Clinical Chemistry 66:1
89–96 (2020)

Reviews

A New Era in Liquid Biopsy: From Genotype to Phenotype

Shana O. Kelley[1*] and Klaus Pantel[2]

BACKGROUND: Liquid biopsy, in which tumor cells and tumor-derived biomolecules are collected from the circulation, is an attractive strategy for the management of cancer that allows the serial monitoring of patients during treatment. The analysis of circulating DNA produced by tumors provides a means to collect genotypic information about the molecular profile of a patient's cancer. Phenotypic information, which may be highly relevant for therapeutic selection, is ideally derived from intact cells, necessitating the analysis of circulating tumor cells (CTCs).

CONTENT: Recent advances in profiling CTCs at the single-cell level are providing new ways to collect critical phenotypic information. Analysis of secreted proteins, surface proteins, and intracellular RNAs for CTCs at the single-cell level is now possible and provides a means to quantify molecular markers that are involved with the mechanism of action of the newest therapeutics. We review the latest technological advances in this area along with related breakthroughs in high-purity CTC capture and in vivo profiling approaches, and we also present a perspective on how genotypic and phenotypic information collected via liquid biopsies is being used in the clinic.

SUMMARY: Over the past 5 years, the use of liquid biopsy has been adopted in clinical medicine, representing a major paradigm shift in how molecular testing is used in cancer management. The first tests to be used are genotypic measurements of tumor mutations that affect therapeutic effectiveness. Phenotypic information is also clinically relevant and essential for monitoring proteins and RNA sequences that are involved in therapeutic response.

The concept of "liquid biopsy" has emerged as a general approach that seeks to integrate information from blood-borne circulating nucleic acids, proteins, cancer cells, and extracellular vesicles to provide a comprehensive picture of tumor progression *(1)*. Liquid biopsy can complement invasive tissue biopsy in many aspects of cancer diagnosis and management (Fig. 1). In the clinic, the analysis of circulating tumor DNA can be used to detect tumor-associated mutations *(2)*. This level of genotypic information is valuable and provides information on potential therapeutic response and tumor progression. Equally important, but not yet broadly used in the clinic, is the phenotypic information that requires the analysis of intact circulating tumor cells (CTCs)[3] *(3)*.

CTCs are recognized as the likely source of deadly metastatic disease in patients *(4)*. CTCs may circulate and seed active metastatic tumors while a primary tumor is present, or disseminated tumor cells (DTCs) may escape a primary tumor and remain dormant for many years *(5–8)*. Intact CTCs are a critical target for liquid biopsy, because they provide information about the molecular makeup of cells within a tumor and carry phenotypic information that is critical for treatment selection. Indeed, many of the most powerful therapeutics to enter the clinic in the past several years are targeted toward aberrant expression of protein or RNA markers that are not reflected in the genotype of the cell. Thus, developing CTC analysis approaches that elucidate the phenotypic properties of a tumor is a key capability for the implementation of comprehensive liquid biopsy tests.

CTCs enter the bloodstream either as single cells or clusters *(9–10)* and must withstand the mechanical forces associated with circulation and evade the immune system to survive and persist long enough to reach a site that represents a suitable environment for the formation of a metastatic lesion. The downregulation of epithelial markers and transition to a more mesenchymal phenotype during the epithelial-to-mesenchymal transition (EMT) may engender sufficient levels of resilience for these cells to reach a target site, although regaining an epithelial phenotype is likely required for the growth of a

[1] Department of Pharmaceutical Sciences, Leslie Dan Faculty of Pharmacy, University of Toronto, Toronto, Canada; [2] Institute of Tumor Biology, University Medical Center Hamburg-Eppendorf, Hamburg, Germany.
* Address correspondence to this author at: Department of Pharmaceutical Sciences, Leslie Dan Faculty of Pharmacy, University of Toronto, Toronto, M5S 3M2, Canada. Fax 416-978-2997; e-mail shana.kelley@utoronto.ca.
Received July 8, 2019; accepted August 22, 2019.
Previously published online at DOI: 10.1373/clinchem.2019.303339

[3] Nonstandard abbreviations: CTC, circulating tumor cell; DTC, disseminated tumor cells; EMT, epithelial-to-mesenchymal transition; EpCAM, epithelial cell adhesion molecule; HER2, human epidermal growth factor receptor 2; EGFR, epidermal growth factor receptor; AR-V7, androgen receptor variant 7; PD-L1, programmed cell death ligand 1; WBC, white blood cell; cfDNA, cell-free DNA; ALK, anaplastic lymphoma kinase; WGA, whole-gene amplification; ER, estrogen receptor; MRD, minimal residual disease; CRPC, castration-resistant prostate cancer; TMB, tumor mutational burden.

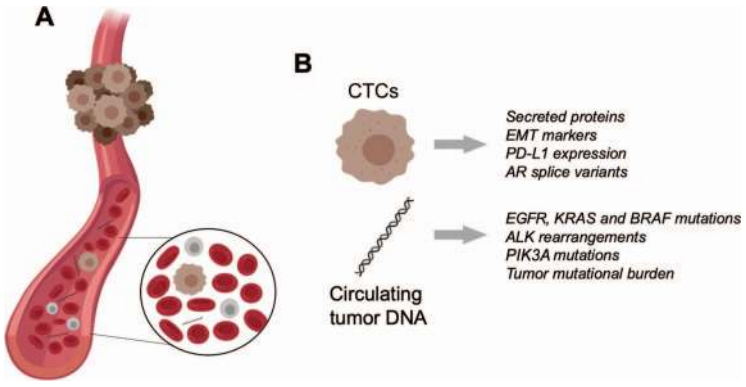

Fig. 1. (A), Liquid biopsy tests, in which tumor cells and biomolecular material is collected noninvasively from the blood, will benefit from the analysis of phenotypic and genotypic information.

(B), The analysis of CTCs facilitates the collection of phenotypic information including EMT status, protein expression levels, and the presence of RNA-level expression alterations. Circulating tumor DNA can be interrogated to profile individual mutations that may affect therapeutic effectiveness or can reveal overall TMB.

metastatic tumor *(11–12)*. A recent study highlighted the importance of retaining epithelial character for the formation of metastatic lesions *(13)*. Thus, in addition to the clinical relevance of phenotypic information collected from CTCs via liquid biopsy, understanding the dynamic properties of these cells is also important to further our understanding of the biology underlying metastasis.

Although technologies that enable the sensitive enumeration of CTCs emerged over a decade ago *(3)*, the elucidation of phenotypic properties of CTCs has remained a challenge. CTCs are exceedingly rare in blood samples and are outnumbered by normal blood cells a billion-fold. A clinical blood sample may contain only 5–10 CTCs and these cells may be heterogeneous, requiring that analysis is performed at the single-cell level *(14)*. New approaches for clinically relevant phenotypic profiling of CTCs must therefore possess a very high level of sensitivity (e.g., a low false-negative rate), specificity (e.g., a low false-positive rate), and the ability to readout protein and RNA levels. The use of this information to understand and correlate heterogeneity among a patient's CTCs, as compared to a primary tumor *(15–17)*, relies on the ability to collect information at the single cell level.

In this review, we discuss recent progress in the use of liquid biopsy testing in the clinic, with an emphasis on testing applications that are becoming increasingly common in mainstream clinical medicine. We discuss the existing and potential applications of genotypic and phenotypic information that can be collected from noninvasive measurements and review new technologies that may provide solutions for the development of clinically actionable tests (Fig. 2).

New Technologies for CTC Phenotype Analysis

When CTCs were first identified as important markers for cancer treatment and management, their enumeration in clinical specimens was pursued as a metric that could be used to evaluate the stage and progression of a tumor. Many of the initial methods applied to CTC enumeration relied on their isolation from blood by use of immunomagnetic labeling of epithelial cell surface markers [e.g., epithelial cell adhesion molecule (EpCAM)]. These markers are uniquely expressed on cancer cells vs normal blood cells and this is therefore a strategy that enables specific capture of CTCs in the presence of a large abundance of blood cells. The FDA (US Food and Drug Administration)-cleared CellSearch platform uses this type of capture combined with immunofluorescence to identify CTCs in clinical specimens and has been employed in many studies to assess disease progression and response to therapy *(18–19)*. This type of testing, however, has not been adopted in the clinic outside of clinical studies because of a lack of clear, actionable results and improvements in patient outcomes *(20)*.

The analytical sensitivity of CellSearch has also been questioned as the platform has undergone extensive testing in research laboratories and the clinic. The standard assay used for CellSearch testing relies on capture via an antibody to EpCAM, an epithelial protein. Because this protein appears to be downregulated as CTCs lose their epithelial phenotype while in the bloodstream, the reliance on this marker may negatively impact capture efficiency. However, the count of $EpCAM^+$ CTCs is closely related to tumor recurrence *(18)*. EpCAM biology is still

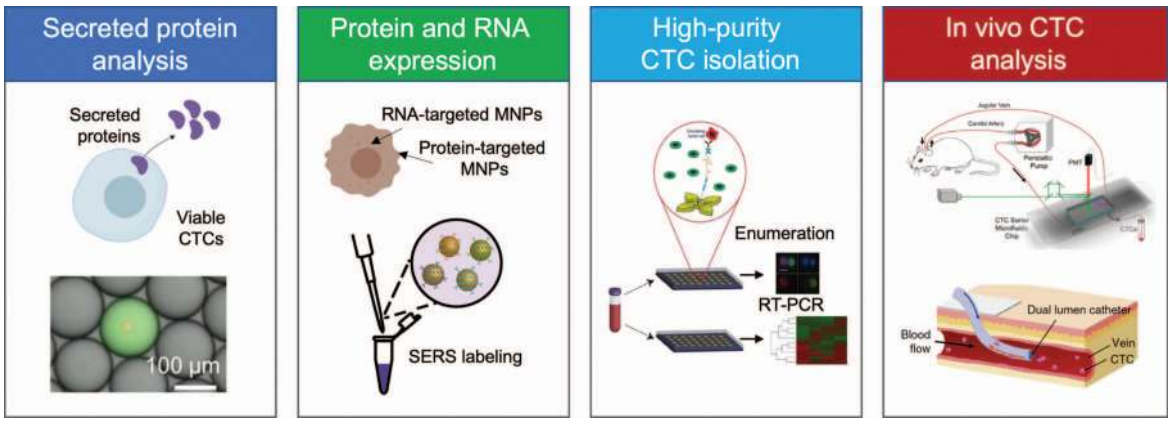

Fig. 2. New technologies for CTC analysis permit the analysis of CTCs at the single-cell level, can facilitate the capture of CTCs with sufficient purity for single-cell level gene expression profiling, and permit in vivo observation of CTC concentrations.

Analysis of a variety of secreted proteins using the Epispot and Vortex platforms has produced important correlations of these factors with patient outcomes [top adapted from Alix-Panabieres and Pantel *(30)*; bottom reproduced from see Dhar et al. *(31)*]. Labeling of surface proteins and intracellular RNAs with magnetic nanoparticles and surface-enhanced Raman spectroscopy (SERS)-active labels has facilitated the visualization of EMT and drug resistance in CTCs [top adapted from Poudineh et al. *(33)*, Labib et al. *(34)* bottom reproduced from Tsao et al. *(59)*]. Significant progress in the development of CTC isolation platforms has permitted the application of single-cell methods expression profiling methods [reproduced from Kominsky et al. *(41)*], and recent breakthroughs related to the development of in vivo systems have been made allowing CTCs to be tracked in real time [reproduced from Hamza et al. *(47)* and Kim et al. *(44)*].

underinvestigated and it appears to have important additional functions beyond homotypic cell adhesion *(21)*.

Many next-generation technologies have sought to improve on the performance of the CellSearch approach and offer high-sensitivity enumeration of CTCs. A variety of microfluidic immunocapture technologies have offered higher levels of performance with tailored capture systems that promote binding of cells to microscale structures and particles *(22–29)*. Other systems have emerged that do not rely on immunocapture and instead harness the size, electrical, or mechanical properties of CTCs to facilitate their capture from clinical blood specimens *(3)*. These approaches produce concentrated CTCs that possess lower purity than immunocapture-based technologies but are advantageous because they are not subject to fluctuations in target markers. However, irrespective of the capture approach, most systems developed to date are still enumeration systems that reveal how many CTCs are present in a blood sample or concentrate these cells for offline analysis. To collect clinically meaningful information from CTCs, technologies that provide direct functional and phenotypic information are needed.

Several recent breakthroughs bring a higher level of information content to CTC analysis. Systems are now available that characterize CTCs at the single cell level and provide information about protein concentrations on the cell surface, secreted proteins, and intracellular RNAs. Single cell analysis is also emerging as a powerful tool that can help elucidate new CTC markers and underlying biology that may contribute to metastasis.

ANALYSIS OF PROTEINS SECRETED FROM CTCs

One of the first breakthroughs in functional profiling of CTCs was the development of the EPISPOT platform that facilitates the detection of viable cells and profiles specific proteins that are secreted or shed from these cells *(30)*. After label-free concentration of CTCs, cells are introduced into a well-based format and antibody-coated membranes are used to collected proteins from the incubation medium. This approach has been used to profile a variety of different tumor markers including CK19 (cytokeratin19), human epidermal growth factor receptor 2 (HER2), MUC1 (Mucin 1), FGF2 (fibroblast growth factor 2), and epidermal growth factor receptor (EGFR). Data collected using this approach have been correlated with patient outcomes in many different cell types. This type of platform is generalizable and can be tailored to answer different questions concerning the presence of different protein factors expressed or secreted by CTCs.

A recently reported approach to specifically measuring matrix metalloprotease activity represents another advance in collecting functional information on proteins secreted from CTCs *(31)*. Here, a bulk concentration step is coupled with the compartmentalization of individual CTCs into droplets in which a reaction with a fluorescent substrate is monitored. Given the proposed

link between matrix metalloprotease activity and the invasiveness of cancer cells, this type of measurement provides a powerful means to assess this critical functional property of CTCs. A recent study published on the application of this method to prostate cancer CTCs established the feasibility of the approach when used with patient samples *(31)*.

ANALYSIS OF INTRACELLULAR AND EXTRACELLULAR PROTEINS EXPRESSED BY CTCs

Profiling protein concentrations displayed on the surface and expressed in the cytoplasm or organelles of CTCs is important to benchmark phenotypic properties of the tumor of origin and to collect information about potential metastatic lesions. Recent advances in microfluidic western blotting methods now permit the analysis of single CTCs using the same antibody-based workflow familiar to the research community *(32)*. CTCs are isolated as a bulk fraction using a label-free approach, placed in microwells, and lysed and then individual proteins are visualized after rounds of probing and stripping. This technique was successfully applied to CTCs isolated from breast cancer patients and enabled the visualization of heterogeneity in the overall population of cells collected.

A different approach to quantifying surface proteins was developed that uses antibody-functionalized magnetic nanoparticles as labeling agents and a microfluidic device to profile concentrations of individual proteins on the surface of CTCs *(24, 33)*. This approach—referred to as magnetic ranking cytometry—facilitates expression analysis at the single cell level, which can be used to visualize EMT during tumor progression in animal models and patients. This technology was also adapted to target intracellular mRNAs and enabled direct detection of the androgen receptor variant 7 (AR-V7) splice variant in individual CTCs *(34)*. Although the initial assays developed on this platform were destructive in nature because immunofluorescence was used to characterize the cells captured in the microfluidic device, a next-generation prismatic–deflection approach allows the recovery of viable cells *(35)*. Cells are deflected along magnetic guides and collected as fractions according to magnetic labeling and can be unequivocally identified as cancer cells using graphene Hall detectors integrated within the microfluidics. This device enabled the isolation and analysis not only of individual viable CTCs but of CTC clusters as well.

Profiling protein markers expressed on the surface of CTCs will likely become an increasingly important area, given the use of immunotherapy and other targeted therapies in advanced cancers. Markers like programed cell death ligand 1 (PD-L1) are typically profiled in tissue biopsies to assess potential for response in lung cancer patients, given the role of this protein in suppressing the T cell response. Although several studies have tested PD-L1 positivity in CTCs *(36)*, it is not yet clear whether CTC PD-L1 concentrations are related to expression in primary or metastatic tumors, but this is now a relationship that can be explored given the availability of quantitative technologies for protein measurement at the single cell level.

AR-V7 is another marker that is important to assess for therapeutic selection. A commercially available test that analyzes the AR-V7 protein in CTCs is now being used in the clinic in North America *(37–38)*. It is not yet established whether quantitative analysis of this marker has clinical utility, but this test is another example of a liquid biopsy test for which phenotypic information collected from CTCs is critical.

TOOLS FOR CTC BIOLOGY AND NEW CLINICAL APPLICATIONS

In addition to the new phenotypic profiling systems mentioned above, other platforms are emerging that will play a vital role in uncovering new CTC phenotypes that may be important for liquid biopsy-based testing. Systems that enable single cell RNA expression profiling and RNA sequencing are powerful for the elucidation of new pathways relevant to the metastatic potential of CTCs. Moreover, devices that enable in vivo capture and analysis of CTCs provide a means to understand the persistence and dynamic behavior of these cells in the bloodstream.

The greatest challenge inherent in isolating CTCs for RNA expression analysis and RNA sequencing is the isolation of high-purity samples. Especially when PCR-based analysis is used, contaminating white blood cells (WBCs) are problematic. Platforms that employ high-performance WBC depletion steps or high-specificity CTC capture are best suited to this application because of the need for high purity samples *(39)*. New sample preparation platforms are also coming online *(40)*, which are critical for single cell handling and the isolation and amplification of exceedingly small quantities of nucleic acids. Enabled by advanced CTC capture and isolation systems, several powerful studies of RNA expression have produced useful insights into CTC biology, including the observation of undifferentiated survival signatures, EMT and noncanonical Wnt signaling in prostate CTCs *(41–42)*, and markers for apoptotic resistance and EMT in breast cancer CTCs *(43)*. Recently, DNA methylation patterns related to CTC clustering were uncovered in studies sequencing single CTCs that connected changes in genes for aggregated cells related to stemness and metastatic potential *(9)*.

Devices that enable analysis of CTCs in situ within living animals, or even patients, are advancing rapidly, and they will enable better access to larger samples of CTCs and permit the analysis of phenotypic dynamics in real time. It is now possible to collect cells directly from the bloodstream using the CellCollector, a device that

can be inserted into a patient through a 20-gauge needle. This device appears to harvest significantly higher numbers of CTCs relative to yields from a typical blood draw. Next-generation systems have also been reported based on ex vivo collection devices *(44)* and flexible magnetic wires that increase CTC recovery efficiency further *(45)*. It is noteworthy, however, that all of these devices use immunoaffinity reagents (i.e., EpCAM) to capture CTCs, and therefore, further work will be needed to broaden the types of CTCs that can be analyzed.

A recent report of a label-free method for identification and eradication of melanoma CTCs using a photoacoustic approach provides a new tool for in vivo monitoring *(46)*. The sensitivity achieved with this method is a major advance, with a single CTC being detectable in a liter of circulating blood. Clusters could also be tracked with this tool, and acoustic waves were also used to ablate CTCs.

A recently reported optofluidic real-time cell sorter represents another important leap forward for in vivo CTC analysis *(47)*. This device was designed to analyze CTCs in genetically engineered mouse models of cancer, in which an optical reporter was engineered into cells with carcinogenic alterations to facilitate specific detection of circulating cells originating from the primary tumor. Cells were collected from the bloodstream at a series of time points and used for single cell RNA sequencing; the results highlighted the heterogeneity in the mice produced from a single model that likely mirrors patient heterogeneity in the clinic.

Liquid Biopsy in the Clinic: Tumor Genotype to Phenotype

CTC analysis allows the determination of therapeutic targets and mechanisms of resistance to cancer therapies at the DNA, RNA, and protein level as well as the performance of drug screening on functional models including cell cultures and xenografts [see review of C. Alix-Panabieres et al. in this issue *(48)*.]

TUMOR GENOTYPE

Resistance-conferring mutations have been largely identified in many types of solid tumors. Mutations in the epidermal growth factor receptor (*EGFR*),[4] KRAS proto-oncogene, GTPase (*KRAS*), and B-Raf proto-oncogene, serine/threonine kinase (*BRAF*) genes have been the prime targets of cell-free DNA (cfDNA) analyses and these analyses have contributed to a better understanding of tumor evolution driven by cancer therapies *(49)*. Genomic rearrangements [e.g., anaplastic lymphoma kinase (ALK) rearrangements in lung cancer] also play a role as therapeutic targets and have been studied using DNA extracted from CTCs *(50)*.

Looking at the genome of single CTCs isolated from the blood of an individual patient is now feasible using FISH (fluorescence in situ hybridization) or whole-genome amplification (WGA) followed by NGS (next-generation sequencing), which has unraveled the frightening degree of intrapatient heterogeneity in patients with solid tumors, e.g., in mutations of the (*PIK3CA*) gene known to confer resistance to estrogen receptor (ER) or HER2 targeting therapies in breast cancer *(21, 51, 52)* or mutations in the *KRAS* gene conferring resistance to EGFR-targeting therapies in colorectal cancer *(19, 53–55)*.

Comparing primary colorectal cancers with CTCs from the same patients has revealed that subclonal mutations present in the primary lesion are overrepresented in the CTCs *(56)*, supporting the view that CTCs are derived from a subset of tumor cells with a particular genotype and not from the bulk of the tumor. Thus, the sole analysis of randomly selected sections of the primary tumor, which is the usual practice in clinical oncology for assessment of therapeutic targets or resistance mechanisms, might lead to false findings in regard to the prevention of metastatic lesions in cancer patients. Rebiopsies of metastatic lesions are possible, but some locations are difficult to access, which has opened a new diagnostic avenue for blood analyses of CTCs and other liquid biopsy analytes like cfDNA *(2)*.

Moreover, sorting of single CTCs also allows the correlation between genotype and phenotype of a single tumor cell. This may enable researchers in the future to assess the genotype of particular subsets of CTCs. For example, CTCs undergoing EMT are thought to exert a higher plasticity to form metastases and it might be therefore of great interest to analyze druggable mutations in these "metastasis-initiator cells." Moreover, it is well-known that ER-positive CTCs and ER-negative CTCs coexist in breast cancer patients with ER-positive primary tumors *(57)*, and recent reports have shown that these CTC subsets have differential genomic aberrations that may pinpoint mechanisms of resistance to endocrine therapies *(58)*.

Acquired resistance to targeted therapy is often seen as originating from selective expansion of preexisting subclonal populations. However, minimal residual disease (MRD) could harbor a small population of quiescent drug-tolerant cells that have survived owing to adaptive activation of alternative molecular pathways *(59)*. Molecular characterization of CTCs during the postsurgical surveillance period could help to identify these pathways of resistance *(60)*.

[4] Human Genes: *EGFR*, epidermal growth factor receptor; *KRAS*, KRAS proto-oncogene, GTPase; *BRAF*, B-Raf proto-oncogene, serine/threonine kinase; *PIK3CA*, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha; *AR*, androgen receptor; *TP53*, tumor protein P53; *ETS* family, ETS proto-oncogene family; *RB1*, RB transcriptional corepressor 1; *PTEN*, phosphatase and tensin homolog.

TUMOR PHENOTYPE

The central role of phenotypic switching in drug resistance is gaining acceptance across many different types of tumors *(59, 61)*. Here, cfDNA analyses cannot provide information, although CTC analysis at the RNA or protein level could help to explore these molecular mechanisms. In breast cancer, CTCs from patients with originally ER+/HER2− breast cancer can interconvert to a HER2+ phenotype under cytotoxic treatment without acquisition of additional genetic aberrations *(61)*. In melanoma, phenotypic switching with acquisition of a less-differentiated phenotype has been reported in CTCs at the time of relapse from targeted therapy *(62)*.

Intrapatient heterogeneity is also mirrored at the gene-expression level of CTCs *(63–65)*, and it appears to be particularly relevant to response to antiandrogen therapy in castration-resistant prostate cancer (CRPC) *(38)* and possibly also other tumor entities. CRPC is the clinical entity for which expressional changes in CTCs have been most widely explored for assessment of treatment resistance to antiandrogen therapies *(20)*. Multiple mechanisms have been reported to contribute to resistance against antiandrogen therapy such as diverse genetic aberrations in androgen receptor (*AR*), tumor protein P53 (*TP53*), ETS proto-oncogene family (*ETS* family), RB transcriptional corepressor 1 (*RB1*), and phosphatase and tensin homolog (*PTEN*), increased expression of androgen receptor, mutations in the ligand binding domain of AR, or production of AR-V7. The detection of AR-V7 in CTCs is currently the most prominent predictive biomarker guiding the treatment choice between AR signaling inhibitors and taxane-based chemotherapy in CRPC *(37, 66)*.

Immune checkpoint inhibitors have opened a new era in immunotherapy, with exceptional long-term remissions in some patients across diverse tumor entities. However, only a fraction of patients respond to this form of therapy and many patients experience severe side effects such as strong autoimmune reactions of the liver and other organs *(67)*. Therefore, biomarkers predicting response and/or severe side effects are urgently needed. Here, CTC analysis would present a real advantage because it allows monitoring of the actual tumor evolution with regard to the expression of target molecules (e.g., PD-L1) of immune checkpoint inhibitor therapies. Pilot studies have suggested that PD-L1 is more frequent on CTCs in metastatic breast cancer compared to the primary lesion *(68)* and it appears to be correlated to EMT in non–small cell lung cancer *(69)*, unfavorable outcome in head and neck cancer *(70)*, and response to anti-PD1 therapy in a phase I trial *(71)*. This information might then be combined with the assessment of tumor mutational burden (TMB) on cfDNA *(72)*. However, despite promising initial results, technical and clinical validation of PD-L1 CTC and TMB assessment *(73)* is still necessary before implementation into clinical practice.

Conclusions

The opportunities of CTC analyses to bring the vision of personalized medicine to reality within the next decade are obvious. Tumor evolution leads to substantial changes in the molecular composition of tumor cells in cancer patients owing to natural or therapy-induced selection of the fittest clones leading to the initiation of metastasis. Thus, the mere analysis of tissue sections of the primary lesion—common practice on diagnostic oncology—is not sufficient. However, rebiopsy of metastatic lesions cannot be achieved in many patients because of the location of the lesion (e.g., in lungs or brain). Moreover, different metastatic lesions in an individual patient have different genomic aberrations *(74–75)* including druggable mutations. Finally, to encompass the dynamic evolution of tumors during therapy, multiple sequential tissue biopsies over time would be required, which is only feasible in a minority of patients, even in academic centers. These limitations have supported the vision that blood-based analysis of tumor cells or tumor cell products such as cfDNA may complement current diagnostics in oncology *(2, 76)*.

Despite the promising results on CTC analyses discussed in his review, the key limitation is the small number of CTCs detectable in a blood sample of approximately 10 mL in most cancer patients (exceptions are patients with small-cell lung cancer or very advanced other tumor types such as prostate or breast cancer). Approaches to overcome this limitation are currently being investigated and include the analysis of much larger blood volumes [e.g., by diagnostic leukapheresis *(76–79)*], the development of in vivo CTC capture devices *(44, 47, 63)*, or noninvasive CTC monitoring by photoacoustic signals through the intact skin *(59)*. The future of CTCs as companion diagnostics to steer cancer therapies will largely depend on the progress of these efforts.

Moreover, it will be important to standardize new methods within international consortia like the European Union/IMI (Innovative Medicines Initiative) consortium CANCER-ID or the ELBS (European Liquid Biopsy Society) in Europe and the US-based BloodPac. To foster the introduction of CTCs into clinical practice, clinical utility of the CTC biomarker has to be demonstrated in a specific context of use (e.g., selection of a specific targeted therapy). Thus, the biomarker development needs to be closely linked to drug development and it requires substantial investments frequently underestimated by industry. This might be one of the reasons for the striking lack of new biomarkers accepted into the clinic despite the plethora of publications on biomarkers (e.g., more than 20000 publications on CTCs in PubMed) in cancer (and other diseases).

Author Contributions: *All authors confirmed they have contributed to the intellectual content of this paper and have met the following 4 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; (c) final approval of the published article; and (d) agreement to be accountable for all aspects of the article thus ensuring that questions related to the accuracy or integrity of any part of the article are appropriately investigated and resolved.*

Authors' Disclosures or Potential Conflicts of Interest: *Upon manuscript submission, all authors completed the author disclosure form. Disclosures and/or potential conflicts of interest:*

Employment or Leadership: K. Pantel, guest editor, *Clinical Chemistry*, AACC.
Consultant or Advisory Role: None declared.
Stock Ownership: None declared.
Honoraria: K. Pantel, Agena, Novartis, Roche, Sanofi.
Research Funding: European Federation of Pharmaceutical Industries and Associations (EFPIA) partners (Angle, Menarini and Servier) of the CANCER-ID programme of the European Union–EFPIA Innovative Medicines Initiative.
Expert Testimony: None declared.
Patents: S.O. Kelley, provisional patent; K. Pantel, EPO patent application No. 2016128125 A1, EPO patent application No.17157020.3–1405.

References

1. Pantel K, Alix-Panabieres C. Circulating tumour cells in cancer patients: challenges and perspectives. Trends Mol Med 2010;16:398–406.
2. Bardelli A, Pantel K. Liquid biopsies, what we do not know (yet). Cancer Cell 2017;31:172–9.
3. Poudineh M, Sargent EH, Pantel K, Kelley SO. Profiling circulating tumour cells and other biomarkers of invasive cancers. Nat Biomed Eng 2018;2:72–84.
4. Pantel K, Brakenhoff RH. Dissecting the metastatic cascade. Nat Rev Cancer 2004;4:448–56.
5. Braun S, Vogl FD, Naume B, Janni W, Osborne MP, Coombes RC, et al. A pooled analysis of bone marrow micrometastasis in breast cancer. N Engl J Med 2005; 353:793–802.
6. Endo H, Inoue M. Dormancy in cancer. Cancer Sci 2019; 110:474–80.
7. Recasens A, Munoz L. Targeting cancer cell dormancy. Trends Pharmacol Sci 2019;40:128–41.
8. Vallette FM, Olivier C, Lezot F, Oliver L, Cochonneau D, Lalier L, et al. Dormant, quiescent, tolerant and persister cells: four synonyms for the same target in cancer. Biochem Pharmacol 2019;162:169–76.
9. Gkountela S, Castro-Giner F, Szczerba BM, Vetter M, Landin J, Scherrer R, et al. Circulating tumor cell clustering shapes DNA methylation to enable metastasis seeding. Cell 2019;176:98–112.e14.
10. Szczerba BM, Castro-Giner F, Vetter M, Krol I, Gkountela S, Landin J, et al. Neutrophils escort circulating tumour cells to enable cell cycle progression. Nature 2019; 566:553–7.
11. Jie XX, Zhang XY, Xu CJ. Epithelial-to-mesenchymal transition, circulating tumor cells and cancer metastasis: mechanisms and clinical applications. Oncotarget 2017;8:81558–71.
12. Chaffer CL, Weinberg RA. A perspective on cancer cell metastasis. Science 2011;331:1559–64.
13. Liu X, Li J, Cadilha BL, Markota A, Voigt C, Huang Z, et al. Epithelial-type systemic breast carcinoma cells with a restricted mesenchymal transition are a major source of metastasis. Sci Adv 2019;5:eaav4275.
14. Alix-Panabieres C, Pantel K. Characterization of single circulating tumor cells. FEBS Lett 2017;591:2241–50.
15. Agnoletto C, Corra F, Minotti L, Baldassari F, Crudele F, Cook WJJ, et al. Heterogeneity in circulating tumor cells: the relevance of the stem-cell subset. Cancers 2019;11:e483.
16. Brown HK, Tellez-Gabriel M, Cartron PF, Vallette FM, Heymann MF, Heymann D. Characterization of circulating tumor cells as a reflection of the tumor heterogeneity: myth or reality? Drug Discov Today 2019;24:763–72.
17. Tellez-Gabriel M, Heymann MF, Heymann D. Circulating tumor cells as a tool for assessing tumor heterogeneity. Theranostics 2019;9:4580–94.
18. Bidard FC, Michiels S, Riethdorf S, Mueller V, Esserman LJ, Lucci A, et al. Circulating tumor cells in breast cancer patients treated by neoadjuvant chemotherapy: a meta-analysis. J Natl Cancer Inst 2018;110:560–7.
19. El-Heliebi A, Hille C, Laxman N, Svedlund J, Haudum C, Ercan E, et al. In situ detection and quantification of AR-V7, AR-FL, PSA, and KRAS point mutations in circulating tumor cells. Clin Chem 2018;64:536–46.
20. Pantel K, Hille C, Scher HI. Circulating tumor cells in prostate cancer: from discovery to clinical utility. Clin Chem 2019;65:87–99.
21. Keller L, Werner S, Pantel K. Biology and clinical relevance of EpCAM. Cell Stress 2019;3:165–80.
22. Adams AA, Okagbare PI, Feng J, Hupert ML, Patterson D, Gottert J, et al. Highly efficient circulating tumor cell isolation from whole blood and label-free enumeration using polymer-based microfluidics with an integrated conductivity sensor. J Am Chem Soc 2008; 130:8633–41.
23. Karabacak NM, Spuhler PS, Fachin F, Lim EJ, Pai V, Ozkumur E, et al. Microfluidic, marker-free isolation of circulating tumor cells from blood samples. Nat Protoc 2014;9:694–710.
24. Mohamadi RM, Besant JD, Mepham A, Green B, Mahmoudian L, Gibbs T, et al. Nanoparticle-mediated binning and profiling of heterogeneous circulating tumor cell subpopulations. Angew Chem Int Ed Engl 2015; 54:139–43.
25. Nagrath S, Sequist LV, Maheswaran S, Bell DW, Irimia D, Ulkus L, et al. Isolation of rare circulating tumour cells in cancer patients by microchip technology. Nature 2007;450:1235–9.
26. Ozkumur E, Shah AM, Ciciliano JC, Emmink BL, Miyamoto DT, Brachtel E, et al. Inertial focusing for tumor antigen-dependent and -independent sorting of rare circulating tumor cells. Sci Transl Med 2013;5: 179ra47.
27. Park MH, Reategui E, Li W, Tessier SN, Wong KH, Jensen AE, et al. Enhanced isolation and release of circulating tumor cells using nanoparticle binding and ligand exchange in a microfluidic chip. J Am Chem Soc 2017; 139:2741–9.
28. Stott SL, Hsu CH, Tsukrov DI, Yu M, Miyamoto DT, Waltman BA, et al. Isolation of circulating tumor cells using a microvortex-generating herringbone-chip. Proc Natl Acad Sci U S A 2010;107:18392–7.
29. Yoon HJ, Kim TH, Zhang Z, Azizi E, Pham TM, Paoletti C, et al. Sensitive capture of circulating tumour cells by functionalized graphene oxide nanosheets. Nat Nanotechnol 2013;8:735–41.
30. Alix-Panabieres C, Pantel K. Liquid biopsy in cancer patients: advances in capturing viable CTCs for functional studies using the EPISPOT assay. Expert Rev Mol Diagn 2015;15:1411–7.
31. Dhar M, Lam JN, Walser T, Dubinett SM, Rettig MB, Di Carlo D. Functional profiling of circulating tumor cells with an integrated vortex capture and single-cell protease activity assay. Proc Natl Acad Sci U S A 2018;115: 9986–91.
32. Sinkala E, Sollier-Christen E, Renier C, Rosas-Canyelles E, Che J, Heirich K, et al. Profiling protein expression in circulating tumour cells using microfluidic western blotting. Nat Commun 2017;8:14622.
33. Poudineh M, Aldridge PM, Ahmed S, Green BJ, Kermanshah L, Nguyen V, et al. Tracking the dynamics of circulating tumour cell phenotypes using nanoparticle-mediated magnetic ranking. Nat Nanotechnol 2017; 12:274–81.
34. Labib M, Mohamadi RM, Poudineh M, Ahmed SU, Ivanov I, Huang CL, et al. Single-cell mRNA cytometry via sequence-specific nanoparticle clustering and trapping. Nat Chem 2018;10:489–95.
35. Aldridge PM, Mukhopadhyay M, Ahmed SU, Zhou W, Christinck E, Makonnen R, et al. Prismatic deflection of live tumor cells and cell clusters. ACS Nano 2018;12: 12692–700.
36. Hofman P, Heeke S, Alix-Panabieres C, Pantel K. Liquid biopsy in the era of immune-oncology. is it ready for prime-time use for cancer patients? Ann Oncol 2019; 30:1448–59.
37. Scher HI, Graf RP, Schreiber NA, Jayaram A, Winquist E, McLaughlin B, et al. Assessment of the validity of nuclear-localized androgen receptor splice variant 7 in circulating tumor cells as a predictive biomarker for castration-resistant prostate cancer. JAMA Oncol. 2018;4:1179–86.
38. Scher HI, Graf RP, Schreiber NA, McLaughlin B, Jendrisak A, Wang Y, et al. Phenotypic heterogeneity of circulating tumor cells informs clinical decisions between AR signaling inhibitors and taxanes in metastatic prostate cancer. Cancer Res 2017;77:5687–98.
39. Lin E, Rivera-Baez L, Fouladdel S, Yoon HJ, Guthrie S, Wieger J, et al. High-throughput microfluidic labyrinth for the label-free isolation of circulating tumor cells. Cell Syst 2017;5:295–304.e4.
40. Pezzi HM, Guckenberger DJ, Schehr JL, Rothbauer J, Stahlfeld C, Singh A, et al. Versatile exclusion-based sample preparation platform for integrated rare cell isolation and analyte extraction. Lab Chip 2018;18: 3446–58.
41. Kozminsky M, Fouladdel S, Chung JS, Wang Y, Smith DC, Alva A, et al. Detection of CTC clusters and a dedifferentiated RNA-expression survival signature in prostate cancer. Adv Sci 2019;6:1801254.
42. Markou A, Lazaridou M, Paraskevopoulos P, Chen S,

Swierczewska M, Budna J, et al. Multiplex gene expression profiling of in vivo isolated circulating tumor cells in high-risk prostate cancer patients. Clin Chem 2018; 64:297–306.
43. Kim HJ, Fouladdel S, Wang Y, Kozminsky M, Burness ML, Paoletti C, et al. Characterizing circulating tumor cells isolated from metastatic breast cancer patients using graphene oxide based microfluidic assay. Adv Biosyst 2019;3:1800278.
44. Kim TH, Wang Y, Oliver CR, Thamm DH, Cooling L, Paoletti C. A temporary indwelling intravascular aphaeretic system for in vivo enrichment of circulating tumor cells. Nat Commun 2019;10:1478.
45. Vermesh O, Aalipour A, Ge TJ, Saenz Y, Guo Y, Alam IS, et al. An intravascular magnetic wire for the high-throughput retrieval of circulating tumour cells in vivo. Nat Biomed Eng 2018;2:696–705.
46. Galanzha EI, Menyaev YA, Yadem AC, Sarimollaoglu M, Juratli MA, Nedosekin DA, et al. In vivo liquid biopsy using cytophone platform for photoacoustic detection of circulating tumor cells in patients with melanoma. Sci Transl Med 2019;11:eaat5857.
47. Hamza B, Ng SR, Prakadan SM, Delgado FF, Chin CR, King EM, et al. Optofluidic real-time cell sorter for longitudinal CTC studies in mouse models of cancer. Proc Natl Acad Sci U S A 2019;116:2232–6.
48. Garrel R, Mazel M, Perriard F, Vinches M, Cayrefourcq L, Guigay J, et al. Circulating tumor cells as a prognostic factor in recurrent or metastatic head and neck squamous cell carcinoma: the CIRCUTEC prospective study. Clin Chem 2019;65:1267–75.
49. Merker JD, Oxnard GR, Compton C, Diehn M, Hurley P, Lazar AJ, et al. Circulating tumor DNA analysis in patients with cancer: American Society of Clinical Oncology and College of American Pathologists joint review. J Clin Oncol 2018;36:1631–41.
50. Pailler E, Oulhen M, Borget I, Remon J, Ross K, Auger N, et al. Circulating tumor cells with aberrant ALK copy number predict progression-free survival during crizotinib treatment in ALK-rearranged non-small cell lung cancer patients. Cancer Res 2017;77:2222–30.
51. Deng G, Krishnakumar S, Powell AA, Zhang H, Mindrinos MN, Telli ML, et al. Single cell mutational analysis of PIK3CA in circulating tumor cells and metastases in breast cancer reveals heterogeneity, discordance, and mutation persistence in cultured disseminated tumor cells from bone marrow. BMC Cancer 2014;14:456.
52. Gasch C, Oldopp T, Mauermann O, Gorges TM, Andreas A, Coith C, et al. Frequent detection of PIK3CA mutations in single circulating tumor cells of patients suffering from HER2-negative metastatic breast cancer. Mol Oncol 2016;10:1330–43.
53. Heitzer E, Auer M, Hoffmann EM, Pichler M, Gasch C, Ulz P, et al. Establishment of tumor-specific copy number alterations from plasma DNA of patients with cancer. Int J Cancer 2013;133:346–56.
54. Buim ME, Fanelli MF, Souza VS, Romero J, Abdallah EA, Mello CA, et al. Detection of KRAS mutations in circulating tumor cells from patients with metastatic colorectal cancer. Cancer Biol Ther 2015;16:1289–95.
55. Kondo Y, Hayashi K, Kawakami K, Miwa Y, Hayashi H, Yamamoto M. KRAS mutation analysis of single circulating tumor cells from patients with metastatic colorectal cancer. BMC Cancer 2017;17:311.
56. Heitzer E, Auer M, Gasch C, Pichler M, Ulz P, Hoffmann EM, et al. Complex tumor genomes inferred from single circulating tumor cells by array-CGH and next-generation sequencing. Cancer Res 2013;73:2965–75.
57. Babayan A, Alawi M, Gormley M, Muller V, Wikman H, McMullin RP, et al. Comparative study of whole genome amplification and next generation sequencing performance of single cancer cells. Oncotarget 2017;8: 56066–80.
58. Paoletti C, Cani AK, Larios JM, Hovelson DH, Aung K, Darga EP, et al. Comprehensive mutation and copy number profiling in archived circulating breast cancer tumor cells documents heterogeneous resistance mechanisms. Cancer Res 2018;78:1110–22.
59. Tsao SC-H, Wang J, Wang Y, Behren A, Cebon J, Trau M. Characterising the phenotypic evolution of circulating tumour cells during treatment. Nat Commun 2018;9: 1482.
60. Pantel K, Alix-Panabieres C. Liquid biopsy and minimal residual disease - latest advances and implications for cure. Nat Rev Clin Oncol 2019;16:409–24.
61. Jordan NV, Bardia A, Wittner BS, Benes C, Ligorio M, Zheng Y, et al. HER2 expression identifies dynamic functional states within circulating breast cancer cells. Nature 2016;537:102–6.
62. Girotti MR, Gremel G, Lee R, Galvani E, Rothwell D, Viros A, et al. Application of sequencing, liquid biopsies, and patient-derived xenografts for personalized medicine in melanoma. Cancer Discov 2016;6:286–99.
63. Gorges TM, Kuske A, Rock K, Mauermann O, Muller V, Peine S, et al. Accession of tumor heterogeneity by multiplex transcriptome profiling of single circulating tumor cells. Clin Chem 2016;62:1504–15.
64. Miyamoto DT, Zheng Y, Wittner BS, Lee RJ, Zhu H, Broderick KT, et al. RNA-Seq of single prostate CTCs implicates noncanonical Wnt signaling in antiandrogen resistance. Science 2015;349:1351–6.
65. Yu M, Bardia A, Wittner BS, Stott SL, Smas ME, Ting DT, et al. Circulating breast tumor cells exhibit dynamic changes in epithelial and mesenchymal composition. Science 2013;339:580–4.
66. Antonarakis ES, Lu C, Luber B, Wang H, Chen Y, Zhu Y, et al. Clinical significance of androgen receptor splice variant-7 mRNA detection in circulating tumor cells of men with metastatic castration-resistant prostate cancer treated with first- and second-line abiraterone and enzalutamide. J Clin Oncol 2017;35:2149–56.
67. Baxi S, Yang A, Gennarelli RL, Khan N, Wang Z, Boyce L, Korenstein D. Immune-related adverse events for anti-PD-1 and anti-PD-L1 drugs: systematic review and meta-analysis. BMJ 2018;360:k793.
68. Mazel M, Jacot W, Pantel K, Bartkowiak K, Topart D, Cayrefourcq L, et al. Frequent expression of pd-l1 on circulating breast cancer cells. Mol Oncol 2015;9: 1773–82.
69. Raimondi C, Carpino G, Nicolazzo C, Gradilone A, Gianni W, Gelibter A, et al. PD-L1 and epithelial-mesenchymal transition in circulating tumor cells from non-small cell lung cancer patients: a molecular shield to evade immune system? Oncoimmunology 2017;6: e1315488.
70. Strati A, Koutsodontis G, Papaxoinis G, Angelidis I, Zavridou M, Economopoulou P, et al. Prognostic significance of PD-L1 expression on circulating tumor cells in patients with head and neck squamous cell carcinoma. Ann Oncol 2017;28:1923–33.
71. Yue C, Jiang Y, Li P. Dynamic change of PD-L1 expression on circulating tumor cells in advanced solid tumor patients undergoing PD-1 blockade therapy. Oncoimmunology 7:e1438111.
72. Wang Z, Duan J, Cai S, Han M, Dong H, Zhao J, et al. Assessment of blood tumor mutational burden as a potential biomarker for immunotherapy in patients with non-small cell lung cancer with use of a next-generation sequencing cancer gene panel. JAMA Oncol 2019;5:696–702.
73. Addeo A, Banna GL, Weiss GJ. Tumor mutation burden-from hopes to doubts. JAMA Oncol 2019;5:934–5.
74. Gerlinger M, Rowan AJ, Horswell S, Math M, Larkin J, Endesfelder D, et al. Intratumor heterogeneity and branched evolution revealed by multiregion sequencing. N Engl J Med 2012;366:883–92.
75. Murtaza M, Dawson SJ, Pogrebniak K, Rueda OM, Provenzano E, Grant J, et al. Multifocal clonal evolution characterized using circulating tumour DNA in a case of metastatic breast cancer. Nat Commun 2015;6:8760.
76. Alix-Panabieres C, Pantel K. Clinical applications of circulating tumor cells and circulating tumor DNA as liquid biopsy. Cancer Discov 2016;6:479–91.
77. Andree KC, Mentink A, Zeune LL, Terstappen L, Stoecklein NH, Neves RP, et al. Toward a real liquid biopsy in metastatic breast and prostate cancer: diagnostic leukapheresis increases CTC yields in a European prospective multicenter study (CTCTrap). Int J Cancer 2018; 143:2584–91.
78. Fehm TN, Meier-Stiegen F, Driemel C, Jager B, Reinhardt F, Naskou J, et al. Diagnostic leukapheresis for CTC analysis in breast cancer patients: CTC frequency, clinical experiences and recommendations for standardized reporting. Cytometry A 2018;93:1213–9.
79. Fischer JC, Niederacher D, Topp SA, Honisch E, Schumacher S, Schmitz N, et al. Diagnostic leukapheresis enables reliable detection of circulating tumor cells of nonmetastatic cancer patients. Proc Natl Acad Sci U S A 2013;110:16580–5.