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REVIEW

# Circulating tumor markers: harmonizing the yin and yang of CTCs and ctDNA for precision medicine

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Current trajectory of clinical care is heading in the direction of personalized medicine. In an ideal scenario, clinicians can obtain extensive diagnostic and prognostic information via minimally-invasive assays. Information available in the peripheral blood has the potential to bring us closer to this goal. In this review we highlight the contributions of circulating tumor cells and circulating tumor DNA and RNA (ctDNA/ctRNA) towards cancer therapeutic field. We discuss clinical relevance, summarize available and upcoming technologies, and hypothesize how future care could be impacted by a combined study.

**Key words:** circulating tumor cells, circulating tumor DNA, circulating tumor RNA, exosomes, cell free DNA, cell free RNA

## Introduction

To date, significant progress has been made in cancer research towards identifying prominent targets for treatments, yet there is no universal cure that can significantly reduce or eliminate cancer. Using any treatment only functions to select for unaffected/resistant tumor cells due to genetic heterogeneity. Tumor biopsies can provide valuable information. However, this approach has many drawbacks such as its invasiveness and inability to capture all the variability in gene expression. Since the primary cause of cancer mortality is metastasis, effectiveness of any therapy can only be measured by the ability to prevent or delay this event. To achieve these goals, accurate, early detection of tumor status is key.

Liquid biopsies rely on screening the peripheral blood by collecting circulating tumor cells (CTCs), and/or circulating tumor DNA and RNA (ctDNA/RNA). The discovery of unique cancer cells' protein (from CTCs), or oncogenic mutations and epigenetic change (from CTC or ctDNA/RNA) can potentially help nullify and counteract the most potent weapon in cancer's arsenal: its genetic evolution to adapt to any treatment. In this review we discuss the importance of studying CTCs and ctDNA/RNA and their impact on clinical care. Overall, we argue for a more prominent use of liquid biopsies for screening CTCs and ctDNA/RNA as means of determining tumor progression and providing personalized therapy.

One of these six fundamental properties of cancers includes tissue invasion and metastasis [1]. Tissue invasion involves a subpopulation of primary cancer cells acquiring new abilities by undergoing a change known as epithelial-to-mesenchymal transition (EMT). The phenotypic result of EMT is increased motility and invasiveness potential, among others. As such, these cells will invade through the tissue surrounding their site of origin until they enter the blood or the lymphatic system, though not all CTCs are mesenchymal and don't always invade and enter circulation due to EMT [2, 3]. CTCs are ambassadors of a localized disease and may remain dormant at metastatic sites and become activated at a later time, resulting in relapse. Captured CTCs can reveal the genetic signature associated with these traits.

The fluctuating levels of ctDNA/RNA can be based on multiple factors including tumor type, progression, therapeutic effect, and metastatic spread [4]. Early ctDNA detection has been found to have a strong correlation with the onset of tumor recurrence and metastasis [5]. This is most likely due to the ability to detect rising levels of ctDNA, coupled with the detection of tumorigenic mutations in susceptible genes [6]. Screening for such mutations has

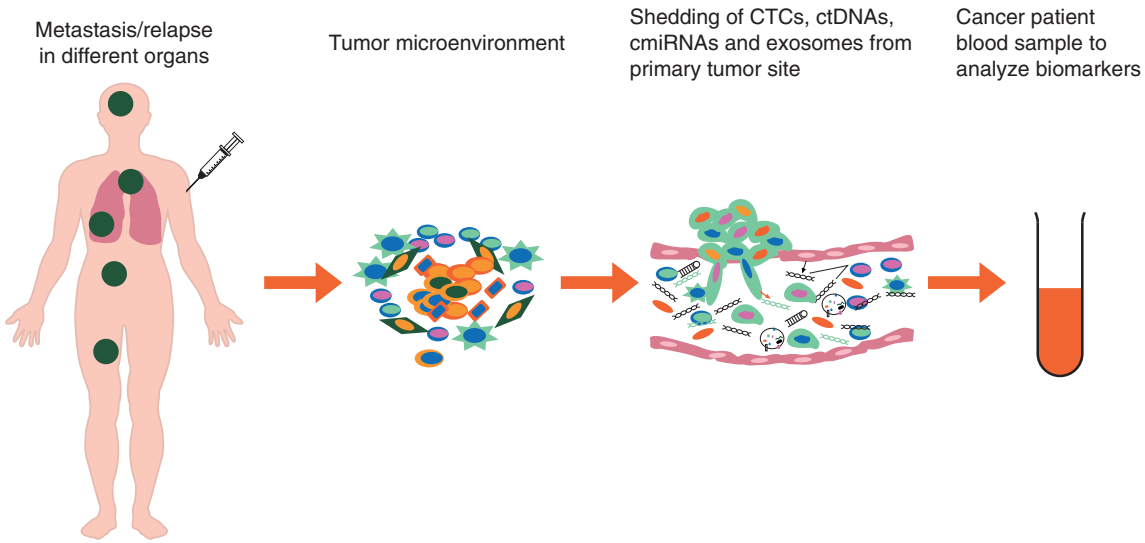


**Figure 1.** Multitude of factors secreted in the circulatory system with the sustained growth of a primary/localized tumor. These can include invasive cells that become CTCs and exosomal and ctDNA/RNA. The CTCs can attach at distant sites and form secondary lesions. A liquid biopsy, obtained from a simple blood draw may help predict such an event though changes in CTCs and ctDNA levels and their genetic and epigenetic constitution.

been helpful in predicting and adjusting to chemotherapeutic resistance [7]. Moving forward, we can expect to see a more prevalent role of ctDNA in clinical care. ctRNA can exist in forms such as miRNAs and long non-coding RNA (lncRNA) and can be free floating, carried by platelets (called tumor educated platelets—TEP), or encased in exosomes [8]. Similarly to ctDNA, ctRNA has been collected from peripheral blood for the purpose of understanding the tumor status. The majority of research regarding ctRNA has been focused on microRNAs (miRNAs or miRs) found in the blood as markers of early detection of multiple tumor types including breast, prostate, colorectal, and NSCLC [9–12]. For the study of tumor metastasis from liquid biopsies, the lncRNA also comes into play, mainly in terms of detecting metastasis and relapse. RNA levels and specific gene transcripts found in the plasma also correlate with tumor aggressiveness [13, 14].

With the information gleaned from CTCs and ctDNA/RNA, we can gain a real-time understanding of the changes that the tumor is undergoing, in the form of CTC numbers, CTC gene expression, possible activating mutations in ctDNA, detection of tumor-promoting miRNA, as well as RNA transcripts captured from the circulation. An illustration of the source and factors contained with a liquid biopsy is shown (Figure 1).

## CTC detection

The basic goal of CTC isolation is to accurately and precisely discriminate between CTCs and various types of cells in the blood. The technological progress towards refining CTC isolation has been primarily driven by a binary approach of antibody-based or size-dependent principles, with many methods relying on microfluidics to achieve their goals.

CTCs are extremely rare and heterogeneous and generally account for 1–10 cells per 10 ml of blood. The scarcity of these cells presents an inherent technological challenge that has been approached in multiple forms. For antibody based selection, the ideal marker is a CTC-associated protein that is not observed on any other type of circulating cells; a marker akin to CD45 for immune cells. The most prominent of antibody-based approach relies on epithelial cell adhesion molecule (EpCAM) [15]. The EpCAM-based CTC isolation has garnered widespread attention and a technology based on this protein and subsequent staining and validation with cytokeratin (CK; positive) and CD45 (negative) has been developed (CellSearch, Veridex, Raritan, NJ). The FDA approval of CellSearch system has led to its proliferative use and sets the benchmark for other CTC isolation technologies.

However, there are fundamental questions about CellSearch technology that have caused many to question the reliability of this test [16]. Both EpCAM and CKs are epithelial markers which are lost after EMT. Therefore, any technology relying on EpCAM for CTC detection is most likely blind against transitioned cells. To address the shortcomings of EpCAM, Plastin3 was identified as a metastasis-specific marker in colorectal cancer (CRC) and breast cancer patients [17–19]. One of the technologies developed by our lab relies on the universally-accepted mesenchymal marker vimentin [20]. This approach relies on the detection of cell surface vimentin (CSV) to isolate CTCs from any type of tumor.

Numerous studies have found CellSearch as a viable prognostic test for overt metastasis, progression-free survival (PFS) and overall survival (OS) in multiple cancers [21–24]. Other isolation techniques claim faster sample processing, better screening using multiple approaches/targets. These and other methods of isolating CTCs are summarized below (Figure 2 and Table 1).

## CTC clinical utility

Multiple clinical trials testing for the strength of CTC counts as a predictor of OS, and PFS have been conducted in various types of tissues. A prospective analysis of 302 non-metastatic, chemonaive

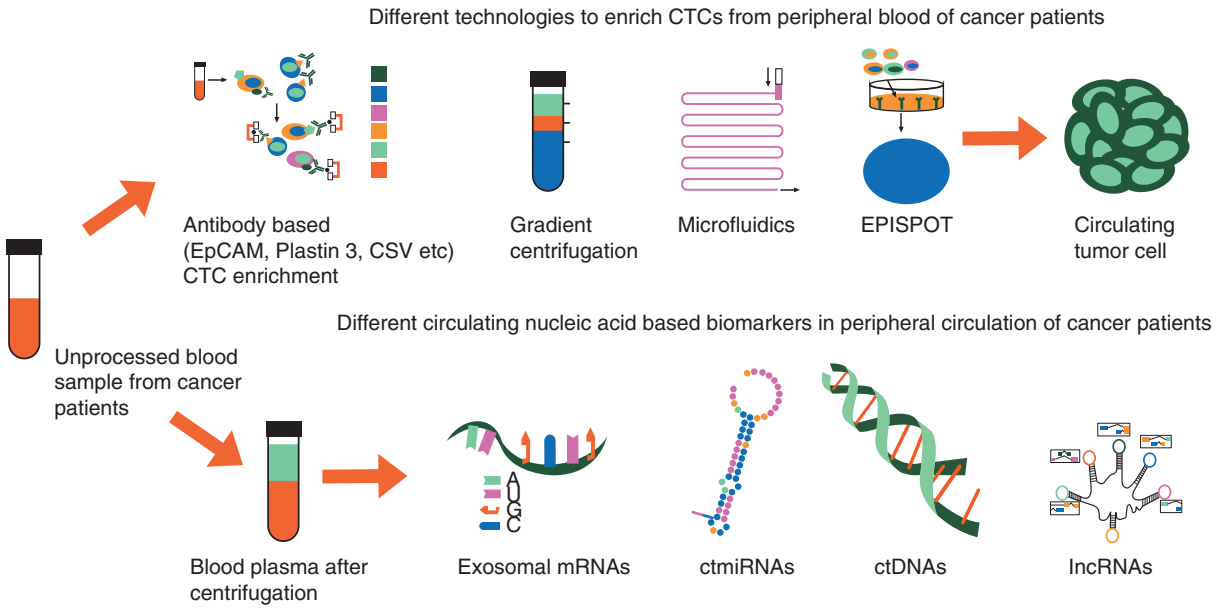


**Figure 2.** CTC isolation methods and an inventory of circulating DNA/RNA components. A blood sample can be processed for CTC isolation using multiple techniques as illustrated. The plasma portion of the sample also contained various types of genetic materials such as circulating tumor mirco RNA, ctDNA, and lncRNA. These molecules may or may not be encased in an exosomal vescicle.

breast cancer patients at stages 1–3 found decreased PFS and OS in 73 patients with detectable (1 or more) CTCs compared with none [56]. Further, the prognosis for PFS and OS was progressively reduced in patients with $\geq$ 2 CTCs, and $\geq$ 3 CTCs when each of these groups were compared with 0 CTC patients. Although these findings support the prognostic potential of CTCs, the investigators failed to find a correlation between CTC counts and pathological lymph-node status. This may be result of small sample size since most of the CTC-positive patients were node negative and estrogen/progesterone receptor positive. In a 118-patient study, CTC detection prior to or after neoadjuvant chemotherapy was an independent prognostic factor of metastatic relapse [57]. Although CTC detection or changes were not observed to be a measure of pathological response. Also, changes in CTC during chemotherapy were not a reliable measure of distant metastasis. In patients with inflammatory breast cancer at stage III of disease progression, CTC counts were found to be viable predictors of disease relapse [58]. The same study also reports that CTCs are not indicators of tumor characteristics, lymph node positivity, therapeutic response, or OS.

The SWOG S0500 study of ~600 metastatic breast cancer patients confirmed that CTC counts have strong prognostic value for OS [59, 60]. However, in patients with baseline and persistent counts of $\geq$5 CTCs there was no benefit between maintaining their initial therapy or changing to an alternate. Although these results suggest reduced power of CTC guided therapy, it may also be attributed to the alternate therapy the patients were prescribed. In the large study of over 2000 early breast cancer patients, the presence of $\geq$1 CTC per 30 ml of blood before beginning treatment was a statistically significant indicator of disease free survival (DFS), OS, and distant DFS [61]. The hazard ratio (HR) of 1 CTC per 30 ml blood for DFS increased from 2.11 to 4.51 if the CTC count increased to $\geq$5 CTC. Similarly, HR for OS increased from 2.18 to 3.60 between CTC counts of $\geq$1 and $\geq$5. Interestingly, neither the SWOG nor the Rack et al., studies found a connection between hormone receptor status and CTC numbers. It's possible that since hormone independence is not an early stage event, the means of collecting CTCs via the CellSearch system (targeting epithelial marker EpCAM) may be omitting the mesenchymal cells which may be more likely to reflect the tumor's receptor status.

In consideration of the scientifically-validated role of CTCs as prognostic markers of OS and PFS in breast cancer, new clinical trials are underway that will utilize CTC counts towards clinical decisions. These trials will assess the value of CTC count-guided decisions about the therapeutic avenues prescribed to patients [62].

Beyond breast cancer, similar results have been observed in other epithelial tumors. A prospective analysis to determine patient OS by using CellSearch measured CTC counts in castration-resistant prostate cancer (CRPC) patients found CTCs to a viable prognostic tool [63]. The study revealed a significant probability of OS in patients with favorable CTC counts (<5), as measured preinitiation of chemotherapy and monthly readings thereafter. Furthermore, patients whose CTC counts switched from unfavorable to favorable fared better OS than those who experienced the opposite effect. As prostate-specific antigen (PSA) change and velocity are common metrics in prostate cancer clinical assessment, the change in PSA (30 and 50%) was significant for OS after 6–8 weeks of therapy but less so compared with favorable and unfavorable CTC counts [63]. A reanalysis of the same data was conducted using more stringent criteria: only first line chemotherapy patients, and an extra 6 months of follow-up data. Here, the CTC counts and lactate dehydrogenase (LDH) but not PSA were shown as indicators of OS time [64]. Similarly, in a 711 patient study of metastatic CRPC, CTC counts of <5 cells/7.5ml blood and LDH of >250 u/l was a significant surrogate for survival over a 2-year period [65]. The SWOG S0421

**Table 1. Prominent CTC isolation and detection techniques**

| Technology | Isolation principle | Comments/notes | References | Developer(s) |
|---|---|---|---|---|
| EpCAM-based | | | | |
| CellSearch | EpCAM-conjugated ferrofluid | EpCAM mAb conjugated to a magnetic bead is used for targeting CTCs. Cells are eluted with magnetic separation and IF labeled. | [25] | Janssen Diagnostics, Raritan, NJ, USA |
| GILUPI CellCollector | EpCAM-tipped steel wire | Steel wire with 2 cm hydrogel tip conjugated to EpCAM. Inserted into arm vein for 30 min. | [26] | GILUPI GmbH, Potsdam, Germany |
| RosetteSep | Negative selection through gradient centrifugation | Precipitate undesired PBMC's by conjugating them with glycohphorin A on RBCs during gradient centrifugation. | [27] | STEMCELL Technologies, Vancouver, BC, Canada |
| Microfluidics | | | | |
| CTC-iChip & Cluster-Chip | Si microarray with ab-coated (EpCAM or others) microposts | Silicone microchip with microposts that can be coated with an antibody of choice (CTC-iChip). Different version of same technology allows for label-free isolation of CTC clusters (Cluster-Chip) which has helped profile the heterogeneous nature of CTCs. | [88, 32] | Dr Daniel A. Haber and Dr Mehmet Toner Labs, MGH, Boston, MA, USA |
| Parsortix | Size-based exclusion using microfluidics. Sterile separate of cells from plasma. | Microfluidics cassette for sterile isolation of CTCs. Isolated cells can be genetically characterized if desired. Also able to separate plasma for separate analysis. | [33, 34] | Angle plc, Surrey, UK |
| Vortex HT/Vortex VTX-1 | Inertial forces of microvortices trap CTC but allow other cells to escape | High capture efficiency (>80%) and sterile isolation of cells. Requires up to seven repeated processing of the same sample for ideal results. Inadequate comparison to CellSearch. | [35] | Vortex Biosciences, Menlo Park, CA, USA |
| ClearCell FX | Microfluidic spiral channel separate CTC by inducing drag | Excludes cells <8–15μm. 70–80% capture efficiency. Comparisons with CellSearch show extremely high differences in favor of ClearCell. | [36, 37] | Clearbridge BioMedics, Singapore |
| Imaging-based | | | | |
| AccuCyte + CyteFinder | Gradient centrifuged buffycoat spread on slide | Mechanical separation of the PBMC layer after gradient centrifugation. Cells are spread on glass slide for staining/imaging. Specialized needle allows for picking individual cells for further study. 8hr processing time per sample. | [38] | Rarecyte, Seattle, WA, USA |
| EPIC/HD-CTC | Nucleated cells from whole blood sample placed on slide, stained and imaged | Nucleated cells are cytospun on to glass slides and immunostained for CD45, CK, DAPI, and a fourth, custom antibody. All cells are imaged and tumor cells (CTCs) are quantified based on positive CK staining. | [39, 40] | Epic Sciences, San Diego, CA, USA |
| Size-based | | | | |
| Isolation by Size of Epithelial Tumor Cells (ISET) | Size-based CTC filtration from whole blood | An 8–11 μm pore size. Blood mixed with proprietary buffer before processing. Can process up to 10ml blood in ∼15m. Higher yield than CellSearch. Validated in multiple tumors by independent groups. | [41–43] | Rarecells Diagnostics SAS, Paris, France |
| ScreenCell size-exclusion device | Size-based, vacuum-driven whole blood screen | Whole blood is drawn by vacuum suction through a filter with 6.5 ± 0.33 μm pore size. Filter can be removed and kept in a tissue culture dish or mounted on slide for staining and imaging. | [44] | ScreenCell, Westford, MA, USA |
| CellSieve | Lithographically made 10 μm thick microfilter with uniform 7 μm sized pores | SU-8 polymer film with 160 000 pores evenly placed on a 9-mm diameter filter. Blood drawn through filter by mechanically controlled syringe pump. Filtered cells backwashed into vial with PBS. More than 90% capture efficiency in spiking assays. | [45, 46] | Creatv MicroTech, Potomac, MD, USA |
| Functional | | | | |
| CSV | CD45 depletion, CSV-based isolation | After CD45+ cell depletion, an in-house developed antibody for CSV is employed for CTC isolation. Isolated cells are immunostained for size and marker-based characterization. | [47] | Dr Shulin Li Lab, MDACC, Houston, TX, USA |

*Continued*

**Table 1.** *Continued*

| Technology | Isolation principle | Comments/notes | References | Developer(s) |
|---|---|---|---|---|
| EPISPOT | CD45 depleted cells cultured on ab-coated NC membrane | Can augment CTCs isolated by other methods. Authors claim greater sensitivity than CellSearch. Clinical trials underway. | [48] | Dr Alix-Panabieres, Universit Medical Centre, Montpellier, France |
| Cell invasion | CTCs ingest fluorescent CAM | Pros: Combines mesenchymal behavior with epithelial phenotype; Cons: Spiked assay recovery rate is low (54 ± 9%) | [49, 50] | Dr Jonathan Rosenberg Lab, UCSF, San Francisco, CA, USA |
| Dielectrophoresis (DEP) | Non-uniform electric field to move cells in liquid and separate by cell types | Isolated cells are viable and sterile. Can augment CellSearch post-isolation. | [51, 52] | DEPArray (Silicon Biosystems, San Diego, CA, USA);ApoStream (APOCELL, Houston, TX, USA) |
| Telomescan (OBP-401) | Label cells with telomerase-controlled GFP using adenovirus | Pros: Clinically validated in multiple tumors; Cons: Some false positive staining in WBC. | [53, 54] | Oncolys BioPharma, Tokyo, Japan |
| Acoustics-based | Acoustic waves on microfluidic flow for physical property based separation | Pros: Label-free isolation from clinical samples. Validated using cell lines and breast cancer clinical samples. More than 80% recovery rate. Promising new technology; Cons: First iteration. Flow rate is low (20 μl/min). Only tested two clinical samples. | [55] | Drs. Dao, Suresh, Huang Labs. MIT, Cambridge, MA, USA; CMU, Pittsburgh, PA, USA; PSU, University Park, PA, USA respectively. |

Ab, Antibody; CAM, Collagen adhesion matrix; CK, Cytokeratin; NC, Nirocellulose.

Phase III trial of 238 patients (out of an initial enrollment of 1038) measured CTC at baseline and 21 days later before beginning second cycle of chemotherapy [66]. In a comparison of patient groups separated between CTCs <or ≥5/7.5 ml blood, the baseline CTC count was reflective of PSA and RECIST response. Further, a CTC enumeration of ≥5/7.5 ml was associated with higher PSA and worse bone pain, among other physiological markers. Surprisingly the tumor volume was not an included metric in the studies mentioned thus far. A meta-analysis of 33 studies out of an initial screening of 768 found that 25 of these 33 showed a favorable role of CTCs in peripheral blood as markers of OS and PFS [23]. In total 5 of 33 were non-favorable for similar criteria, while the other 3 studies were inconclusive. CTCs detected in bone marrow were not predictors of OS, but did correlate to disease/relapse-free survival. Although there is now ample evidence to support the presence of baseline CTC ≥ 5/7.5 ml of blood as a prognostic indicator of poor OS and PFS, the glaring issue of predicting the driving force in PCA, hormone independence, via CTC enumeration/analysis is still lacking. RT-qPCR analysis of CTCs captured from two groups of 31 enzalutamide or abiraterone-treated metastatic CRPC patients found AR-V7 transcripts in CTCs of 39 and 19% patients, respectively [67]. The TMPRSS2-ERG transcripts were also screened in a 41 patient cohort of post-chemotherapy CRPC phase II trial [68]. 15/41 (37%) patients that expressed the fusion gene in their CTCs had a baseline median of 17 CTCs per 7.5 ml blood, however the presence of this transcript did not affect survival over 4 years.

A meta-analysis of 53 melanoma clinical trials found that CTC positivity rates between various stages ranged from 32% (Stage I) to 47.4% (Stage IV), while the 5-year mortality increased from 10% at stage I to 90% at stage IV [69]. The greatest differences occurred between stages I and IV with minor increases in the middle stages. A multicenter study of CTC counts in patients with metastatic CRC found that 3 or more CTCs were a significant predictor of PFS and OS using baseline and post-therapy counts [70]. Analysis of metastatic neuroendocrine neoplasm patients starting long-term chemotherapy found that the absence of CTCs at baseline and after the first cycle of treatment was a strong predictor of OS and progressive disease [71]. This was also true with patients showing a ≥50% decline in CTC counts between baseline and first measurement post treatment. A summary of all phase II-IV clinical trials using CTCs as an outcome measure is shown (Table S1, available at *Annals of Oncology* online).

The availability of newer devices for immobilizing/capturing CTCs for genetic analysis has opened up a new arena of genetic studies of these elusive tumor cells. Aceto et al., used the CTC-iChip for RNA sequencing on a breast CTC cluster and discovered plakoglobulin as the key contributor towards maintaining CTC clusters [72]. The whole exome sequencing of CTCs from two prostate cancer patients revealed 70% overlap with mutations found in lymph node metastasis and primary tissue [73]. Similarly, there was concordance between cfDNA and CTCs in metastatic breast cancer patients [74]. CTCs showed heterogeneity in the *TP53*, *PIK3CA*, *ESR1*, and *KRAS* genes. Notably, the

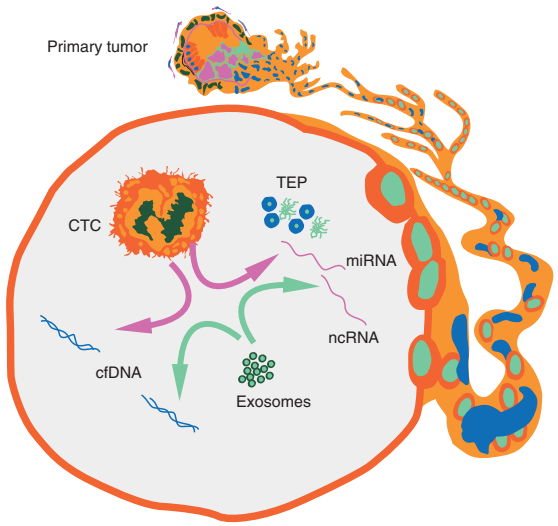


**Figure 3.** A liquid biopsy contains wealth of information relevant to determining tumor status, metastatic potential, and likelihood of relapse. Some of the contributing factors to making such an assessment include CTC counts, CTC genetic profile and protein expression, levels of ctDNA/RNA and the presence or absence of known mutations or epigenetic signatures. A thorough analysis of liquid biopsies from cancer patients screening for these factors can reveal essential information for personalized care.

*ESR1* and *KRAS* mutations were not observed in primary tumor tissue, indicating a sub-population of cells within the tumor. Another study of breast cancer CTCs revealed prevalent mutations in *TP53*, *PGFRRA*, *ERBB2*, and *KIT* though authors noted significant 'intro- and inter-patient heterogeneity' [75]. This pattern of varying mutations in CTCs was also recorded in metastatic HCC patients [76]. Perhaps in an effort to understand the relevance of the differing mutations in CTCs not observed in primary tumors, Dr Caroline Dive's group pioneered a unique method known as CTC-derived xenograft (CDX) models [27, 77, 78]. Essentially, these are CTCs captured from fresh liquid biopsies that are immediately injected subcutaneously into NSG mice. The resulting tumors exhibited similar genomic signature to CTCs and reflected comparable drug/therapeutic response as the source NSCLC and melanoma tissues [77, 79]. Overall, the genomic analysis of CTCs is still more cost and time prohibitive compared to ctDNA analysis, which shares significant overlap with CTC DNA.

## Circulating tumor DNA

Circulating DNA can either be borne from tumor cells, exosomes or be found as cell free DNA (cfDNA) also referred to as ctDNA though cfDNA can include non-tumor related DNA [80, 81]. Until recently, the technology was typically not sensitive enough to detect circulating DNA [82]. Circulating cell-free tumor DNA is found in predicable fragment lengths of the range of 140–170 bp, due to its original format as a histone-packaged nucleosome. These DNA fragments maintain epigenetic marks from the original tumor, a contributing factor motivating ctDNA analysis [83].

## Circulating tumor RNA

The circulating transcriptome consists of both coding and non-coding RNA, such as miRNA [84]. When compared with other forms, microRNAs are usually more stable in circulation due to their small size [85]. Key sources of circulating RNA include CTCs, tumor cell exosomes and microsomes, and TEPs [8].

Some studies have also focused on single-cell sequencing of the transcriptome [86]. Various other studies have concentrated on stabilizing and analyzing cell free RNA (cfRNA) [87]. In one study, investigators focused on plasma levels of cfRNA and telomere-specific reverse transcriptase mRNA, showed both to be associated with tumor response in rectal cancer patients who received preoperative chemoradiotherapy [88]. Multiple studies have also identified miRNA profiles for both adenomas and colon cancer [89]. cf-microRNAs present in the peripheral blood of cancer patients may reflect their influence on the biology of the tumor as well as therapeutic interventions and help with diagnosis, prognosis and response to therapy [90, 91]. Multiple miRNAs profiles have been revealed for different cancers that vary with tissue origin. A number of data bases have been developed such as miRCancer Database mircancer.ecu.edu, microrna.org, mirbase.org as well as ncbi.nlm.nih.gov/nuccore to name a few. These databases catalog the various tumor specific miRNAs profiles and potential outcomes. The clinical relevance of miRs and other RNA in cancer is highlighted by numerous current and ongoing clinical trials (Table S2, available at *Annals of Oncology* online ).

## Exosomes

The content of exosomes includes proteins, lipids, DNA, mRNA, and LincRNA [92]. Exosomes are secreted by several cell types, including cancer cells; examples of exosome-modified processes include cellular immunity, response to infection, and tumorigenesis. Exosomes have been reported to promote tumorigenesis in many cancer types, in particular through transfer of miRNAs [93, 94]. Remarkably, tumor-derived exosomes contain their own miRNA associated machinery and display cell-independent capacity to process precursor miRNAs into mature miRNAs. This phenomenon mediates an efficient and rapid silencing of mRNA in target cells, thus promoting oncogenesis.

The function of exosomes in tumor progression can be explained in part by the capacity of tumor-derived exosomes to modulate the host microenvironment in a distant site [95–97]. These findings have raised the concept of the pre-metastatic niche. For example, melanoma-derived exosomes injected in naïve mice have been shown to induce neoangiogenesis at pre-metastatic niche sites before the presence of any tumor cells [93]. Similarly, pancreatic ductal adenocarcinoma-derived exosomes were shown to induce liver pre-metastatic niche formation in mice and consequently increase liver metastatic burden [98]. Uptake of these tumor-derived exosomes by the liver resulted in TGF-β secretion and upregulation of fibronectin production. This resulting microenvironment then enhanced the recruitment of bone marrow derived macrophages. It was also noted that macrophage migration inhibitory factor was highly expressed in tumor-derived exosomes and its blockade prevented the formation of this liver pre-metastatic niche and thus inhibited metastasis.

A recent study by Hoshino et al. [99] demonstrated that various types of tumor-derived exosomes fuse preferentially with resident cells at their predicted destination. This concept of metastatic organotropism argues that exosomes taken up by organ-specific cells prepare and prime the pre-metastatic niche. Proteomic analysis of exosomes revealed distinct integrin expression patterns perhaps indicating the predilection for exosomes to target specific cells types or organs. Integrins α6β4 and α6β1 were associated with lung metastasis while exosomal integrins αvβ5 was linked to liver metastasis. Targeting these integrins in turn decreased exosome uptake as well as lung and liver metastasis.

In the case of blood cancers, a pathogenic role for exosomes has been demonstrated for multiple myeloma and leukemias, in particular through the transfer of miRNAs, mRNA and proteins [93, 94]. Roccaro et al. [94] showed that the protein content of exosomes isolated from myeloma bone marrow stromal cells was enriched for oncogenic proteins, cytokines, and kinases, including IL-6, CCL2, and fibronectin. This supports the notion that bone marrow stromal cells support tumorigenesis not only through paracrine mechanisms like growth factors [100, 101] but also through direct transfer of microRNAs and proteins from exosomes, thus creating a favorable niche for the expansion of the malignant clone. In AML, analysis of exosome content has the potential to be used as a biomarker of disease as mRNAs that encode proteins including NPM1, FLT3, CXCR4, MMP9, and IGF-1R were detected in exosomes isolated from patient-derived AML blasts and leukemic cell lines [100]. Characterization of these exosomes showed that the mRNA content reflected the FLT3 and NPM1 allelic diversity of the cell population from which they were isolated. Thus, analysis of exosome content may provide actionable clinical information.

Exosome formation by tumor cells may also affect response to therapy. For example, in patients with B-cell lymphoma, tumor-derived exosomes expressed CD20 to counter targeting immunotherapy with rituximab *in vitro* and *in vivo* [99, 101]. Pharmacologic blockade of ATP-binding cassette transporter A3 using the cyclooxygenase type-2 inhibitor indomethacin inhibited exosome release, thereby reducing the sump effect of CD20-bearing exosomes and enhancing the efficacy of rituximab [99]. Together, the above studies provide insights into the mechanisms by which exosomes contribute to the pathobiology of cancer. Continued investigation of the properties of exosomes is warranted to better understand their role.

## ctDNA/RNA clinical utility

A key advantage of a liquid biopsy includes being much less invasive than primary tumor biopsies. This enables multiple clinical applications, including characterization of molecular profiles in lieu of tissue, monitoring response to therapy, detection of minimal residual disease, and tumor evolution with therapy.

For positive selection biomarkers, where the presence of a mutation is associated with response to targeted therapy, the performance of ctDNA is already sufficient for integration into clinical care. In Europe, the regulatory labeling for gefitinib was updated to allow use of ctDNA for assessment of activating EGFR mutations in patients where a tumor sample is not available for testing. Rociletinib (CO-1686), an inhibitor of EGFR T790M, is undergoing priority review by the FDA with both tissue and plasma-based testing incorporated into the initial studies [102]. Although not yet included in the regulatory label for agents in the United States, similar ctDNA tests are being offered for clinical use by a number of companies and are generally accepted as sufficient for treatment eligibility by insurance providers.

Negative selection biomarkers of KRAS or NRAS mutations for EGFR inhibitors in CRC have been slower to be adopted for routine clinical care, in part due to concerns about false negatives and the high accessibility of tumor tissue for testing. Nevertheless, efforts are underway to deploy ctDNA testing due to improved turnaround time and specimen availability. The increasing clinical utilization of ctDNA testing currently is also driven by patients and physicians attempting to identify targeted therapies for off-label use or clinical trial enrollment, which has been enabled by the development of clinical assays using next generation sequencing (NGS) [80].

The half-life of non-exosomal ctDNA is estimated to be several hours, and therefore provides opportunities for tumor monitoring, and therapeutic and surgical response [103, 104]. In CRC, e.g. patients who are later demonstrated radiographically to have responding disease have a drop of >90% of the ctDNA levels after the first 2 weeks of treatment [104]. This rapid clearance has been shown to stratify risk of recurrence after resection with curative intent. In breast cancer, patients with detectable ctDNA after resection had a 25-fold higher hazard of recurrence [105]. These biomarkers are now being integrated into prospective clinical trials. A summary of phase 2 through 4 clinical trials incorporating ctDNA is listed in Table S3, available at *Annals of Oncology* online .

Serial blood draws throughout the identification, monitoring and treatment phases of the tumor continuum can provide a circulating profile of macromolecules. Such a snapshot of circulating genome and transcriptome can be more representative of the different regions of a heterogeneous tumor. This helps support the identification and emergence of therapeutic resistance much earlier than solid tissue biopsies. An illustration of the information that can be potentially extracted from a liquid biopsy is shown (Figure 3). A variety of clinical studies are attempting to harness the power and utility of liquid biopsies. Currently available clinical data and ongoing trials encompass a number of tumor sites each with their own specific tumor characteristics and informative biomarker subsets [106, 107]. These hypothesis-driven, investigator-initiated studies may show potential benefit of early detection and identification of genetic mutations. We believe with technological progress and prolific use of such techniques holds great promise for the future of cancer treatment and individualized care.

## Combined approach and future perspectives

Here we have discussed three sources of biomarkers for personalized therapy. Moving forward, we envision that each of these biomarkers can be incorporated for a thorough, minimally-invasive means of assessing prognostic outlook, therapeutic response, and risk of relapse. For example, a trial of 62 metastatic PCA patients resistant to anti-androgen therapy found that the presence of the

androgen receptor (AR) splice variant AR-V7 in CTC was associated with castration resistant PCA (CRPC) [67]. Previous research has found a significant correlation with plasma DNA between PCA subgroups based on disease spread and tumor stage and that these levels were reflective of CTC levels [108]. With the recent development of single cell western technology by Amy Herr's group as well as the new device for similar application (Milo—ProteinSimple, San Jose, CA, USA), it is also possible to extract translational changes [109]. Similarly, a recent study of AR copy number in the serum of 59 advanced CRPC patients found significant correlation between copy number gain with baseline PSA, alkaline phosphatase, LDH levels, as well as PFS and OS [110]. Further, elevated levels of multiple miRNAs have been associated with metastatic PCA, compared with healthy controls [111]. miR-141 is one such biomarker which is concentrated in exosomes, compared with serum and is elevated in PCA patients [112]. In a hypothetical scenario, these independent studies could all be conducted on a single liquid biopsy whereby the individual CTCs could be subjected to either whole genome analysis, FISH, or specific CNVs using NGS. Additionally, the levels of target miRNA, or cfDNA/RNA transcripts in plasma can be screened for driver mutations. As evidenced by Dr Dive's recent work, the heterogeneity of a patient's CTC population reflects the disease status [27]. Since these tumors take months to develop and analyze, the single-cell western blot approach can provide instant results which can be later confirmed/scrutinized using CDX models. The variety of approaches listed in this paragraph highlight the currently available robust, reliable, and validated means of extracting information from a liquid biopsy. Overall, we envision that growing knowledge of CTCs, cfDNA/RNA, and exosomes will provide an increased resolution to the snapshot of tumor status obtained through liquid biopsies.

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