [46]. Furthermore, circulating MP levels demonstrated potential for the diagnosis and prognosis of advanced NSCLC [47].
Qualitative information through the molecular characterization of EV constituents, including nucleic acids and proteins (Figure 1), are the most readily obtained [42]. The RNA content of EVs, including both coding and noncoding (nc)RNAs, has been widely studied [43]. The DNA content of exosomes recently gained attention as a biomarker source in a study in which mutations in KRA S and TP53 were detected in serum exosomes from patients with pancreatic cancer [14]. In another study, the identification of exosomal KRAS mutations proved better than CA 19-9 levels for the prognostic stratification of patients with pancreatic ductal adenocarcinoma (PDAC) [48].

EVs carry proteins in their lumen and membrane, and numerous reports have been published demonstrating the important role of EV proteins as possible cancer biomarkers [42]. Melo et al. demonstrated the ability of circulating exosomal glypican-1 (GPC1) to distinguish PDAC from healthy donor samples with a reported accuracy of 100% [45]. More recently, Moon et al. demonstrated the suitability of EV Del-1 [49] and fibronectin [50] as biomarkers for early breast cancer diagnosis. Furthermore, it was demonstrated that the levels of circulating CD138+ MPs increase in MM, and a significant prognostic potential for CD138+ MPs in predicting risk of relapse and therapeutic response in individual patients was observed [46]. Finally, the levels of
Table 2. Examples of CTC Isolation, Enrichment, and Identification Strategies

<table>
<thead>
<tr>
<th>Category</th>
<th>Principle</th>
<th>Technique/platform</th>
<th>Refs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biological</td>
<td>Surface marker expression</td>
<td>EpCAM+ Enrichment/CellSearch</td>
<td>[36]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>EpCAM+ plus other surface markers</td>
<td>[113]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CD45+ depletion</td>
<td>[114]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GmbH cell collector (in vivo capture)</td>
<td>[117]</td>
</tr>
<tr>
<td></td>
<td>Surface marker expression: microfluidics</td>
<td>CTC-Chip</td>
<td>[115]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NanoVelcro</td>
<td>[116]</td>
</tr>
<tr>
<td>Physical</td>
<td>Size: filtration</td>
<td>ISET™</td>
<td>[118]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Metacell®</td>
<td>[119]</td>
</tr>
<tr>
<td></td>
<td>Size: microfluidics</td>
<td>Parsortix™</td>
<td>[120]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Spiral inertial microfluidic chip</td>
<td>[122]</td>
</tr>
<tr>
<td></td>
<td>Density</td>
<td>Differential centrifugation</td>
<td>[123]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MagDense</td>
<td>[124]</td>
</tr>
<tr>
<td></td>
<td>Dielectric properties</td>
<td>DEP</td>
<td>[125]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DEP-LFFF</td>
<td>[126]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DEPArray</td>
<td>[127]</td>
</tr>
<tr>
<td>Functional</td>
<td>CAM digestion</td>
<td>Vita-Assay™</td>
<td>[128]</td>
</tr>
<tr>
<td>assays</td>
<td>Protein release during culture</td>
<td>EPISPOT assay</td>
<td>[129]</td>
</tr>
<tr>
<td></td>
<td>Telomerase expression</td>
<td>TelomeScan®</td>
<td>[130]</td>
</tr>
</tbody>
</table>

annexinV+ EpCAM+ asialoglycoprotein receptor-1 (ASGPR1)+ circulating MPs are diagnostic of hepatocellular carcinoma and cholangiocarcinoma [51], and the levels of CD147+ EpCAM+ MPs are predictors of CRC [52].

Technologies for EV Isolation and Analysis, and Recent Advances

One important limitation to the clinical application of EVs as liquid biopsies is the lack of standardized protocols for sample handling and EV isolation and analysis, which could impact reproducibility in the clinical setting [53]. Currently used EV isolation procedures often comprise many biospecimen-handling steps and can subject EVs to different types of physical and chemical insult, which may damage EVs and/or modify their biological and physical properties. Another factor influencing the reproducibility of EV studies is the lack of standardized guidelines defining EV nomenclature and definition, and the control experiments needed for validation. To overcome these limitations, a comprehensive collection of guidelines and recommendations was recently updated by the International Society for Extracellular Vesicles [54].

Similar to CTCs, conventional EV isolation strategies exploit physical (density and size) and biological (expression of surface markers) properties [55]. Density-based approaches, such as differential centrifugation and/or ultracentrifugation and density gradient centrifugation, are the most commonly used methods for EV isolation. Among these, differential ultracentrifugation is considered the gold standard technique, especially for exosome purification. Although widely used, these techniques rely on expensive equipment, are time-consuming, and do not guarantee pure yields, often resulting in a compromise between purity and recovery [55].
Size-based techniques include filtration and size-exclusion chromatography (SEC). Filtration can result in high yields and purity, but again is limited in terms of the adherence of EVs to the filters and vesicle damage due to high pressure [56]. SEC allows superior recovery of EVs compared with ultracentrifugation [56].

Immunoaffinity capture methods use magnetic beads conjugated to antibodies recognizing EV surface markers. An advantage of these methods is that they produce EV fractions with high purity, allowing the isolation of specific subsets of EVs based on the surface marker used. By contrast, this feature could be a limitation because it may overlook potentially important EV subpopulations lacking the expression of the selected marker [55]. A novel development of immunoaffinity capture is its integration into microchips allowing in situ immunoassay analysis [57].

Another commonly used isolation method, especially for exosomes, is polymer precipitation. This method involves the use of polymers [e.g., polyethylene glycol (PEG)] to reduce the solubility of EVs to precipitate them with a rapid low-speed centrifugation. Although producing high recovery rates, this methodology has low purity [58].

Recently emerging methodologies for EV isolation use electric fields. Lewis et al. developed an alternating current electrokinetic (ACE) chip capable of performing exosome capture from whole blood and in situ immunofluorescent analysis in 30 min. The authors validated this chip by assessing the suitability of GPC-1 and CD63 levels as diagnostic markers of PDAC [59].

Finally, another promising category of potential novel approaches to EV isolation relies on microfluidics [58]. The available microfluidic approaches are based on different EV properties, such as nanoscale size-based filtration [60], antibody-functionalized microfluidic channels [61], and spiral inertial microfluidic devices [62]. In a recent report, Ko et al. developed a magnetic nanopore-sorting platform that has been used to isolate specific cancer-derived EVs. They used this system to identify, in a mouse model of PDAC, a miRNA signature to train a machine-learning algorithm for the classification of distinct cancer states [63]. Microfluidics technologies are set to boost the development of lab-on-a-chip systems for fast, cost-effective, integrated isolation and analysis of EVs, towards the development of EV-based point-of-care diagnostics.

Together with isolation methods, EV detection methods are also experiencing advances in development, especially with regards to the analysis of protein cargo. Common analytical technologies include WB, ELISA, mass spectrometry (MS), and flow cytometry (FCM) [64]. With the exception of FCM, these techniques focus primarily on bulk EV analysis, without assessing their individual variability [58]. FCM is currently used for single-MP characterization [46,51,52], but fails to analyze single exosomes due to their small size. Exosome FCM analysis currently involves the binding of multiple exosomes to larger beads [45]. Recently, Kibria et al. developed a micro-FCM platform that was capable of assessing the expression of CD47 in single circulating exosomes from patients with breast cancer [6]. Another technique capable of allowing single-EV protein phenotyping, at a higher size resolution than current flow cytometers, is a variation of nanoparticle tracking analysis (NTA) in which fluorescent antibodies are used to identify EVs expressing a given marker [65]. Despite the better size resolution of NTA, a great advantage of FCM is its relatively higher multiplexing capacity.

**Circulating Tumor RNA**

The fraction of circulating cell-free RNA originating from cancer cells is referred to as ctRNA. The existence of extracellular RNA was first documented in 1978 [66] and the first report of its
potential as a cancer biomarker was published several years later [67]. Compared with DNA, RNA is a relatively unstable molecule, the naked half-life of which in plasma is ~15 s [68]. Its stability is enhanced by its association with proteins [69], proteolipid complexes [67], and EVs [44].

Clinical Significance of ctRNA as a Cancer Biomarker, and Current Limitations

Nearly all known classes of RNA have been found in systemic circulation and, to a certain extent, each has the potential to serve as a cancer biomarker [70]. Similar to other components of the tumor circulome, ctRNA is a source of quantitative and qualitative information. In fact, although expression profiles of coding and ncRNAs represent the most important source of information, the identification of tumor-specific fusion transcripts or alternative splice events is also possible [71]. The most important classes of ctRNA potentially suitable as biomarkers are mRNAs, miRNAs, and long ncRNAs (lncRNAs) (Figure 1). Their analysis is performed with techniques ranging from qRT-PCR or dPCR-based assessment of single or small panels of RNAs to the comprehensive characterization of RNA (especially miRNAs) signatures via RNA-Seq [70].

Circulating exosomal mRNA has been used to investigate the mutational status of KRAS and BRAF in patients with CRC [72], and exosomal EGFRvIII mRNA has potential for the diagnosis of EGFRvIII-positive high-grade gliomas [73]. In another report, the detection of androgen receptor splice variant 7 (AR-V7) in plasmatic exosomes by ddPCR was shown to be a good predictor of resistance to hormonal therapy in prostate cancer [74]. Numerous lung cancer-related gene fusions are also readily identified in both vesicular and nonvesicular mRNA and have value as biomarkers [75]. Among the nonvesicular fraction of ctRNAs, circulating human telomerase reverse transcriptase (hTERT, catalytic subunit of the telomerase complex) mRNA demonstrated greater diagnostic and prognostic accuracy than PSA for prostate cancer [76].

With regards to miRNAs, plasma exosomal miR-196a and miR-1246 levels have the potential for the early diagnosis of pancreatic cancers [77], and panels of miRNAs have been shown to be reliable biomarkers for the diagnosis [78] or prognosis [79] of lung cancer. More recently, a serum exosomal miRNA signature was proven to be an innovative tool for the differential diagnosis of gliomas [80].

A novel promising source of RNA biomarkers are IncRNAs. For example, plasma exosome LINC00152 levels have been linked to gastric cancer [81], and the combination of two mRNAs and one IncRNA in serum exosomes has diagnostic potential for CRC [82]. Furthermore, serum exosomal HOTAIR IncRNA has applicability in the diagnosis and prognosis of glioblastoma multiforme [83]. More recently, a panel of five circulating IncRNAs was studied as promising diagnostic biomarkers for gastric cancer [84].

To date, the most important limitations to the implementation of ctRNAs in the clinical setting involve the preanalytical and analytical steps. Although circulating RNAs are protected by the association with different molecules and structures, they are unstable in plasma if stored at 4 °C, and limited by the speed of extraction [85]. Furthermore, different extraction protocols have different recovery rates, and there is currently no consensus on an optimal extraction protocol [85]. Again, lab-on-a-chip devices appear to offer a potential solution to this issue, allowing rapid and integrated purification and analysis of samples while minimizing their handling. A proof-of-concept of this is the microdevice developed by Potrich et al., which is capable of selectively extracting miRNAs from cell culture supernatants and allows in situ reverse transcription and qPCR analysis [86]. Another example is the Integrated
Comprehensive Droplet Digital Detection (IC 3D) system, a microfluidic platform capable of quantifying extremely low concentrations of miRNAs directly from plasma in 3 h [87].

**Tumor-Educated Platelets**

TEPs are perhaps the latest components of the tumor circulome to be considered for biomarker analysis. The concept of ‘platelet education’ by cancer refers to the presence of specific RNA signatures in platelets from patients with cancer. This was first reported in 2010 and 2011 with the observations that: (i) in patients with metastatic lung cancer, 197 platelet genes were downregulated and several genes were differentially spliced compared with controls [88]; and (ii) in glioma, cancer-derived microvesicles are actively taken up by platelets and transfer their RNA content, harboring a cancer-characteristic RNA signature revealed by microarray [89]. In 2015, Best et al. characterized TEPs extracted from a patient cohort across six cancer types via RNA-Seq, distinguishing patients with localized or metastatic tumor from healthy individuals with 96% accuracy and locating the anatomical position of the tumor with 71% accuracy. This paved the way for ‘pan-cancer and multiclass cancer diagnostics’ [90]. In more recent work, the same group applied particle-swarm optimization (PSO)-enhanced algorithms to platelet RNA-Seq libraries to generate a panel of biomarkers capable of distinguishing patients with lung cancer from healthy individuals and from those with lung inflammatory conditions [91].

**Controversies over the Use of Liquid Biopsies in Cancer Management**

Despite reports demonstrating the potential of liquid biopsies in addressing current needs in cancer management, numerous controversies remain on their utility. This is particularly true for ctDNA and CTCs, which have already found application in clinical management. In a recent report, Torga and Pienta [92] compared the performance of two commercially available NGS-based ctDNA tests for metastatic prostate cancer, finding an astonishingly low concordance (7.5% patients studied) between the two tests. The discordance was attributed to issues in study design and sample analysis, highlighting the current limitations of preanalytical and analytical standardization in the field. Furthermore, with few exceptions, most of the ctDNA assays available have limited evidence of clinical validity and utility in advanced cancer. Likewise, their utility in early-stage cancers, treatment monitoring, or MRD detection remains to be established [93]. Additionally, with regards to CTCs, although their clinical validity has been demonstrated, particularly for their prognostic capacity in metastatic disease, evidence of their clinical utility is still missing, preventing their implementation into standard clinical practice [94]. Numerous large-scale clinical trials are clearly needed, and some are currently in progress to address this unmet need.

**Concluding Remarks and Future Directions**

Current standards for patient stratification and treatment selection include the analysis of tumor genetic alterations from tissue biopsies. Despite their undoubted value, tissue biopsies have important limitations, being highly invasive procedures that fail to capture tumor clonal heterogeneity. Liquid biopsies, comprising the analysis of circulating tumor-derived factors (the tumor circulome), are gaining exceptional attention as a valuable alternative. The tumor circulome is a source of different classes of tumor-derived biological components. Novel technologies are being developed to further improve the analysis of the tumor circulome, with the aim of fully exploring the complexity of the information obtainable from a simple blood draw.

The studies reviewed here underline the potential of liquid biopsies, and the development of novel technologies allows researchers to characterize each single component of the tumor circulome with increasing precision. Liquid biopsies are being positioned as a game-changing
Table 3. Limitations of Liquid Biopsies*

<table>
<thead>
<tr>
<th>Liquid biopsy component</th>
<th>Limitation</th>
<th>Solution</th>
<th>Refs</th>
</tr>
</thead>
<tbody>
<tr>
<td>ctDNA</td>
<td>Low sensitivity of mutation detection (when MAF is low)</td>
<td>Unique molecular identifiers</td>
<td>[26]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Nuclease digestion of non-mutated DNA</td>
<td>[27]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sampling of alternative body fluids</td>
<td>[28]</td>
</tr>
<tr>
<td></td>
<td>Low predictive value of single and/or small sets of mutations</td>
<td>Analyze large mutations sets and/or associate mutations with other classes of biomarker (e.g., proteins)</td>
<td>[29]</td>
</tr>
<tr>
<td></td>
<td>Lack of standardized preanalytical handling protocols, sample degradation, poor reproducibility</td>
<td>Layout of standardization guidelines</td>
<td>[30]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Automated purification and/or analysis chips minimizing sample handling</td>
<td>[5]</td>
</tr>
<tr>
<td>CTCs</td>
<td>Poor efficiency of isolation from blood because of marker rarity, fragility, physical, and phenotypic heterogeneity</td>
<td>Combined use of different methodologies for enrichment and/or isolation (e.g., CellSearch + Parsortix)</td>
<td>[121]</td>
</tr>
<tr>
<td>EVs</td>
<td>High variability between isolation techniques; lack of standardized protocols</td>
<td>Comprehensive standardization guidelines (e.g., MISEV 2018)</td>
<td>[54]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Automated purification and/or analysis chips (e.g., ACE chips)</td>
<td>[59]</td>
</tr>
<tr>
<td></td>
<td>Lack of single-EV protein expression analysis techniques (especially for exosomes)</td>
<td>Development of high-resolution flow cytometers</td>
<td>[6]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fluorescence-based nanoparticle tracking analysis</td>
<td>[65]</td>
</tr>
<tr>
<td>ctRNAs</td>
<td>Preanalytical handling variability, RNA instability</td>
<td>Layout of sample handling standardization guidelines</td>
<td>[85]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Automated purification and/or analysis chips minimizing sample handling</td>
<td>[87]</td>
</tr>
</tbody>
</table>

*Abbreviation: MISEV, minimal information for studies of extracellular vesicles.

tool in personalized cancer management. However, their clinical application has been comparatively slow, hampered by multiple technical challenges (listed in Table 3). As a consequence, several problems still need to be resolved to firmly establish the role of liquid biopsies in the clinical setting (see Outstanding Questions). The lack of standardization of preanalytical and analytical variables is a significant limitation in the field. A liquid biopsy ideally should be cost-effective, fast, reproducible, and ensure sample integrity. One approach via which this can be achieved is through automated chip-based devices allowing for the analysis of biomarkers from whole blood without the need for lengthy and costly purification steps. While complex chip systems, such as the ACE chip, remain costly, alternative polymeric microfluidic devices, such as the spiral microfluidic chip used for CTC isolation, are, in comparison, cost-effective [95]. Although much work is still needed to comprehensively define the future role of liquid biopsies in cancer diagnosis, monitoring, and prognosis, the promising results reported so far testify to the potential of this approach in changing the current paradigms of cancer management.

**Resources**

[www.who.int/news-room/fact-sheets/detail/cancer](www.who.int/news-room/fact-sheets/detail/cancer)
References


61. Kanwar, S.S. et al. (2014) Microfluidic device (ExChip) for on-chip isolation, quantification and characterization of circulating exosomes. Lab Chip 14, 1891–1900


64. Liu, C. et al. (2018) Recent advances in extrosomal protein detection via liquid biopsy biosensors for cancer screening, diagnosis, and prognosis. AAPS J. 20, 41


76. March-Villabona, J.A. et al. (2012) Cell-free circulating plasma hTERT mRNA is a useful marker for prostate cancer diagnosis and is associated with poor prognosis tumor characteristics. PLoS One 7, e43470

77. Xu, Y.F. et al. (2017) Plasma exosome miR-196a and miR-1246 are potential indicators of localized pancreatic cancer. Oncotarget 8, 77028–77040


112. Hyun, K.A. et al. (2016) Epithelial-to-mesenchymal transition leads to loss of EpCAM and different physical properties in circulating tumor cells from metastatic breast cancer. Oncotarget 7, 24677–24687
121. Lampignano, R. et al. (2017) A novel workflow to enrich and isolate patient-matched EpCAM(high) and EpCAM(low/negative) CTCs enables the comparative characterization of the PIK3CA status in metastatic breast cancer. Int. J. Mol. Sci. 18, E1885
123. Ramirez, A.B. et al. (2017) RareCyte(R) CTC analysis step 1: AccuCyte(R) sample preparation for the comprehensive recovery of nucleated cells from whole blood. Methods Mol. Biol. 1634, 163–172