Chapter

CTCs as Liquid Biopsy: Where Are We Now?

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Abstract

A few years ago, the analysis of circulating tumor cells (CTCs) in the blood of patients with cancer was defined by the term "real-time liquid biopsy." Blood samples can be obtained and analyzed at the time of diagnosis and repeatedly during the systemic treatment. The analysis of the *liquid biopsy* has provided new insights into the biology of metastasis with important implications for the clinical management of cancer patients. In this review, we updated all technical strategies developed to improve enrichment, detection, and characterization of CTCs. We also focused on their biological properties as well as on their clinical relevance in different cancer types. At the end, we opened the discussion to all the other circulating biomarkers used as *liquid biopsy*.

Keywords: circulating tumor cells, *liquid biopsy*, clinical relevance, circulating biomarkers, precision medicine

1. Introduction

A few years ago, the analysis of circulating tumor cells (CTCs) in the blood of patients with cancer was defined by the term "*liquid biopsy*" [1]. Blood samples can be obtained and analyzed at the time of diagnosis and during the systemic treatment. Detection of CTCs in circulation gives important information on the molecular properties of tumor lesions. This information contributes to the early detection of metastatic lesions and participates in the personalized treatment of cancer patients such as prognostic evaluation, stratification of patients for targeted therapies, real-time monitoring of treatment efficacy, identification of therapeutic target, and resistance mechanism.

The analysis of the *liquid biopsy* has provided new insights into the biology of metastasis with important implications for the clinical management of cancer patients (**Figure 1**). Numerous clinical studies and meta-analyses including large cohorts of patients have shown that the number of CTCs is an important indicator of the risk of progression or death in patients with metastatic solid cancer (e.g., breast, prostate, colon, etc.) [2–6].

Despite the remarkable advances made in recent years, so far, *liquid biopsy* analyses are rarely implemented in routine patient testing. In-depth investigation of CTCs remains technically challenging. CTCs occur at the very low concentrations of one tumor cell in the background of millions of blood cells. Their identification and characterization require extremely sensitive and specific analytic methods. Moreover, up to now, results obtained with liquid biopsy analysis did not lead yet to

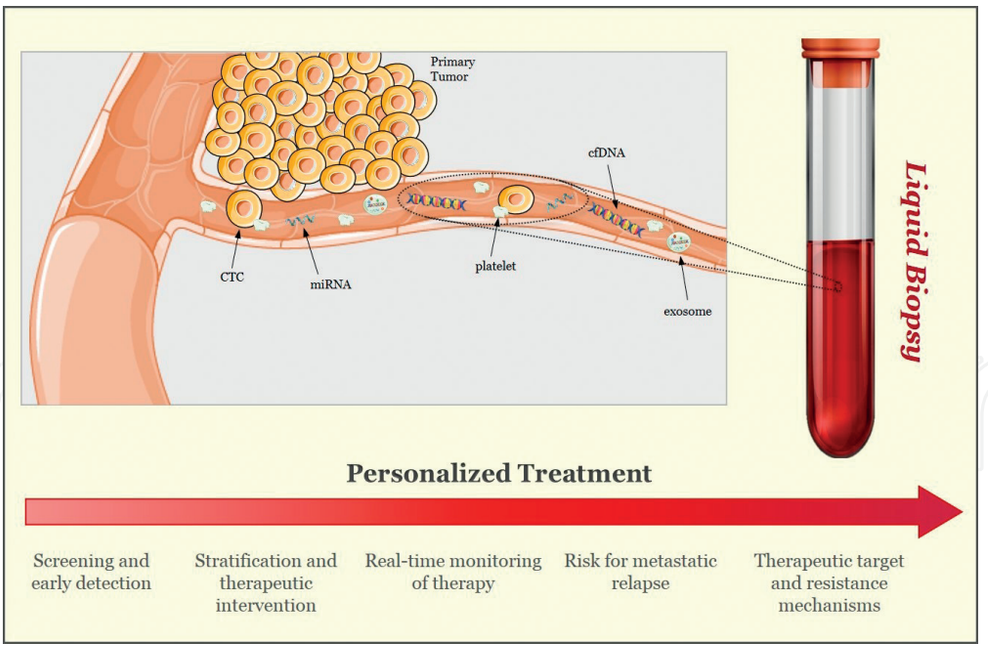


Figure 1.
From the blood sample toward the precision medicine in cancer patients.

validated guidelines for treatment and patient management. Nevertheless, technical advances and encouraging clinical studies demonstrated that *liquid biopsy* holds great promise for revolutionizing cancer diagnostics in a soon future.

Here, we will outline the advantages and challenges of CTCs as *liquid biopsy* in oncology by discussing the strategies for enrichment, detection, and characterization linked to the biology of these cells. Moreover, the potential of CTC analysis for clinical utility will be argued as well as other circulating biomarkers.

2. Technical strategies for enrichment, detection, and characterization of CTCs

At the moment, in-depth investigation of CTCs still remains technically challenging as they are every rare events in blood circulation. Their identification and characterization require extremely sensitive and specific analytic methods, which are usually a combination of enrichment and detection procedures (**Figure 2**). The different strategies to analyze CTCs are described in this chapter, and all the advantages/disadvantages plus the commercial status are summarized in **Table 1**.

2.1 Strategies for CTC enrichment

Up to date, a large panel of technologies was designed to enrich CTCs from the surrounding normal hematopoietic cells. These enrichment methods rely on different properties of CTCs: (a) biological properties (e.g., surface protein expression) and (b) physical properties (e.g., size, density, electric charges, and deformability).

Biological properties are mainly used in immunological procedures with antibodies against either tumor-associated antigens (positive selection) or common leukocytes antigen CD45 (negative selection). Positive enrichment typically attains high cell purity, which depends on antibody specificity. Among the current positive systems, most of the technologies targeted the epithelial cell adhesion molecule (EpCAM) antigen, as the FDA-cleared CELLSEARCH® system which is frequently compared for all new CTC detection methods as the gold standard. However,

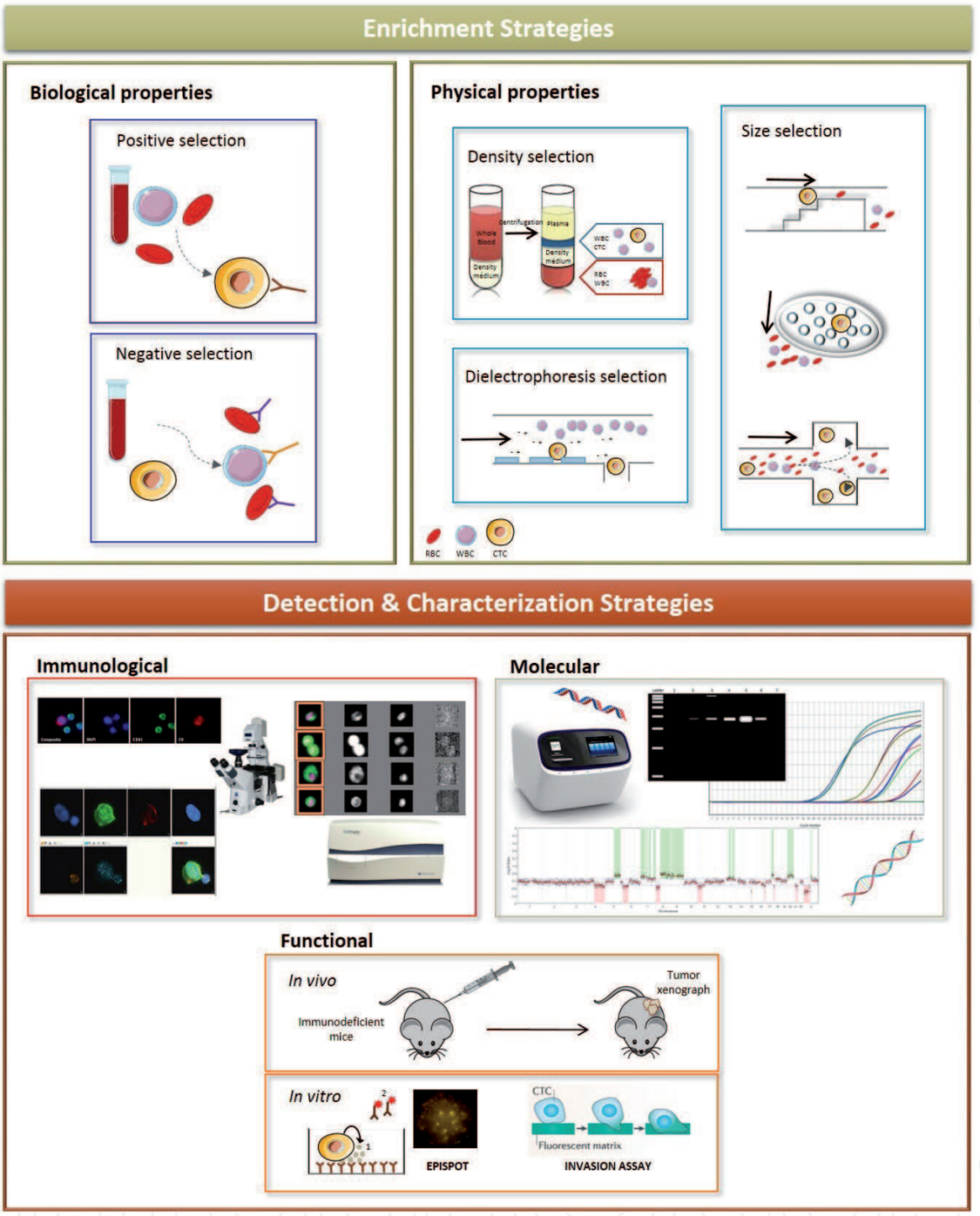


Figure 2.
Strategies for enrichment, detection, and characterization of CTCs.

capturing CTCs lacking EpCAM expression has involved the use of cocktails of antibodies against various other epithelial cell surface antigens (e.g., EGFR, MUC1) or against tissue-specific antigen (e.g., PSA, HER2) and against mesenchymal or stem-cell antigens (e.g., Snail, ALDH1) [7]. Positive selection of CTCs requires an assumption about the unknown nature of CTCs in an individual blood sample. This bias is avoided by negative selection in which the blood sample is depleted of unwanted cells. Indeed, negative enrichment targets and removes background cells, such as leukocytes, using antibodies against CD45 (which is not expressed on carcinomas or other solid tumors) and other leukocyte antigens, to achieve a CTC-enriched sample. Moreover, negative enrichment technologies evade some of the pitfalls of positive enrichment; for example, CTCs are not tagged with a difficult-to-remove antibody, they are not activated or modified via an antibody-protein

SUBCATEGORIES	CRITERIA	TECHNOLOGIES	ADVANTAGES	DISADVANTAGES	COMMERCIAL STATUS
Enrichment methods					
Biological properties					
Positive selection	Immunomagnetic EpCAM	CellSearch®	Robust - Reproducible - Automated	Miss CTCs lacking or with very low expression of EpCAM	Silicon BioSystems-Menarini FDA approved - IVD
		MACS	No centrifugation step		Miltenyi RUO
		MagSwepper			Not commercialized
	Immunomagnetic Antibodies cocktail	AdnaTest	Based on a panel of proteins expressed on CTCs		AdnaGen RUO
	Microfluidic EpCAM	Ephesia Chip			Not commercialized
		IsoFlux™	Automated	Miss CTCs lacking or with very low expression of EpCAM	Fluxion RUO
		CTC-iChip	Combination of biological and physical properties		Not commercialized
	Microfluidic Antibodies cocktail	OncoCEE	Based on cocktail of Abs to capture CTCs		Biocept RUO
	Surface capture	CytoTrapNano™			CytoLumina RUO
	In vivo	CellCollector®	Screen a large volume of blood	Cells can not be removed from the device for the an easy CTC detection	Gilupi CE approved
Negative sélection	Immuno complexes	RosetteSep™ CTC Enrichment	No bias based on protein marker expression nor size of CTCs	Leucocyte contamination	STEMCELL Technologies RUO
	Immunomagnetic	EasySepTM			STEMCELL Technologies RUO
		MACS			Miltenyi RUO
	Microfluidic	CTC-iChip			Not commercialized
Physical properties					
Density selection		Ficoll-Paque®	Fast and cheap No specific equipment needed	Heterogeneity of the density of CTCs	GE Healthcare
		OncoQuick®			Greiner Bio-One
Size based selection	Microfiltration	ISET®	Automated	Heterogeneity of the size and deformability of CTCs	Rarecells RUO
		ScreenCell®			ScreenCell RUO
		CellSieve™			Creatv MicroTech RUO
		Parsortix™	Automated		Angle RUO
	Inertial focusing	VTX-1	Automated		Vortex Biosciences RUO
		ClearCell® FX		Need of RBC lysis before enrichment	Clearbridge BioMedics RUO
Dielectrophoretic charge		ApoStream®		Need a pre enrichment step with ficoll gradient centrifugation	APOCELL RUO
Cluster selection		Cluster-Chip			Not commercialized
Detection / Characterization method					
Immunological					
	Imaging	CellSearch®	Standardized - Robust - Reproducible - Automated	Limited for the protein characterisation - only one channel free	Silicon BioSystems FDA approved - IVD
		IsoFlux Cytation Imager			Fluxion RUO
		CytoTrapNano™	Only one equiment for enrichment and detection step		CytoLumina RUO
		Ikoniscope®	Hight resolution imaging Fish tests associated	Standard microscopy	Ikonisys
		Ariol system	Hight resolution imaging Software for clinical analysis		Leica Biosystems
	FAST technology	FASTCell™	No need of enrichment method before		SRI International
		CytoTrack CT11™	No need of enrichment method before Pick up of cells		CytoTrack
		EPIC	Only lysis of RBC as enrichment step	No equipment avaliable Only the service is commercialized	EPIC Science
	Flow Cytometry	Different kind of cytometer	Sorting capacity for some cytometer	Less sensitive	
	Dielectrophoresis microscopy	DEPArray	Sorting capacity at single cell level		Silicon BioSystems RUO
Nucleic acid based					
	mRNA	screenCell®	Specific panels for each cancer types	No CTC observation	AdnaGen RUO
		Genesis System	High throughput technology Microscopy avaliable for CTC observation		Celsee RUO
	RNA sequencing DNA mutation DNA methylation DNA sequencing	Specific to each lab and research studies			Not commercialized
Functional					
	CTC culture	EPISPOT	Detection of viable CTCs	No possibility to recover CTCs for downstream analysis	Not commercialized
		EPIDROP	Detection of the total number of CTCs plus viables CTCs at the single cell level		Not commercialized
		Vita-Assay™	Detection of viable CTCs		Vitatex RUO
	In vivo	PDX/CDX models	Specific to each lab and research studies		Not commercialized

Table 1.
Advantages, disadvantages, and commercial status of technologies for enrichment, detection, and characterization of CTCs.

interaction, and antibody selection does not bias the subpopulation of CTCs captured. However, these advantages come at the cost of purity, as negative enrichment strategies typically have a much lower purity than positive enrichment [8–10] and require a suitable CTC detection step.

These last years, numerous marker-independent techniques have been developed for CTC isolation and detection. Label-free enrichment process based on physical properties, such as density, size, deformability, and electric charge, have come to avoid molecular bias induced by variability of cell biomarker expression associated with tumor heterogeneity. Mostly used, size and density technologies like microfiltration technologies, based on the precedent that CTCs generally exhibit a larger morphology than leukocytes, or microfluidic devices using inertial focusing to separate CTCs from blood are developed by several companies such as ScreenCell® [11], ISET® [12], CellSieve™ [13, 14], Parsortix™ [15], or Vortex [16]. Such technologies or approaches have the advantages of being less complicated, sometimes rapid, and require minimal equipment. However, some of these approaches may be prone to clogging, and the release of the CTCs into suspension for further analysis is challenging.

2.2 Strategies for CTC detection

After enrichment, the CTC fraction still contains a substantial number of leukocytes, and CTCs need to be specifically identified at the single-cell level by a robust and reproducible method that can distinguish them from normal blood cells.

Immunological technologies are the most frequent methods used for CTC detection using a combination of membrane and/or intracytoplasmic anti-epithelial, anti-mesenchymal, and anti-tissue-specific marker or antitumor-associated antibodies [7]. However, many CTC assays use the same identification step as the CELLSEARCH® system: cells are fluorescently stained for cytokeratins (CK), the common leukocyte antigen CD45, and a nuclear dye (DAPI).

Nucleic acid-based CTC detection methods are the most widely used alternatives to immunological assays to identify CTCs. These techniques identify specific tumor DNA or mRNA to confirm the presence of CTCs indirectly [17]. Detection involves designing specific primers supposedly associated with CTC-specific genes. These genes either code for tissue-, organ-, or tumor-specific proteins or, more specifically, contain known mutations, translocations, or methylation patterns found in cancer cells [18]. These methods have the highest sensitivity but lack specificity, owing to the potential of captured noncancerous cells to generate false-positive signals, thus decreasing the overall accuracy. Considering the genetic heterogeneity of CTCs, multiplex PCR, such as the AdnaTest kit (AdnaGen AG), could overcome this limitation [19, 20].

Furthermore, functional assays that exploit aspects of live cellular activity for CTC detection have the particularity to focus on the discovery of the "metastasis-competent cells." The functional epithelial immunospot (EPISPOT) assay was introduced for in vitro CTC detection and focuses only in viable CTCs [21]. This technology assesses the presence of CTCs based on secretion, shedding, or release of specific proteins during 24–48 h of short-term culture [22]. More recently, Tang et al. described a high-throughput metabolic-based assay for rapid detection of rare metabolically active tumor cells in pleural effusion and peripheral blood of lung cancer patients [23]. In vivo, important information can be obtained by transplantation of patient-derived CTCs into immunodeficient mice: tumors that could grow after xenotransplantation of enriched CTCs have the characteristics of metastasis-initiator cells [8].

2.3 Strategies for CTC characterization

CTCs hold the key to understand the biology of metastasis and provide a biomarker to noninvasively measure the evolution of tumor subclone during treatment

and disease progression. Improvements in technologies to yield purer CTC populations make better cellular and molecular investigation. Characterization of CTCs allows better insight into tumor heterogeneity, within most assays, including immunofluorescence, array CGH, next-generation sequencing (NGS) of both DNA and RNA, and fluorescence in situ hybridization.

Protein analyses on single CTCs are currently performed by immunostaining with antibodies directed against protein of interest. Multiple labeling is possible but usually restricted to a few proteins of interest for tumor cell biology and cancer therapy. This may help to identify signaling pathways relevant to metastasis development and treatment responses. In breast cancer patient, the HER2 status of CTCs could be assessed and shows discrepancies with primary tumor status [24, 25]. More recently, immune checkpoint regulators such as programmed death-ligand 1 (PD-L1) have become exciting new therapeutic targets and could be used for *liquid biopsy* in future clinical trials on patients undergoing immune checkpoint blockage [26, 27].

Immunological detection and characterization offer the advantage of allowing isolation of stained CTCs for subsequent molecular characterization. While manual isolation by micromanipulation of CTCs is possible [28], it is rather arduous and time-consuming. An alternative automated single-cell selection device has been therefore developed. The DEPArray™ technology based on a dielectrophoresis strategy by trapping single cells in DEP cages [29] is designed for single-cell recovery of CTCs. Multiple clinical studies have used DEPArray™ to detect and recover single CTCs for subsequent genetic analyses [30–32].

Among single-cell sequencing to identify genomic and transcriptomic characteristics of CTCs, most studies have focused on genomic analyses and carried out whole genome amplifications (WGAs) to increase the amount of DNA, which is subsequently subjected to the analyses of specific mutations and copies number variations using conventional and next-generation sequencing technologies [28, 33, 34]. As an example, CTCs with mutated KRAS genes will escape anti-EGFR therapy, and their early detection might help to guide therapy in individual patients. Besides isolation of single CTCs, a 3–4 log units enrichment step are enough to detect CTCs based on recently developed highly sensitive technologies (e.g., droplet digital PCR) [35].

Another approach is fluorescence in situ hybridization (FISH) analysis of single CTCs identified by immunocytochemistry [36, 37]. Such an immuno-FISH approach can be combined with automated detection of CTCs and might be easier to implement in future clinical diagnostics. Recently, padlock probe technology, which enables in situ analysis of AR-V7 in CTCs, showed that 71% (22 of 31) of CRPC patients had detectable AR-V7 expression ranging from low to high expression [38]. Patients with AR-V7-positive circulating tumor cells (CTCs) have greater benefit of taxane-based chemotherapy than novel hormonal therapies, indicating a treatment-selection biomarker [39, 40].

Finally, these last years, many teams tried to obtain CTC lines by culturing CTCs *ex vivo*. The establishment of in vitro cultures and permanent lines from CTCs has become a challenging task. Indeed, CTC lines could be used to identify proteins and pathways involved in cancer cell stemness and dissemination and also to test new drugs to inhibit metastasis-competent CTCs. *Ex vivo* CTC cultures have been established for breast [41, 42], prostate [43], lung [44], colon [22], and head and neck cancer [45]. To our knowledge, permanent CTC lines have been described only from circulating colon cancer cells: one before (CTC-MCC-41) [22, 46] and eight after the initiation of the anticancer treatment [47].

3. Biology of CTCs

3.1 Epithelial to mesenchymal plasticity

Epithelial to mesenchymal transition (EMT), which is characterized by the downregulation of epithelial proteins and upregulation of mesenchymal proteins, is a complex process that supports the migratory capacity of epithelial tumor cells and is thought to play a crucial role in promoting cancer metastasis. EMT led to increased motility via rearrangements of cellular contact junctions and loss of cell adhesion (i.e., E-cadherin, N-cadherin, claudins), plus epithelial cell morphology through cytoskeleton modification (i.e., cytokeratin, vimentin, fibronectin, etc.) [48]. This invasive phenotype enables cancer cells to pass through the basal membrane and endothelial barriers of blood vessels to reach bloodstream. However, it is still unclear what degree of EMT is needed in tumor cells to attain the circulation.

Despite the wealth of experimental data, the exact role of EMT in cancer patients remains more controversial. Over the past 10 years, the development of sensitive technologies that allow the detection and molecular characterization of CTCs helped to shed new light into the importance of EMT for human tumor cell dissemination [7, 49]. All these data lead now to a new trend, focused on plasticity of tumor cell: epithelial to mesenchymal plasticity (EMP) associated with stemness. This process is today considered as a central actor of the metastatic cascade, providing tumor cells the ability to adapt to the different microenvironments encountered during metastatic spread to colonized organs (i.e., adjacent stroma, blood, newly colonized organs).

CTCs with mesenchymal and stemness features can be attributed in some clinical studies to higher disease stages and metastasis [50–52] and even to therapy response and worse outcome [53–55]. However, the published studies addressing the impact of mesenchymal-like CTCs show heterogeneity with regard to assay specificity, size of cancer and control groups, and endpoint parameters.

To conclude, evaluation of the EMT and stem-cell markers in CTCs may provide information of clinical interest, and using these markers to classify CTCs can elucidate CTC heterogeneity. Nevertheless, studies still suffer from lack of standardized procedures and small sample sizes. Therefore, larger well-designed clinical trials are needed to further illuminate the potential values of EMT markers in CTCs.

3.2 Anoikis resistance

In normal tissue, adhesion to appropriate extracellular matrix proteins is essential for survival. Loss of this adhesion induces cell death which has been termed "anoikis." Anoikis is a physiologically relevant process for tissue homeostasis and development because it prevents detached epithelial cells from colonizing elsewhere, thereby inhibiting dysplastic cell growth or attachment to an inappropriate matrix [56]. Dysregulation of anoikis, such as anoikis resistance, is a critical mechanism in tumor metastasis. If cells acquire oncogenic signals that are able to overcome this machinery, they gain the ability to survive outside their normal environment in the absence of adhesion to the extracellular matrix. The tumor cells that acquire anoikis resistance can survive detachment from their primary site, traveling through the circulatory and lymphatic systems to disseminate to ectopic locations [57]. Different studies have shown that the death receptor pathway of caspase activation mediates anoikis; thus, defects in this pathway such as overexpression of the caspase-8 inhibitor FLIP can turn cell resistant to anoikis. Similarly, resistance

to anoikis can be conferred by roadblocks in the mitochondrial pathway, such as overexpression of the Bcl-2 family of anti-apoptotic proteins [57].

The investigation of molecular mechanisms involved in cancer cell survival while they are leaving the adherent microenvironment of the tumor to the circulatory system is important to understand the process by which cancer can spread to distant organs, as well as to design new therapeutics to inhibit the spread of the disease.

3.3 Escape to the immune system

Once in the bloodstream, CTCs face several natural obstacles that hinder the metastatic process. One of the main obstacles that CTCs face in the blood is the attack of the immune system. Lots of work was done to understand mechanisms involve in the battle between the immune system's capabilities to fight cancer and the immune-suppressive processes that promote tumor growth. Several biomarkers showed up from this work; for example, in colorectal cancer, immune escape was observed by the upregulation of CD47, a "don't eat me signal" that prevents CTCs from macrophage and dendritic cell attack [58]. The most clinically advanced biomarkers are the programmed death-1 (PD-1) and its ligand (PD-L1). PD-L1 expressed in tumors has been highlighted to function as a key component of the cancer-immunity cycle by preventing the immune system from destroying cancer cells. PD-1 receptor is a surface protein expressed on activated T-cells, and its ligand PD-L1 is expressed on the surface of antigen-presenting cells. The formation of the PD-1/PD-L1 complex induces a strong inhibitory signal in the T-cell, which leads to a reduction of cytokine production and a suppression of T-cell proliferation [59]: the immune system is misled by the cancer cells expressing PD-L1 and does not destroy them. That understanding led to the development of immune checkpoint inhibitor therapies, antibodies against both PD-1 and PD-L1, and remarkable clinical responses which have been seen in several different malignancies including, but not limited to, melanoma, lung, kidney, and bladder cancers [59].

However, CTCs can use several mechanisms to survive in the circulatory system. For example, these cells can couple to reactive platelets. Several hypotheses propose that the surface coating of platelets may serve as a shield against immune assault or that platelets may load the major histocompatibility complex to CTCs to imitate host cells and therefore avoid immune surveillance [60]. The aggregation of CTCs with platelets, stromal fibroblasts, and leukocytes leads to the formation of floating complexes and increases the survival of CTCs in the bloodstream by avoiding anoikis and killing by immune cells [61]. In addition, the vascular endothelial growth factor (VEGF), secreted by platelets, is able to affect the maturation of dendritic cells that play a key role in antigen presentation [62].

3.4 CTC microemboli

An alternative mechanism for metastasis has emerged from recent studies, the collective migration of tumor cells by clusters of CTCs. CTC clusters are defined as groups of tumor cells (more than two or three cells, varied among studies) that travel together in the bloodstream. Thus, in the blood circulation, CTCs can be found both as single tumor cells and clusters of tumor cells in patients with an advanced stage of the cancer. Study using mouse models with tagged mammary tumors demonstrates that these clusters arise from oligoclonal tumor cell groupings and not from intravascular aggregation events [63]. Moreover, CTC clusters have 23- to 50-fold increased metastatic potential. Even fewer in number, clusters of CTCs possess much higher metastatic potential than individual CTCs.

Patients with CTC microemboli or clusters in their bloodstream have significantly worse overall and progression-free survival than those with only individually migrating single CTCs [63]. The prognostic value of CTC clusters can be estimated by clinical observations.

Current studies have partially elucidated the reasons for CTC clusters to have higher potential of metastasis. First, tumor cells within CTC clusters showed prolonged survival and decreased apoptosis [64]. Second, the physical specialty of CTC clusters allows for a greater likelihood of it residing in distant organs. Microvasculature of viscera can retain large CTCs; thus, it can retain CTC clusters more easily [65].

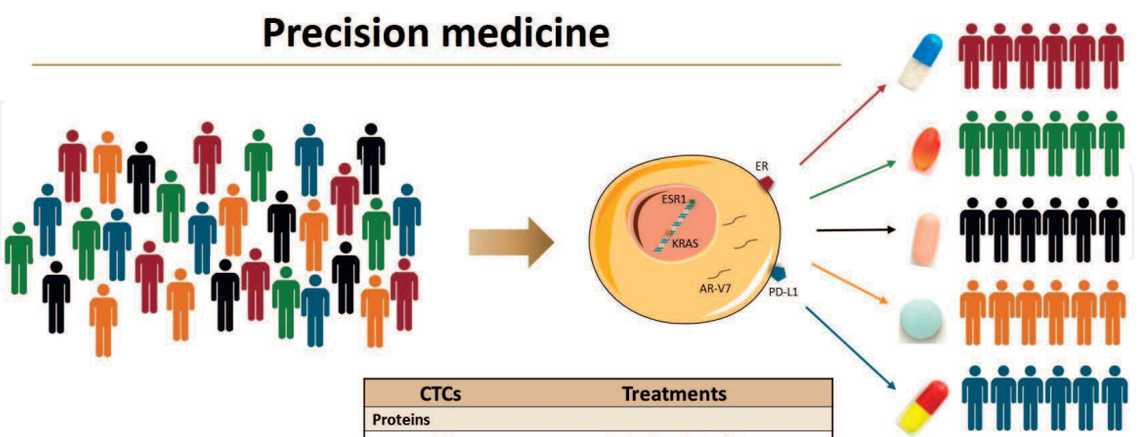
4. Clinical relevance of CTCs

Despite many clinical validation studies, CTCs have not been included yet into the clinical guidelines (e.g., ASCO guidelines at http://www.asco.org/practice-guidelines/quality-guidelines/guidelines). Although CTC enumeration can improve current tumor staging and contribute to the early assessment of therapy effects, the clinical utility of CTCs remains to be addressed in interventional studies (i.e., its capacity to decide adopting or to rejecting a therapeutic action).

In this chapter, we highlighted the clinical relevance of CTCs in breast, prostate, colon, and lung cancer. **Figure 3** illustrates how CTCs as *liquid biopsy* can guide clinicians to personalized medicine.

4.1 Breast

More advanced studies, regarding clinical utility of CTCs, are related to metastatic breast cancer (MBC). Sequential CTC enumeration has been shown in a large multicenter prognostic study to be superior to conventional serum protein markers (CA-15-3, CEA) for early detection of therapy failure in MBC [5]. However, in the



CTCs	Treatments
Proteins	
ER	Endocrine therapies
Her2-neu	Her2 targeted therapies
PD-L1	Immunotherapies - PD-L1 inhibitors
ARNm	
AR-V7	Resistance to endocrine therapies
DNA mutation	
KRAS	EGFR targeted therapies
BRAF	BRAF targeted therapies
PI3K	PI3K pathway inhibitors Resistance to EGFR therapies
ESR1	Resistance to endocrine therapies
T790M	Third generation of Tyrosine Kinase Inhibitors

Figure 3.
CTC as liquid biopsy for precision medicine.

interventional trial SWOG 0500 (NCT00382018), although the prognostic significance of CTCs was confirmed, the CTC-driven switch to an alternate cytotoxic therapy was not effective in prolonging overall survival for MBC patients with persistently increased CTCs after 21 days of therapy [66]. The inconvenient of these kinds of interventional biomarker-driven studies is the fact that the result is dependent of the therapy efficacy. This strategy can only work if there is an efficient therapy for the cohort identified by the test.

Another promising approach is the stratification of patients to chemotherapy or hormonal therapy based on CTC enumeration like in the interventional STIC CTC METABREAST clinical trial (NCT0 1,710,605) for MBC patients [67]. Besides CTC enumeration, stratification based on CTC phenotype might become also an important strategy. Stratification of MBC patients based on HER2 status of CTCs is currently tested in the DETECT III trial [67].

Other possible uses for CTC detection include prognostication in early stage patients, identifying patients requiring adjuvant therapy. The SUCCESS study provides strong evidence of the prognostic relevance of CTCs in early breast cancer before and after adjuvant chemotherapy in a large patient cohort [68]. This study outlines the potential of the CTC analysis at primary diagnostic to evaluate individual risk and points that they may use for treatment management in early stage of cancer. These data have been confirmed by Bidard et al. who conducted a meta-analysis in nonmetastatic breast cancer patients treated by neoadjuvant chemotherapy (NCT) to assess the clinical validity of CTC detection as a prognostic marker [69]. They showed that CTC count is an independent and quantitative prognostic factor in early breast cancer patients treated by NCT. *Liquid biopsy* complements current prognostic models based on tumor characteristics and response to therapy. Moreover, Trapp et al. demonstrated recently that the presence of CTCs 2 years after chemotherapy was associated with decreased OS and DFS. Based on these results, active individualized surveillance strategies for breast cancer survivors based on biomarkers should be reconsidered [70].

4.2 Prostate

For men with metastatic castration-resistant prostate cancer (mCRPC), the CELLSEARCH® system method for CTCs enumeration is the only FDA-cleared CTC test available clinically. The CTC count has been shown to provide prognostic value and was associated with treatment response in mCRPC patients, in several recent studies [4, 71–73], indicating a clear value as a patient-level indicator of survival. However, despite increasing evidence that CTCs could be used to monitor disease progression in mCRPC [18], CTC use is still limited to clinical trials in academic centers. Clinical utility of CTCs, reflecting the ability of this test to favorably change outcomes, is still an unmet clinical need in prostate cancer [74]. The first interventional clinical trial in prostate cancer that will show the clinical utility of CTCs should start in 2019 (TACTIK project—NCT03101046).

Moreover new data suggest that CTCs may harbor genetic information (such as the androgen receptor splice variant 7, AR-V7) relevant to changing clinical management and predicting treatment sensitivity or resistance to cancer therapies such as enzalutamide, abiraterone, and taxane-based chemotherapies [39].

Regarding nonmetastatic cancer patient, a recent European TRANSCAN study CTC-SCAN investigated the feasibility of detecting CTCs in nonmetastatic high-risk prostate cancer (PCa) patients by combining the CELLSEARCH® platform, the in vivo CellCollector® capture system, and the EPISPOT assay. The observed correlation, with established risk factors and the persistence of CTCs 3 months after surgery, suggested a potential clinical relevance of CTCs as markers of

minimal residual disease (MRD) in PCa [75]. CTC-based liquid biopsies have the potential to monitor MRD in patients with nonmetastatic prostate cancer although follow-up evaluations are now required to assess how to provide independent prognostic information. A new European project (Transcan—PROLIPSY) will assess whether CTCs in combination with exosomes and ctDNA as noninvasive *liquid biopsy* allow the diagnosis of prostate cancer and the evaluation of its aggressiveness.

4.3 Colon

In 2008, Cohen et al. demonstrated the independent prognostic and predictive value of CTCs for patients initiating chemotherapy for metastatic colorectal cancer (mCRC) [2]. Since this first publication defining a cutoff of three CTCs, different meta-analyses have confirmed that baseline levels of CTC count is an important prognostic factor for PFS and OS in patients with mCRC [76–78].

Despite the strong evidence of a prognostic significance of CTC count, there is no solid evidence demonstrating the interest of CTC count for therapeutic strategy, and this biomarker is rarely used in the management of patients with mCRC. However, patients with high CTC counts recruited in a phase II study could benefit from a more intense chemotherapeutic regimen [79]. These preliminary data require validation in randomized trials. Moreover, Lalmahomed et al. failed to show a prognostic effect of CTCs for early relapse after the resection of colorectal liver metastases [80].

4.4 Lung

The role of CTCs in non-small cell lung cancer (NSCLC) has been addressed in several clinical trials. More specifically, the prediction of the outcome of patients with early and advanced NSCLC based on the CTC enumeration has been explored. The CTC count with the CELLSEARCH® system in advanced NSCLC patients who received standard chemotherapy was associated with a shorter PFS and OS, but standardize cutoff could not be observed [81–84]. Furthermore, analysis of CTCs from patients with metastatic NSCLC identified the expected EGFR-activating mutation in CTCs from 11 of 12 patients (92%) and in matched free plasma DNA from 4 of 12 patients (33%) [85]. The T790 M mutation, which confers drug resistance, was revealed in CTCs from patients who had received tyrosine kinase inhibitors, suggesting the strong potential gain of noninvasive liquid biopsy. Moreover, serial increases in CTC counts were associated with tumor progression, with the emergence of additional EGFR mutations in some cases. Recently, KRAS and EGFR mutations, relevant for treatment decisions, could be detected in CTCs and in the corresponding primary tumors of the same patients [86].

5. Other circulating biomarkers as *liquid biopsy*

Even if the term "*liquid biopsy*" was originally used for CTC analysis, currently, it includes all different circulating biomarkers like circulating cell-free DNA (cfDNA), microRNA (miRNA), and exosomes that are shed into the bloodstream by tumors and/or metastatic deposits, as well as tumor-educated platelets which are described to have a role in tumor metastasis. Like CTCs, all these other circulating biomarkers need to be validated in clinical trials. **Table 2** summarizes observational and interventional clinical trials on breast, lung, prostate, and colorectal cancer registered in *clinical.gov* (A) and the applications (B).

A.

Biomarkers / Cancer types	CTCs	Circulating DNA	Exosomes	microRNA	TEPs
Breast	**816**	**43**	**5**	**6**	**0**
Interventional	755	24	4	3	
Observational	61	19	1	3	
Lung	**792**	**77**	**9**	**0**	**0**
Interventional	733	25	5	-	
Observational	59	52	4	-	
Prostate	**515**	**25**	**8**	**2**	**0**
Interventional	475	10	2	0	
Observational	40	15	6	2	
Colorectal	**528**	**55**	**4**	**2**	**0**
Interventional	474	18	3	1	
Observational	54	37	1	1	

B.

CLINICAL TRIALS	CTCs	Circulating DNA	Exosomes	microRNA	TEPs
Cancer screening	X	X			
Pronostic value	X	X	X	X	
Treatment response	X	X	X	X	
Protein marker characterization	X				
Mutation analysis	X	X			
Biologial process understanding	X	X	X	X	

Table 2.
*(A) Number of observational and interventional clinical trials (clinical.gov) involving **liquid biopsy** in the main cancer types and (B) the applications of each circulating biomarkers (CTCs, circulating DNA, exosomes, microRNA, and TEPs).*

5.1 Circulating tumor DNA

Apoptotic and necrotic tumor cells are known to discharge cell-free nucleic acid fragments into the bloodstream of cancer patients. Although most circulating DNA is believed to originate from nonmalignant cells, an increased level of cfDNA was observed in blood of patients with late stage cancer [87]. Among the pool of total cfDNA, there is circulating tumor DNA (ctDNA) which cannot be specifically isolated from the total pool but can be detected by tumor-specific mutations [88]. In general, cfDNA can be analyzed from plasma by targeted or untargeted approaches. The targeted approaches involve the detection of known genetic changes, e.g., "druggable" mutations, with impact on therapy decisions [89]. The interest of cfDNA was demonstrated by Douillard et al. [90] by determining the EGFR mutational status in NSCLC and can represent a substitute for tissue biopsies when these are not available. Moreover, in 2016, the detection of EGFR gene mutations in cfDNA using the cobas EGFR Mutation Test v2 achieved FDA approval as a companion diagnostic for erlotinib, becoming the first blood-based biopsy test approved for implementation in clinical decisions [91].

However, despite the evidence of potential clinical utility and even if it has been recommended (e.g., by the FDA) that the blood could be analyzed first to reduce the number of invasive biopsies in cancer patients, the lower sensitivity of ctDNA analyses prevents its use in clinical management for the moment, and the

primary tumor analysis still remains the gold standard in NSCLC diagnostics of EGFR mutations.

5.2 MicroRNAs

MicroRNAs (miRNAs, miR-x), consisting in approximately 22 nucleotides, represent another potential blood biomarker in oncology. These noncoding small RNAs are master regulators of genic expression and consequently of many cellular processes. Alterations in the expression of microRNA genes have been shown to play in important role in human malignancies. These alterations can be caused by a variety of mechanisms, including deletions, amplifications, or mutations involving microRNA loci, by epigenetic silencing or by dysregulation of transcription factors targeting specific microRNAs [92]. The three major detection techniques for circulating cell-free miRNA (cfmiRNA) analysis, following RNA extraction, comprise quantitative RT-PCR, microarray analyses, and deep sequencing. The assessment of cfmiRNA has been suggested for early diagnosis, prognosis, therapy monitoring, and therapeutic response prediction in different cancer types (e.g., lung, breast, colon, prostate, and ovary cancers and melanoma), as reviewed by Armand-Labit and Pradines [93].

5.3 Exosomes

Tumor and normal cells are known to release microvesicles such as exosomes (40–150 nm) into the circulation, discharging cellular content. Currently, one challenge for the analyses of circulating cell-free nucleic acids in blood is their instability. Thus, due to their protective environment, the exosomes represent a valuable source for analysis of proteins, DNA, RNA, miRNA, lipids, and metabolites [94]. Ultracentrifugation, density-based separation, or immune-affinity capture using magnetic beads coated with anti-EpCAM antibodies can be used to isolate exosomes [95]. They are important regulators of the cellular niche, and their altered characteristics in many diseases, such as cancer, suggest their importance for diagnostic and therapeutic applications and as drug delivery vehicles. Hoshino et al. demonstrated that the composition of exosomal integrins could predict organ-specific metastasis and that tumor-derived exosomes participate in preparing the pre-metastatic niche [96]. Correspondingly, the same group shows that a pro-metastatic phenotype of bone marrow progenitor cells is promoted by education through melanoma exosomes [97].

5.4 Tumor-educated platelets

A new emerging class of components for *liquid biopsy* is tumor-educated platelets (TEPs). These anucleated blood cells (second most abundant cell type in circulation) could be educated by tumor cells by the transfer of tumor-associated biomolecules, mostly RNA. Platelets are isolated by centrifugation and RNA can be subjected to RT-PCR [45]. Performing mRNA sequencing on TEPs, Best and his colleagues showed that cancer patients with different tumor types could be discriminated from healthy individuals with 96% accuracy and that the primary tumor was correctly located with a precision of 71% [98]. Studies have shown that platelet count and platelet size can already provide clinically relevant information about the presence of cancer [99]. High platelet count is associated with increased mortality in a variety of cancers.

Furthermore, biomarkers (MET or HER2 expression/KRAS, EGFR, and PIK3CA mutations) were identified in surrogate TEP mRNA profiles, which might be tested

in future studies as potential predictors for targeted therapies. Recently, Diem et al. showed that elevated pretreatment platelet-to-lymphocyte ratios correlate with a reduced response rate to nivolumab anti-PD-L1 immunotherapy in NSCLC [100], indicating that circulating platelets may enhance a pro-tumorigenic effect in the presence of an antitumor immune response.

6. Conclusion

CTC as *liquid biopsy* represents a promising approach for personalized treatment in oncology. Lots of efforts have been made to overcome technical challenges for enrichment, detection, and characterization of these tumor cells. Nevertheless, low number (or even absence) of CTCs can weaken the reliability of CTC-based assays in some patients with current detection techniques. This points the need for further technological advances and procedure standardization. To introduce CTC tests into clinical trials, an intense validation of the technical aspects of the applied assays is currently executed in Europe by the EU-funded CANCER-ID network (www.cancer-id.eu) that will be continued by the European *Liquid Biopsy* Society (ELBS).

Additionally, an extensive work has been made to understand biological processes of cancer dissemination and metastasis, underlying different aspects for CTCs survival in bloodstream. This knowledge could improve pharmaceutical drug researches and therapeutic strategies for better clinical management of cancer patients.

Beside CTC analysis several other circulating biomarkers are under investigations and demonstrate real valuable data. It is now well accepted that there is not a perfect unique biomarker and that combining different circulating biomarkers can bring a huge benefit for precision medicine for cancer patients.

In conclusion, *liquid biopsy* diagnostics might help to focus the current cancer screening modalities, which would reduce side and healthcare costs. However, despite promising first results and the enormous interest by diagnostic companies and the public press, disease monitoring and early detection of cancer face serious challenges of both sensitivity and specificity.

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