REVIEW

Clinical Applications of Circulating Tumor Cells and Circulating Tumor DNA as Liquid Biopsy

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ABSTRACT "Liquid biopsy" focusing on the analysis of circulating tumor cells (CTC) and circulating cell-free tumor DNA (ctDNA) in the blood of patients with cancer has received enormous attention because of its obvious clinical implications for personalized medicine. Analyses of CTCs and ctDNA have paved new diagnostic avenues and are, to date, the cornerstones of liquid biopsy diagnostics. The present review focuses on key areas of clinical applications of CTCs and ctDNA, including detection of cancer, prediction of prognosis in patients with curable disease, monitoring systemic therapies, and stratification of patients based on the detection of therapeutic targets or resistance mechanisms.

Significance: The application of CTCs and ctDNA for the early detection of cancer is of high public interest, but it faces serious challenges regarding specificity and sensitivity of the current assays. Prediction of prognosis in patients with curable disease can already be achieved in several tumor entities, particularly in breast cancer. Monitoring the success or failure of systemic therapies (i.e., chemotherapy, hormonal therapy, or other targeted therapies) by sequential measurements of CTCs or ctDNA is also feasible. Interventional studies on treatment stratification based on the analysis of CTCs and ctDNA are needed to implement liquid biopsy into personalized medicine. *Cancer Discov; 6(5); 479–91. ©2016 AACR.*

INTRODUCTION

Early in the formation and growth of a primary tumor (e.g., breast, colon, lung, or prostate cancer), cells are released into the bloodstream. These circulating tumor cells (CTC) can be enriched and detected via different technologies that take advantage of their physical and biologic properties. CTC analyses are considered a real-time "liquid biopsy" for patients with cancer (1). Research on CTCs is a very dynamic field with more than 17,000 articles listed in PubMed as of December 2015. More recently, the term "liquid biopsy" has also been adopted for the analysis of circulating cell-free tumor DNA (ctDNA) released from apoptotic or necrotic tumor cells (2). The development of sensitive molecular assays has allowed researchers to screen ctDNA in blood plasma for tumor-specific aberrations; thus, ctDNA and CTC approaches have become competing biomarkers (3). However, we propose that the information obtained from both sources, CTCs and ctDNA, is different, complementary, and depends on the context of use (4).

Following a short discussion of the biology and detection technologies, the present review focuses on the clinical applications of CTCs and ctDNA as liquid biopsy in patients with cancer.

THE BIOLOGY BEHIND THE LIQUID BIOPSY CONCEPT

CTCs

Tumor cells are released from the primary tumor and/or metastatic sites into the bloodstream. The time of CTCs in the bloodstream is short (half-life: 1–2.4 hours; ref. 5). Whether the release of CTCs into the bloodstream is a random process or predetermined by a specific biologic program is still a matter of debate. Nevertheless, the conditions in the bloodstream are harsh for epithelial tumor cells, and it is likely that CTCs might undergo a strong selection process (6). This is consistent with the observation that apoptotic CTCs or fragmented CTCs are frequently found in the peripheral blood of patients with cancer (7). The clearance of surviving CTCs happens through extravasation into secondary organs. For example, in patients with colorectal cancer, comparative analysis of CTCs in the mesenteric and peripheral veins showed that the liver captures tumor cells released by the primary carcinoma (8), which is consistent with a wealth of animal data on this subject (6). As the dissemination of tumor cells to distant organ

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sites necessitates a treacherous journey through the vasculature, it is fostered by close association with activated platelets and macrophages (9). Thus, the tumor cells form heteroaggregates that support attachment to the endothelium and thereby contribute to metastasis (10). Moreover, the migration of metastatic cells in circulation is often dependent upon gradients of chemokines, including CXCR4, CCR4, CCR7, and CCR9, that direct tumor cells through the vasculature (11).

The detection of CTCs in patients, months or years after primary tumor resection, indicates that tumor cells can recirculate from secondary metastatic sites into the bloodstream (5, 12). Whether and how these CTCs contribute to metastatic spread and progression remains unclear. Previous experimental results in a mouse model suggest that tumor cells can recirculate back to the primary site, a process called "tumor self-seeding" (13). To the best of our knowledge, there is no direct proof that this also happens in patients with cancer; however, it is interesting to note that the detection of disseminated tumor cells (DTC) in the bone marrow of patients with breast cancer is correlated not only to metastatic relapse but also to locoregional relapse with potentially more aggressive metastatic variants (14). Future comparative genomic analyses of CTCs and DTCs together with primary and metastatic lesions from the same patients might provide more insights.

Over the past 10 years and in most of the current assays, CTCs have been detected through the use of epithelial markers such as EPCAM and cytokeratins that are not expressed on the surrounding mesenchymal blood cells (15). However, epithelial tumor cells can undergo an epithelial-to-mesenchymal transition (EMT) that results in a reduced expression of epithelial markers and an increased plasticity and capacity for migration and invasion, as well as a resistance to anoikis, which are hallmarks required for CTC survival and dissemination (15). It has been previously suggested that this transition might affect tumor cells with stem cell–like properties in particular (16). Thus, it can be envisaged that the founder cells of overt metastases (i.e., "metastatic stem cells") among CTCs have a reduced expression of epithelial markers. However, the present view is that tumor cells with a partial EMT, also called the "intermediate phenotype," might have the highest plasticity to adapt to the conditions present in secondary sites (17). This view is supported by the recent work of Cayrefourcq and colleagues, who showed that, after establishing the first colon CTC line from a patient with colon cancer, colon CTCs strongly expressed EPCAM as well as cytokeratins simultaneously with the expression of SNAIL, ALDH1, and CD133 (18). Moreover, Baccelli and colleagues showed that CTCs from patients with breast cancer xenografted into immunodeficient mice expressed detectable amounts of EPCAM and cytokeratins next to CD44, CD47, and the MET oncoprotein (19).

ctDNA

Tumor DNA can be released from primary tumors, CTCs, micrometastasis, or overt metastases into the blood of patients with cancer. The majority of such ctDNA is derived from apoptotic and necrotic tumor cells that release their fragmented DNA into the circulation. Recent studies have indicated that viable tumor cells can release microvesicles (exosomes) that might contain double-stranded DNA (20, 21), but this subject is still debated. Cell-free DNA (cfDNA) is also released by dying nonmalignant host cells, and this normal cfDNA dilutes the ctDNA in patients with cancer, in particular in situations when tissue-damaging therapies such as surgery, chemotherapy, or radiotherapy are administered. The DNA fragment length might provide some information on the origin of cfDNA (22, 23), but we still need to know more about the biology behind the release of ctDNA into the circulation. Moreover, the clearance of cfDNA is also not fully understood. cfDNA has a short half-life of 16 minutes (23) and is cleared through the liver and kidneys (24). Renal dysfunction affects cfDNA clearance (25), which might be a confounding factor modulating the concentration of ctDNA in some patients with cancer.

Several reports indicate that ctDNA can even be taken up by host cells, and this uptake affects the biology of these cells (26). Whether "genometastasis" also occurs in patients with cancer remains the subject of future investigations. Interestingly, Trejo-Becerril and colleagues reported observations on the ability of cfDNA to induce *in vitro* cell transformation and tumorigenesis by treating NIH3T3 recipient murine cells with the serum of patients with colon cancer and supernatant of SW480 human cancer cells (27). Cell transformation and tumorigenesis of recipient cells did not occur if serum and supernatants were depleted of DNA, which supports the hypothesis that cancer cells emit into the circulation biologically active DNA to foster tumor progression. Thus, ctDNA may be explored as a new target for antitumor therapy aimed to deplete this "oncogenic DNA," an idea already published decades ago (28).

TECHNOLOGIES FOR CTC AND ctDNA DETECTION

CTCs

Recent progress has been made in the development of various devices to enrich and detect CTCs, including the discovery and validation of new CTC markers (15). It is important to note that the CTC field focused on the biology of tumor dissemination and in particular on EMT affecting tumor cells with potential stem cell–like properties (16). As a consequence, many groups optimized new devices to select and detect CTCs that underwent EMT (15).

As reviewed recently (15), CTC assays usually start with an enrichment step that increases the concentration of CTCs by several log units and enables an easier detection of single tumor cells. Subsequently, CTC can be detected in different ways. In principle, CTCs can be positively or negatively enriched on the basis of biologic properties (i.e., expression of protein markers) or on the basis of physical properties (i.e., size, density, deformability, or electric charges). Positive or negative CTC enrichment can also be achieved on the basis of a combination of physical and biologic properties in the same device. CTCs can then be detected using immunologic, molecular, or functional assays (15). In the past, many research teams focused on functional tests using CTC cultures/cell lines and xenografts (29, 30). These *in vitro* and *in vivo* models can be used to test drug susceptibility. However,

in order to contribute to personalized medicine, the efficacy of establishing CTC cultures and xenografts needs to be enhanced. So far, hundreds of CTCs are needed to establish a cell line or xenograft, which limits this approach to few patients with advanced disease.

The focus on new technical developments based on discoveries on CTC biology has slowed down the introduction of CTCs into clinical diagnostics. However, new important insights into the biology of CTCs combined with various innovative technologies have been reported (31), and technical platforms for combined enrichment, detection, and characterization of CTCs are on the horizon.

ctDNA

Highly sensitive and specific methods have been developed to detect ctDNA, including BEAMing Safe-SeqS, TamSeq, and digital PCR to detect single-nucleotide mutations in ctDNA or whole-genome sequencing to establish copy-number changes. In principle, technologies can be divided into targeted approaches that aim to detect mutations in a set of predefined genes (e.g., *KRAS* in the context of EGFR blockade by antibodies) or untargeted approaches (e.g., array-CGH, whole-genome sequencing, or exome sequencing) that aim to screen the genome and discover new genomic aberrations, e.g., those that confer resistance to a specific targeted therapy (32). The strengths and limitations of these technologies have been recently discussed (33). In general, targeted approaches have a higher analytic sensitivity than untargeted approaches, despite strong efforts to improve detection limits (33). Recently, we have seen an emergence of ultrasensitive technologies able to detect the smallest amounts of ctDNA in the "sea" of normal cfDNA, which are needed for early detection of cancer or minimal residual disease (34).

SCREENING AND EARLY DETECTION OF CANCER

Studies on cancer screening start usually with comparisons of patients with cancer with controls (healthy individuals or patients with benign diseases). Subsequent cohort studies are cumbersome and require large study populations and extended follow-up times. Focusing on patients with high risk of developing cancer [e.g., patients with chronic obstructive pulmonary disease (COPD)] is a good strategy to speed up this validation process.

CTCs

Ilie and colleagues reported that CTCs could be detected in patients with COPD without clinically detectable lung cancer (35). The study included 168 (68.6%) patients with COPD and 77 subjects without COPD (31.4%), including 42 control smokers and 35 nonsmoking healthy individuals. Patients with COPD were monitored annually by low-dose spiral CT. CTCs were detected in 3% of patients with COPD (5 of 168 patients). The annual surveillance of the CTC-positive COPD patients by CT-scan screening detected lung nodules 1 to 4 years after CTC detection, leading to prompt surgical resection and histopathologic diagnosis of early-stage lung cancer, whereas no CTCs were detected in the control smoking and nonsmoking healthy individuals. Interestingly, CTCs detected in patients with COPD had a heterogeneous expression of epithelial and mesenchymal markers. These preliminary findings need to be validated in larger cohorts, and sources that may lead to unspecific findings in non-cancer patients, such as the release of epithelial cells into the blood of patients with inflammatory bowel diseases (36), need to be identified.

ctDNA

Detection of cancer by monitoring ctDNA has received great attention (37, 38). The greatest technical challenge is the identification of very low amounts of ctDNA in blood samples with variable amounts of cfDNA and the choice of the right panel of cancer-specific genomic aberrations. Recently, the Johns Hopkins team used digital polymerase chain reaction–based technologies to evaluate the ability of ctDNA to detect tumors in 640 patients with various cancer types (39). ctDNA was identified in only 48% to 73% of patients with localized cancers: colorectal cancer, gastroesophageal cancer, pancreatic cancer, and breast adenocarcinoma. Although remarkable, these detection rates are not satisfactory for early cancer detection. ctDNA was often present in patients without detectable CTCs (39). However, CTCs were not enriched but simply determined in blood pellets with an enormous background of leukocytes, an approach with a very low sensitivity rarely used in modern CTC diagnostics.

To date, many teams are in an ongoing race for more sensitive ctDNA technologies. For example, the team of Maximilian Diehn has developed a new approach called "cancer personalized profiling by deep sequencing" (CAPP-Seq; ref. 34). CAPP-Seq was implemented for non–small cell lung cancer (NSCLC) with a design covering multiple classes of somatic alterations that identified mutations in >95% of tumors with 96% specificity for mutant allele fractions down to approximately 0.02%. Although ctDNA was detected in the majority of patients with stage II–IV disease, only 50% of patients with early-stage NSCLC (stage I) were identified. The abundance of ctDNA in the cfDNA fraction in patients with stage I tumors was measured to be approximately 10-fold lower than that in patients with more advanced disease. This difference is not unexpected and might also be observed in other tumor entities. Thus, further improvements to the technology, including the ability to analyze larger blood volumes, are required to reach an acceptable sensitivity for early cancer detection.

Besides sensitivity, the specificity of the results also poses some additional challenges. Cancer-associated mutations occur with increasing age even in individuals who never develop cancer during their lifetime. For example, clonal hematopoiesis with somatic mutations was observed in 10% of persons older than 65 years of age and was a strong risk factor for subsequent hematologic cancer. However, the absolute risk of conversion from clonal hematopoiesis to hematologic cancer was modest (1.0% per year; ref. 40). Thus, the detection of cancer-associated mutations on cfDNA might not indicate that the individual tested already has cancer or will develop cancer in her/his lifetime, but it might induce substantial anxiety and extensive diagnostic procedures with side effects like radiation.

ESTIMATION OF THE RISK FOR METASTATIC RELAPSE (PROGNOSTIC INFORMATION)

CTCs

There is a plethora of studies showing the prognostic significance of CTCs in patients with various types of solid tumors, in particular in breast cancer (41). Here, we focus on patients with early-stage disease who have no clinical or radiologic signs of overt distant metastases [tumor–node–metastasis (TNM) stage M_0]. Interestingly, the 2010 TNM classification has already included a new stage called cM_0(i+), where "i+" refers to the detection of isolated tumor cells in blood, bone marrow, and lymph nodes. However, this classification is rarely used in clinical practice, partly because the CTC counts in M_0 patients are very low, which has raised doubts about their usefulness as a reliable marker. Nevertheless, there is increasing evidence that the determination of CTC counts before or after initial surgery in M_0 patients is a reliable indicator of an unfavorable prognosis.

Rack and colleagues recently published the largest multicenter study thus far (42). CTCs were analyzed in 2,026 patients with early breast cancer before adjuvant chemotherapy and in 1,492 patients after chemotherapy using the CellSearch System. Before chemotherapy, CTCs were detected in 21.5% of patients, with 19.6% of node-negative and 22.4% of node-positive patients showing CTCs ($P < 0.001$). The presence of CTCs was associated with poor disease-free survival (DFS; $P < 0.001$), breast cancer–specific survival ($P = 0.008$), and overall survival (OS; $P = 0.0002$). CTCs were confirmed as independent prognostic markers in multivariable analysis. The prognosis was worst in patients with at least five CTCs per 30 mL blood (DFS: HR = 4.51). After chemotherapy, 22.1% of patients were CTC positive, and the persistence of CTCs showed a negative prognostic influence. Thus, CTC detection both before and after adjuvant chemotherapy is linked to an increased risk of relapse in primary breast cancer.

In prostate cancer, unselected groups of newly diagnosed patients without overt metastases have a good prognosis, which requires extended follow-up evaluations of 10 years or more. To circumvent this problem, several groups have focused on high-risk patients with high Gleason scores or high prostate-specific antigen (PSA) serum levels. Loh and colleagues analyzed CTCs using CellSearch and found only a small number of CTCs in high-risk patients (43). Thalgott and colleagues evaluated high-risk patients undergoing neoadjuvant chemotherapy/hormonal therapy and radical prostatectomy and observed that patients with persistent CTCs post-RP developed biochemical recurrence (44).

Promising studies demonstrating significant correlations between CTC counts and metastatic relapse have been observed in other tumor entities, such as colorectal cancer (8), bladder cancer (45, 46), liver cancer (47), and esophageal cancer (48). Taken together, larger cohorts need to be analyzed using more sensitive CTC assays to further explore the clinical relevance of CTCs in nonmetastatic cancer patients. In particular, longitudinal blood analyses during the period between primary surgery and relapse seem to be interesting to discover the kinetics of minimal residual disease, which could provide valuable insights into the biology of cancer dormancy (6, 49).

ctDNA

An exciting challenge is the shift from analysis of patients in advanced stages with high loads of ctDNA to early-stage patients who are treated with curative intent. In cancer types for which adjuvant therapy has marginal benefit in a pathologically staged population or in a lower-risk population where adjuvant therapy is not currently offered, there is the prospect that patients with detectable ctDNA (or CTCs) might be targeted for recruitment into trials to ascertain the benefit of intervening with adjuvant therapy then as opposed to waiting for the emergence of overt metastases. This might extend the benefit of adjuvant therapy, as opposed to only monitoring its utility in populations who are already going to receive adjuvant therapy as a current standard. Of course, randomized trials would be needed to demonstrate utility, but this may be one of the most impactful settings for liquid biopsy measurements.

In paired ctDNA and primary breast cancer samples, Shaw and colleagues identified focal high-level DNA amplifications clustered in numerous chromosome arms, some of which harbored genes with oncogenic potential, including *USP17L2* (*DUB3*), *BRF1*, *MTA1*, and *JAG2* (50). Up to 12 years after diagnosis, these amplifications were still detectable in some of the follow-up plasma samples, indicating the presence of "occult" micrometastatic disease (50). In estrogen receptor (ER)–positive breast cancer, mutations of *PIK3CA* are frequent genomic alterations (51). Applying a digital PCR assay, Oshiro and colleagues detected that serum samples from 23% of *PIK3CA*-mutant patients were positive for this mutation, and mutant *PIK3CA* ctDNA mutation status was a significant and independent prognostic factor for patients with breast cancer (52).

In a postsurgery surveillance study by Reinert and colleagues, ctDNA levels were quantified using droplet digital PCR in 151 plasma samples from six relapsing and five nonrelapsing patients with colorectal cancer (53). Relapses could be detected months ahead as compared to conventional follow-up. More recently, Tie and colleagues exploited ctDNA analysis to evaluate tumor burden and predict response to standard chemotherapy in patients with early-stage colorectal cancer (54). By sequencing a panel of 15 genes frequently altered in colorectal cancer, the report highlighted how mutation levels in blood can anticipate response to therapy.

IDENTIFICATION OF THERAPEUTIC TARGETS AND RESISTANCE MECHANISMS

CTCs

Studying CTCs offers a wealth of information on therapeutic targets and resistance mechanisms at the protein, RNA, and genome levels.

Protein Level

ER is a key target in breast cancer, and primary tumors are classified as ER-positive and ER-negative based on a cutoff value of 1% immunostained cells in the analyzed section, and hormonal therapy is stratified based on this result. However, ER-positive breast tumors can harbor ER-negative CTCs, which might be able to escape endocrine therapy (55).

Recently, Paoletti and colleagues developed a multiparameter CTC-Endocrine Therapy Index (CTC-ETI), which may predict resistance to endocrine therapy in patients with HR-positive metastatic breast cancer (MBC; ref. 56). The CTC-ETI combines enumeration and CTC expression of four markers: ER, BCL2, HER2, and Ki67. The clinical implications of CTC-ETI are being evaluated in an ongoing prospective trial.

Another target, the *HER2* oncogene, which is amplified and overexpressed in approximately 20% of primary carcinomas, has become a key target in breast cancer. There is increasing evidence that overt distant metastases and CTCs have discrepant HER2 statuses compared to the primary tumor in up to 30% of cases (57). In particular, the presence of HER2-positive CTCs in patients with HER2-negative primary tumors (58, 59) has induced multicenter trials aimed at investigating whether these patients will benefit from HER2-targeting therapies (see below).

More recently, immune checkpoint regulators such as PD-L1 have become exciting new therapeutic targets leading to long-lasting remissions in patients with advanced malignancies (60). However, in view of the remarkable costs and the toxicity profiles of these therapies, predictive biomarkers able to discriminate responders from nonresponders are urgently needed. Mazel and colleagues provided first evidence that PD-L1 is frequently expressed on CTCs (>60% of patients) in patients with hormone receptor–positive, HER2-negative breast cancer (61). Immunoscores for PD-L1 expression classify different subpopulations of CTCs. The established CTC/PD-L1 assay can be used for liquid biopsy in future clinical trials of patients undergoing immune checkpoint blockade (62).

In prostate cancer, PSA and prostate-specific membrane antigen (PSMA) are upregulated following androgen receptor (AR) activation and AR suppression, respectively. An interesting report by Miyamoto and colleagues indicated that PSA/PSMA-based measurements are surrogates for AR signaling in CTCs, and this information might help to predict the outcome of AR-based therapy (63).

RNA Level: mRNA and microRNA

Several groups have developed protocols for RT-PCR analysis of mRNA expression in CTCs targeting specific genes or signatures (15, 64). Recent studies focusing on prostate cancer demonstrated that mRNA analysis of CTCs could reveal substantial information on drug sensitivity and resistance. Most hormone-dependent prostate cancers become resistant to treatment after 1 to 3 years and resume growth despite hormone therapy (65). Castration-resistant prostate cancer (CRPC) is defined by disease progression despite androgen-deprivation therapy. Several drugs have recently emerged for the treatment of CRPC (e.g., enzalutamide or abiraterone). However, a proportion of men do not benefit from these agents, and a clearer understanding of the mechanisms underlying resistance to these drugs would facilitate selection of alternative therapies for such patients. Recent studies indicated that the mRNA expression of ARv7, a truncated form of AR that lacks the ligand-binding domain but remains constitutively active, in CTCs could predict failure of antiandrogen therapy with enzalutamide and abiraterone (66, 67). ARv7-positive patients may retain sensitivity to taxanes (68–71), and ARv7 may therefore become a treatment-selection marker in metastatic CRPC. Besides ARv7, additional mechanisms of resistance have been recently discovered by RNA sequencing of CTCs in metastatic CRPC, underlying the power of expression analysis of single CTCs (72).

MicroRNAs (miRNAs) are key regulators of gene expression and have emerged as potentially important diagnostic markers and targets for anticancer therapy. Recently, Gasch and colleagues described a robust *in situ* hybridization (ISH) protocol, incorporating the CellSearch CTC detection system, enabling clinical investigation of important miRNAs such as miR-10b on a cell-by-cell basis (73). In a proof-of-principle study, this method was used to demonstrate heterogeneity of miR-10b on a CTC-by-CTC basis in patients with breast, prostate, or colorectal cancer.

DNA Level

Mutations in genes encoding therapeutic targets or signalling proteins downstream of the target can affect the efficacy of targeted drugs. For example, mutations in EGFR affect anti-EGFR therapy in lung cancer, and mutations in KRAS—a protein downstream of EGFR—block the efficacy of anti-EGFR therapy in colorectal cancer. Recently, the analysis of hundreds of CTCs obtained from patients with colorectal cancer revealed high intrapatient and interpatient heterogeneity of KRAS (74, 75). CTCs with mutated *KRAS* genes will escape anti-EGFR therapy, and their early detection might help to guide therapy in individual patients.

In prostate cancer, mutations in the gene encoding AR were identified in CTC-enriched peripheral blood samples from patients with CRPC (76). *AR* amplifications enable the tumor cells to profit from the minute amounts of residual androgens in patients receiving drug-induced castration therapy, whereas *AR* mutations can result in tumor cells that are refractory to androgen blockade.

In breast cancer, resistance to HER2-targeting therapies is a key issue, and activation of the PI3K pathway, e.g., by mutations in the *PIK3CA* gene, plays a pivotal role in this process (77–79). Schneck and colleagues analyzed CTCs using the SNaPshot method and found *PIK3CA* mutations in enriched CTC pools of 15.9% of patients with metastatic breast cancer (80). Other authors isolated single CTCs and reported strong heterogeneity in the *PIK3CA* mutational status among CTCs from individual patients (81–83). These single CTC analyses resulted in higher detection rates of *PIK3CA* mutations; however, more information about the occurrence of these mutations in the context of therapeutic interventions is urgently needed.

In melanomas, targeted therapies have started to change the dismal prognosis of metastatic disease, although resistance occurs rapidly in most treated patients. BRAF has become one of the lead targets for therapy with specific inhibitors, and mutations in the *BRAF* gene are important predictors of sensitivity to therapy. Recently, dynamic changes of *BRAF* mutations were detected in CTCs and ctDNA (84, 85), which may guide BRAF-directed therapies in the future.

In colorectal cancer, Heitzer and colleagues also performed screening for druggable mutations in CTCs (86) using massive parallel sequencing of a panel of 68 colorectal cancer–associated genes. Mutations in known driver genes (e.g., *APC*, *KRAS*, or *PIK3CA*) found in the primary tumor and metastasis

were also detected in corresponding CTCs. Interestingly, some CTCs revealed a high level of amplification of *CDK8* as a potential new therapeutic target for CDK inhibitors (87).

Taken together, these investigations demonstrate that CTC analyses at the DNA, RNA, and protein levels may have an important future impact for better understanding of resistance to therapy in patients with cancer.

ctDNA

Therapeutic targets are usually proteins, but ctDNA analyses can reveal important information on genomic aberrations affecting the efficacy of targeted drugs, including mutations of the *EGFR* gene in lung cancer, mutations of the *KRAS* gene in colorectal cancer, *TP53* and *PIK3CA* mutations in breast cancer, and *AR* gene mutations in prostate cancer. We discuss these ctDNA analyses in the next section, as most of them have been used for monitoring therapies in patients with cancer.

REAL-TIME MONITORING OF THERAPIES

CTCs

The clinical validity of CTC quantification (CellSearch method) for monitoring of chemotherapy was assessed in patients with metastatic breast cancer by undertaking a pooled analysis of individual patient data (88). This landmark analysis was based on data provided by 17 international centers, including 1,944 eligible patients from 20 studies. Remarkably, CTC counts improved the prognostication of metastatic breast cancer when added to full clinicopathologic predictive models, whereas serum tumor markers did not (88), despite the fact that these serum markers are frequently used in clinical practice.

Similarly, Scher and colleagues assessed whether CTC counts (assessed by CellSearch) could be an individual-level surrogate for survival in patients with metastatic CRPC in the context of COU-AA-301, a multinational, randomized, double-blind phase III trial of abiraterone acetate plus prednisone versus prednisone alone in patients with metastatic CRPC previously treated with docetaxel (89). The biomarkers were measured at baseline and 4, 8, and 12 weeks, with 12 weeks being the primary measure of interest. The study showed that a biomarker panel containing CTC number and LDH level was a surrogate for survival at the individual-patient level, whereas changes in PSA serum levels, used in clinical practice to assess therapeutic efficacy, were not relevant.

Thus, both studies demonstrate that changes in CTC counts measured with a standardized assay can contribute to the early assessment of therapy effects.

ctDNA

In colorectal cancer, acquired resistance to EGFR-specific antibodies is associated with the emergence of RAS pathway mutations, and these mutations have been detected on ctDNA before disease progression can be documented by standard imaging (90–92). Interestingly, patients with colorectal cancer who acquired resistance to EGFR antibodies displayed a heterogeneous pattern of mutation in *KRAS, NRAS, BRAF*, and *EGFR* (93, 94). ctDNA could also be used to track clonal evolution and targeted drug responses (95). Of note, the proportion of *KRAS*-mutated alleles dynamically increased and decreased in the presence and absence of the anti-EGFR drug (95). Along the same lines, Mohan and colleagues performed whole-genome sequencing of ctDNA in patients with colorectal cancer treated with anti-EGFR therapy (96) and found several copy-number changes in all samples, including loss of the chromosomal 5q22 region harboring the *APC* gene and loss of chromosome arms 17p and 18q as well as amplifications in known genes involved in the resistance to EGFR blockade, such as *MET, ERBB2*, and *KRAS* (96). Thus, these studies indicate that clonal evolution during targeted therapies might be detected by analysis of ctDNA.

In breast cancer, using whole-genome sequencing, Bettegowda and colleagues detected ctDNA in more than 75% of patients with advanced cancer and 50% of patients with localized breast cancer (39). Furthermore, Dawson and colleagues identified somatic genomic alterations in serially collected plasma from patients with metastatic breast cancer (97). Tumor-associated cfDNA was detected in most patients with breast cancer in whom somatic genomic alterations were identified. The ctDNA levels showed a greater dynamic range, and greater correlation with changes in tumor burden, than did CA15-3 or CTCs detected by the CellSearch system. However, the dropout rate was considerable, and the analyses were restricted to a small cohort of patients. Recently, Madic and colleagues performed another comparative evaluation of CTCs and ctDNA in patients with triple-negative breast cancer (98). *TP53* mutations were found in more than 80% of tumor and ctDNA samples. CTC counts detected with the CellSearch system were correlated with overall survival, whereas ctDNA levels had no prognostic impact. Murtaza and colleagues also tracked genomic evolution on ctDNA of metastatic breast cancer patients in response to therapy (32), and demonstrated that ctDNA mutation levels increased in association with acquired drug resistance, including an activating mutation in PIK3CA following treatment with paclitaxel, a truncating mutation in the ER coactivator mediator complex subunit 1 (MED1) following treatment with tamoxifen and trastuzumab, and, following subsequent treatment with lapatinib, a splicing mutation in GAS6, the ligand for the tyrosine kinase receptor AXL (32). Taken together, these exciting proof-of-principle studies need further validation using larger cohorts and more modern CTC assays.

In prostate cancer, there is a clear focus on aberrations related to resistance to antiandrogen therapies. Heitzer and colleagues started to show the feasibility of genome-wide analysis of ctDNA in patients with metastatic prostate cancer (99). The genome-wide profiling of ctDNA revealed multiple copy-number aberrations, including those previously reported in prostate tumors, such as losses in 8p and gains in 8q. High-level copy-number gains in the *AR* locus were observed in patients with CRPC. More recently, Azad and colleagues found that *AR* amplification was significantly more common in patients progressing on enzalutamide than on abiraterone or other agents (100). Besides *AR* gene amplification, Joseph and colleagues showed that the AR F876L mutant, a missense mutation in the ligand-binding domain of the AR, is detectable in plasma DNA from ARN-509–treated patients with progressive CRPC, suggesting that selective outgrowth of this mutant might be a relevant mechanism of second-generation antiandrogen

resistance that can be potentially targeted with next-generation antiandrogens (101). Along the same lines, Romanel and colleagues developed a targeted next-generation sequencing approach amenable to plasma DNA, covering all *AR* coding bases and genomic regions that are highly informative in prostate cancer (102). Whereas *AR* copy number was unchanged from before treatment to progression and no mutant *AR* alleles showed signals for acquired gain, emergence of T878A or L702H AR amino acid changes in 13% of tumors at progression on abiraterone was observed. Patients with *AR* gain or T878A or L702H before abiraterone are less likely to have a decline in PSA and had a significantly worse overall and progression-free survival. Taken together, these studies indicate that *AR* gene aberrations detected on ctDNA predict outcome of antiandrogen therapy.

STRATIFICATION AND THERAPEUTIC INTERVENTION BASED ON LIQUID BIOPSY

The blood-based stratification of targeted therapies in clinical intervention trials is probably the ticket for the introduction of liquid biopsy analysis into clinical practice. However, the caveat is that the diagnostic approach is now held hostage by the efficacy of the therapy. In the following sections, we discuss some studies based on CTC and ctDNA analysis that will exemplify this issue.

Chemotherapy Switch Based on CTC Enumeration

In the screening part of the SWOG 0500 (NCT00382018) clinical trial, metastatic patients treated with first-line chemotherapy (combined or not with targeted therapy) had their CTC count determined before cycles 1 and 2. Patients with persistently elevated CTCs (≥5 CTCs/7.5 mL) after one cycle (i.e., around days 21–28) were at higher risk of early cancer progression and were randomized between continuation of the first-line chemotherapy (until classic evidence of clinical or radiologic progression) or early switch to another chemotherapy regimen, before any radiologic progression (103). This study confirms the prognostic significance of CTCs in patients with MBC receiving first-line chemotherapy. For patients with persistently increased CTCs after 21 days of first-line chemotherapy, early switching to an alternate cytotoxic therapy was not effective in prolonging overall survival. For this population, there is a need for more effective treatment than standard chemotherapy (104). The randomized phase II study by Georgoulias and colleagues indicated that trastuzumab decreases the incidence of clinical relapses in patients with early breast cancer presenting chemotherapy-resistant CTCs (105).

Stratification of Patients to Chemotherapy or Hormonal Therapy Based on CTC Enumeration

In the STIC CTC METABREAST clinical trial (NCT 01710605), about 1,000 patients with HR^+ M^+ breast cancer were randomized between clinician choice and CTC count-driven choice. In the CTC arm, patients with high CTC counts (≥5 CTCs/7.5 mL) received chemotherapy, whereas patients with low counts (<5 CTCs/7.5 mL) received endocrine therapy as first-line treatment. The only difference between the CTC and standard arms was the rates of hormone therapy versus chemotherapy-based treatment. This pivotal trial has been designed to show noninferiority of the CTC arm for progression-free survival (primary clinical endpoint) and superiority of the CTC arm for the medico-economics study (co-primary endpoint; ref. 103). The study started in 2012 and is still recruiting patients; more than 660 patients have been included as of December 2015.

Stratification of Patients Based on HER2-Phenotyping of CTCs

The following studies are still ongoing at the time of manuscript submission, and we will therefore briefly discuss the study designs.

In the DETECT III study, 1,426 patients with metastatic breast cancer and with up to three chemotherapy lines for metastatic disease are tested for $HER2^+$ CTCs. In all patients, the HER2 status of the primary tumor and metastatic lesions has to be negative. At least one $HER2^+$ CTC/7.5 mL of blood has to be detected in these patients. Two hundred twenty-eight patients meeting the inclusion criteria are randomized between two arms receiving standard therapy or standard therapy plus lapatinib. Standard treatment consists of chemotherapy or endocrine therapy that is either approved for combination with lapatinib or has been investigated in clinical trials. Patients with bone metastases are treated with denosumab in both arms. The primary endpoint of the DETECT III study is progression-free survival; secondary endpoints are overall survival, overall response rate, clinical benefit rate, and the dynamic of CTCs.

Based on the fact that HER2 can be "gained" in CTCs at metastatic stage in $HER2^-$ primary breast cancers, the CirCe trial is a second interventional phase II study. It uses the *HER2*/CEP17 ratio measurement by FISH for *HER2* amplification assessment. In this single-arm study, patients with *HER2*-amplified CTCs receive an anti-HER2 drug without combined chemotherapy; response rate will be the study's main endpoint.

Finally, the Treat CTC trial is a randomized phase II trial for patients with *HER2*-nonamplified primary breast cancer with ≥1CTC/15 mL of blood after completion of (neo-)adjuvant chemotherapy and surgery. Before randomization, a central review of both the HER2 status of the primary tumor and the CellSearch CTC images is performed. Eligible patients are randomized in a 1:1 ratio to either the trastuzumab or the observation arm. Patients randomized to the trastuzumab arm receive a total of six injections every 3 weeks. Patients randomized to observation are observed for 18 weeks. The primary endpoint will compare CTC detection rate at week 18 between the two arms, whereas the secondary endpoint will compare recurrence-free interval.

Stratification of Patients Based on *EGFR* Mutation Analysis of ctDNA

Recently, the first ctDNA test for *EGFR* mutations in NSCLC has been approved, which is an important step toward clinical implementation of liquid biopsy. Approval was based on a phase IV, open-label, single-arm study (NCT01203917) aimed to assess efficacy and safety/tolerability of first-line gefitinib in Caucasian patients with stage IIIA/B/IV, *EGFR* mutation–positive NSCLC. The study

included *EGFR* mutation analysis in matched tumor and plasma samples and concluded that (i) first-line gefitinib was effective and well tolerated in Caucasian patients with *EGFR* mutation–positive NSCLC and (ii) ctDNA could be considered for mutation analysis if tumor tissue is unavailable (106).

CONCLUSION AND PERSPECTIVES

Analyses of CTCs and ctDNA have paved new diagnostic avenues and are to date the cornerstones of liquid biopsy diagnostics (Fig. 1). "To what extent they might replace tumor biopsies in the future" remains the subject of speculation. For primary diagnosis of tumors that are not easy to biopsy, such as lung cancer, and for restaging/molecular analysis of metastatic lesions, liquid biopsy might provide an alternative. Moreover, liquid biopsy diagnostics might help to focus the current cancer screening modalities to the population at higher risk, which would reduce side effects (e.g., radiation in mammography) and health care costs. However, despite a few promising first results and the enormous interest by diagnostic companies and the public press ("cancer detection from a drop of blood"), early detection of cancer faces serious challenges of both sensitivity and specificity. In contrast, monitoring of CTCs

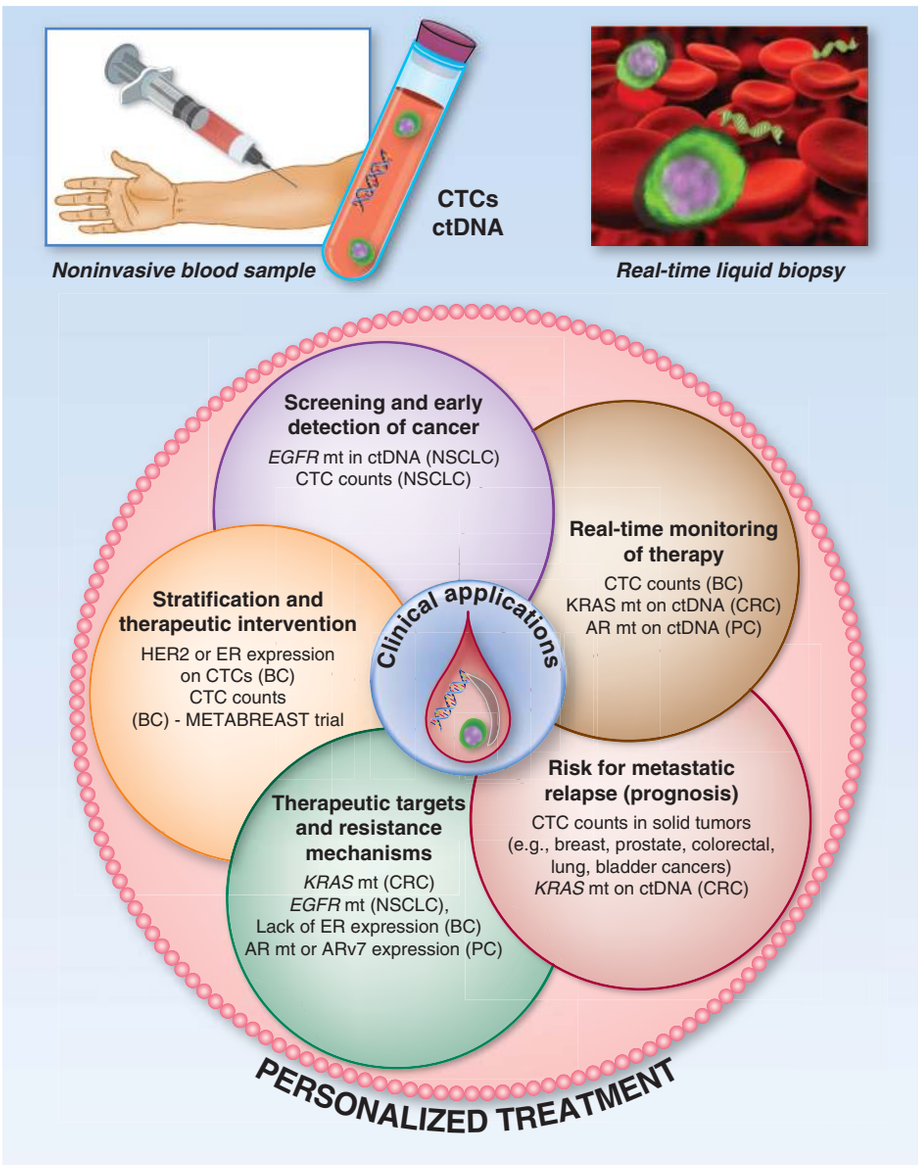

Figure 1. Clinical applications of CTCs and ctDNA as liquid biopsy for personalized medicine. Blood samples can be sampled repeatedly to predict relapse in M_0 patients or metastatic progression in M_1 patients, monitor the efficacy of therapies and understand potential resistance mechanisms. Before therapy, patients can be stratified to the most effective drugs, whereas after initiation of treatment persistence in increases of CTCs/ctDNA indicates resistance to therapy, and this information may allow an early switch to a more effective regimen before the tumor burden is excessive and incurable. mt, mutation; BC, breast cancer; PC, prostate cancer; CRC, colorectal cancer.

and ctDNA during systemic therapy of cancer patients is an application that might be easier to achieve and closer to introduction into clinical practice. Analyses of ctDNA and CTCs for mutations conferring response or resistance to targeted therapies have already been demonstrated in several studies to be feasible in patients with advanced-stage cancer. More tests for mutations in genes encoding therapeutic targets and/or the corresponding resistance genes will follow in the near future. In particular, NSCLC is an interesting tumor entity for this application because various mutations directing specific targeted therapies in small cohorts of responsive patients have been recently identified, and biopsies are not easily obtained in a considerable number of patients (107).

The challenging switch from patients with advanced-stage cancer to early-stage disease with lower ctDNA concentrations has begun with promising results. The inconsistent detection of mutations in ctDNA by the earlier studies could be improved by advanced genomic approaches that have higher sensitivity to identify rare mutations in matched ctDNA and tumor tissue samples. In the CTC arena, promising correlations between CTC counts and relapse have already been observed with the EPCAM-based CellSearch system in several tumor entities. In the future, more sensitive CTC assays will be tested in clinical trials, and the precision of detecting minimal residual cancer might even be increased using more efficient capture systems and enlarging the detection marker panels,

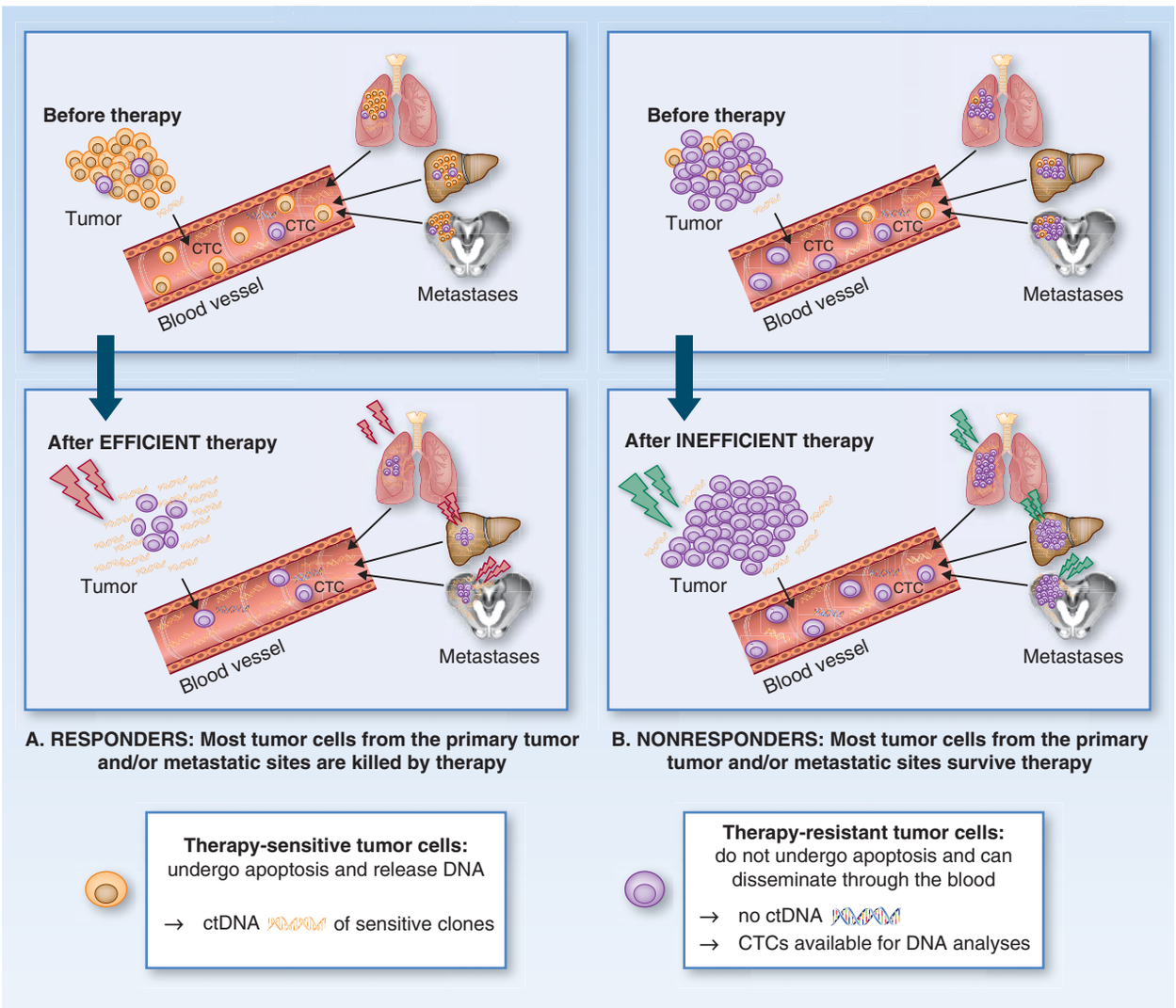

Figure 2. Impact of efficient and inefficient therapies on CTCs and ctDNA. **A,** if the therapy is efficient, patients are good responders, and many sensitive tumor cells in the primary tumor and/or metastases are destroyed by the treatment. As an immediate result, therapy-sensitive tumor cells (in orange) undergo apoptosis and release their tumor DNA (ctDNA) into the peripheral blood. However, the small resistant subclones of viable tumor cells (in purple) will grow and release CTCs into the blood without releasing their DNA. A small fraction of these cells may eventually undergo apoptosis and release small amounts of their DNA (purple) into the circulation. **B,** if the therapy is inefficient, patients are nonresponders and harbor few therapy-sensitive tumor cells (in orange) but many therapy-resistant tumor cells (in purple) before therapy. The therapy will eliminate the sensitive tumor cells, whereas the resistant cells will survive, grow, and disseminate through the bloodstream in increasing numbers without releasing high amounts of tumor DNA (in purple).

hopefully without losing assay specificity. In this context, it should be emphasized that mesenchymal markers such as vimentin are expressed on blood cells in various degrees and are therefore not suitable for CTC detection unless combined with other markers, excluding blood cell origin. Interestingly, tumor cells can show a cell-surface expression of vimentin, which might also be used as a CTC marker (108).

Although ctDNA concentrations generally show a higher dynamic range than CTCs detected with current assays (97), they might not always represent the burden of viable tumor cells (109). In any case, the total amount of CTC versus ctDNA available for analysis might not be a limiting factor, because more sensitive CTC and ctDNA assays are currently in development. The key question is whether the information obtained by CTC and/or ctDNA analysis predicts clinical response to therapy and survival in a given cancer. So far, the number of patients included in ctDNA studies is much smaller than the respective number for CTC-based studies. For example, in the ctDNA breast cancer study by Dawson and colleagues (97) only 30 patients were investigated, whereas the recent CTC study by Rack and colleagues included thousands of patients with breast cancer (42). Moreover, analysis of CTCs can address tumor heterogeneity at the DNA, RNA, and protein levels, whereas computational analyses of ctDNA results can address tumor heterogeneity as well but are restricted to genomic aberrations. Previous work already shows that several drug targets or drug-resistance mechanisms can be detected only by mRNA or protein expression analyses (e.g., ARv7 or PD-L1). Single-cell RNA sequencing of CTCs is now feasible, and we are confident that it will become an important tool in defining new drug targets. However, RNA is less stable than DNA, and precautions need to be taken to maintain RNA integrity in viable CTCs within the blood sample.

Interpretation of the clinical results might be hampered by the fact that we still know little about the dynamic biology of CTC and ctDNA release. ctDNA represents mainly the genome of dying tumor cells, but viable tumor cells drive cancer progression and cause therapy resistance (Fig. 2A and 2B). Thus, the selection of the appropriate time points for ctDNA screening will be crucial to detect those ctDNA species that are derived from the resistant tumor cell clones. In this context, the parallel determination of viable CTCs might provide additional information required to tailor the therapy to the individual need of the cancer patient.

Beyond primary mutations in tumors, CTCs and ctDNA are successful in detecting secondary, acquired drug resistance mutations and mechanisms, which appear to be more heterogeneous than the primary mutations. Several publications on ctDNA have focused on secondary, acquired drug resistance mutations and demonstrated that the ability to detect them prior to progression was visible by imaging or conventional tumor marker analyses (e.g., *KRAS* or *EGFR* mutations). Similarly, CTC studies could demonstrate the emergence of resistant clones (e.g., *HER2* amplifications/overexpression or *PIK3CA* mutations) that were not initially observed. However, it cannot be overlooked that "acquired" drug resistance is in some cases due not to new mutations but to the emergence of very small subclones initially overlooked in the primary tumor (86).

Future clinical trials should focus on interventional studies to demonstrate the clinical utility of liquid biopsy. It needs to be emphasized that even the best liquid biopsy analysis for therapy targets or resistance mechanisms cannot guarantee that the patient will show a durable and life-prolonging response to the respective therapeutic agents. The clinical utility of liquid biopsy analysis (like any other diagnostic approach) needs to be proven in randomized clinical intervention studies in which therapy decisions are based on liquid biopsy analysis and established endpoints such as time to progression or overall survival are used. However, more reliable CTC and ctDNA assays validated in international ring experiments are needed to serve as companion diagnostics in these trials. A European IMI consortium of more than 35 institutions from academia and industry (called CANCER-ID; ref. 110) is now focusing on the validation of liquid biopsy assays. In addition to CTCs and ctDNA, circulating exosomes (111, 112) or blood platelets (113) might become promising candidates as novel blood-based biomarkers. Future studies are required to directly compare the pros and cons of these technologies for any particular indication.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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