Liquid Biopsy: From Discovery to Clinical Application

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ABSTRACT Over the past 10 years, circulating tumor cells (CTC) and circulating tumor DNA (ctDNA) have received enormous attention as new biomarkers and subjects of translational research. Although both biomarkers are already used in numerous clinical trials, their clinical utility is still under investigation with promising first results. Clinical applications include early cancer detection, improved cancer staging, early detection of relapse, real-time monitoring of therapeutic efficacy, and detection of therapeutic targets and resistance mechanisms. Here, we propose a conceptual framework of CTC and ctDNA assays and point out current challenges of CTC and ctDNA research, which might structure this dynamic field of translational cancer research.

Significance: The analysis of blood for CTCs or cell-free nucleic acids called "liquid biopsy" has opened new avenues for cancer diagnostics, including early detection of tumors, improved risk assessment and staging, as well as early detection of relapse and monitoring of tumor evolution in the context of cancer therapies.

INTRODUCTION

The liquid biopsy concept was introduced for circulating tumor cells (CTC) 10 years ago (1) and rapidly extended to circulating tumor DNA (ctDNA; ref. 2) and other tumor-derived products such as circulating cell-free RNA (noncoding and messenger RNA; ref. 3), extracellular vesicles (4), or tumor-educated platelets (ref. 5; Fig. 1). Research on the two key components of liquid biopsy assays, CTCs and ctDNA, is a very active field, with more than 26,070 publications listed under the key phrase "CTC" and more than 5,720 for "ctDNA" in PubMed in September 2020 (i.e., on average 30 to 40 new publications each week for CTCs in 2020). These liquid biomarkers are used in more than 557 clinical trials registered at the NCI website (http://clinicaltrials.gov; 325 for CTCs and 232 for ctDNA; among them, 7 involving both biomarkers). Strong evidence for CTCs and ctDNA as prognostic markers has been documented in many tumor entities including breast, prostate, lung, and colorectal cancers (6–10). In ongoing interventional studies, the clinical utility of CTCs and ctDNA for treatment decisions is being evaluated (11). In particular, the use of CTCs and ctDNA as real-time liquid biopsy has received attention over the past years (12).

However, the diversity of published assays using different principles for detection and characterization of CTCs and ctDNA is confusing to the cancer research community. Both CTCs and ctDNA occur at very low concentrations in the peripheral blood, which poses a serious challenge for any analytic system. This review will focus on the current clinical applications of both liquid biomarkers in patients with solid tumors and will discuss the unresolved issues in CTC and ctDNA research. In this review, we focus on studies using blood for CTC and ctDNA analyses, but the liquid biopsy concept can be expanded to other body fluids, including urine, cerebrospinal fluid (CSF), bone marrow, saliva, or sputum (Fig. 2).

TECHNOLOGIES FOR CTC AND ctDNA DETECTION

Several recent articles have reviewed the technologies used for CTC and ctDNA analyses (13–15). We will therefore only briefly describe the principles of CTC and ctDNA assays (Fig. 3).

CTCs

Various devices have been developed to enrich and detect CTCs, with a focus on devices able to select and detect CTCs that underwent epithelial–mesenchymal transition (EMT) and lack expression of EPCAM as the most frequently used cell surface protein used for CTC enrichment of blood from

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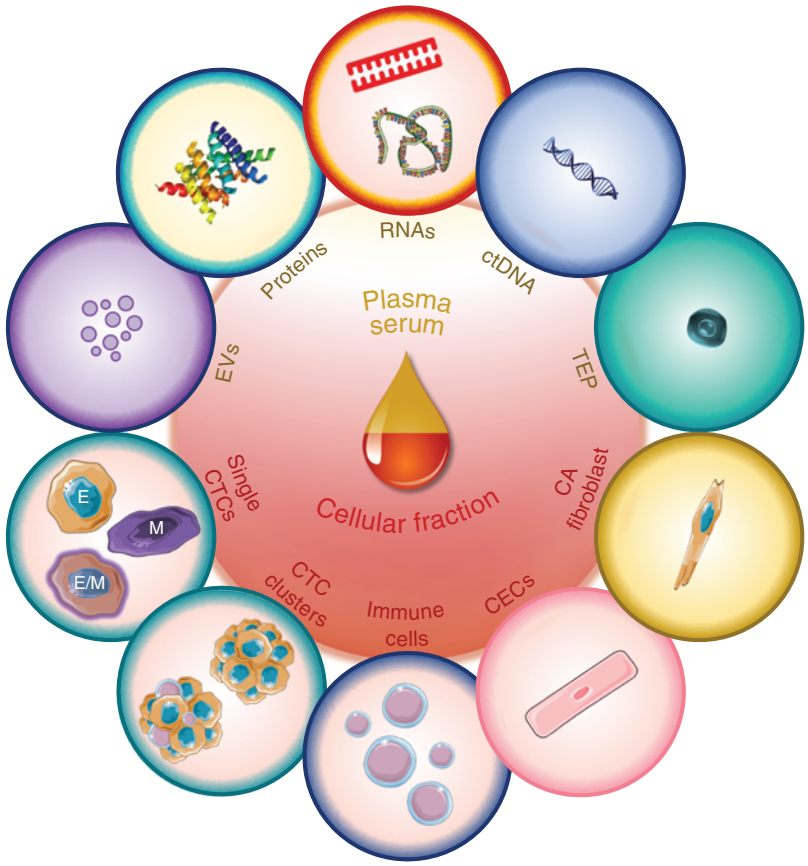

Figure 1. Circulating biomarkers as liquid biopsy for precision medicine. The drop of blood symbolizes the blood sample from which after centrifugation the plasma/serum (yellow, top) fraction is separated from the cellular fraction (red, bottom). In a non-invasive blood sample, different complementary circulating biomarkers can be detected, isolated, and characterized. In the plasma or serum (top), EVs (such as exosomes), proteins, circulating cell-free RNA (noncoding and messenger RNA), ctDNA, and TEPs can be detected. In the cellular fraction (bottom), we can detect (i) the tumor cell fraction with CTCs (single CTCs and CTC clusters constituted with only CTCs or with CTCs escorted by immune cells, such as neutrophils) and the (ii) the nontumor cell fraction (immune cells, CECs, or CA fibroblasts). E, epithelial; M, mesenchymal; EV, extracellular vesicle; CA fibroblast, cancer-associated fibroblast; CEC, circulating endothelial cell; TEP, tumor-educated platelet.

patients with carcinoma (16). However, EPCAM stays in the race, as EPCAM-based enrichment for CTC detection has provided a reliable prognostic tool in different cancers (17). Different subsets of CTCs have various transcriptional programs that can be revealed at the single-cell level by current advances in single-cell sequencing technologies, in particular RNA sequencing (RNA-seq; refs. 18, 19). This technology is crucial to clarify the biology of CTCs. RNA-seq of single CTCs has been used in prostate cancer (20), breast cancer (21, 22), and pancreatic cancer (23) and led to the identification of prognostic signatures, metastatic drivers, and drug target and resistance mechanisms.

CTC assays start with an enrichment step that increases the concentration of CTCs by several log units and enables an easier detection of single tumor cells (13). CTC can be enriched on the basis of biological properties (i.e., expression of protein markers) or physical properties (i.e., size, density, deformability, or electric charges), and these enrichment principles can be combined to optimize the yield of CTCs. After enrichment, an identification step is required to detect CTCs surrounded by the remaining leukocytes at the single-cell level using immunologic, molecular, or functional assays (24). During the past years, research teams have established CTC cultures, CTC lines (25–27), and CTC xenografts (28–30) as models for functional tests (31–33). These *in vitro* and *in vivo* models can be used to test drug susceptibility (34). However, 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 a few patients with advanced disease.

New technical developments will lead to integrated platforms for a combined enrichment, detection, and characterization of CTCs. Moreover, new devices for the *in vivo* detection and/or capture of CTCs (35–37) have opened a new avenue for CTC research, which may overcome the limitation of low CTC counts present in a usual 7.5-mL blood tube. Besides the FDA-cleared CELLSEARCH system that uses EPCAM-coated magnetic particles for CTC enrichment, these new devices include, for example, assays for label-independent size-based enrichment (38) and photoacoustic detection (35), as well as a temporary indwelling intravascular aphaeretic system (36) and an EPCAM-coated *in vivo* capture wire (37).

Finally, the epithelial ImmunoSPOT (EPISPOT) assay has been used for CTC detection in blood and bone marrow samples for 19 years and has been validated at the clinical level for several different cancers, including breast (39), colon (10), prostate (37), and head and neck (40) cancers as well as melanoma (41). This test is currently being further developed into a more sensitive liquid microdroplet format [known as EPISPOT in a DROP (EPIDROP)] that enables the detection of CTCs at the single-cell level (13).

With the EPIDROP, CTCs are immunostained before individual encapsulation in fluid microdroplets, and therefore the total number of CTCs ($EPCAM^+$ or $EPCAM^-$) and the functional CTCs can be enumerated. Indeed, viable CTCs can be distinguished from apoptotic CTCs, and $EPCAM^+$ versus $EPCAM^-$ CTCs, enabling the assessment of EMT status. In

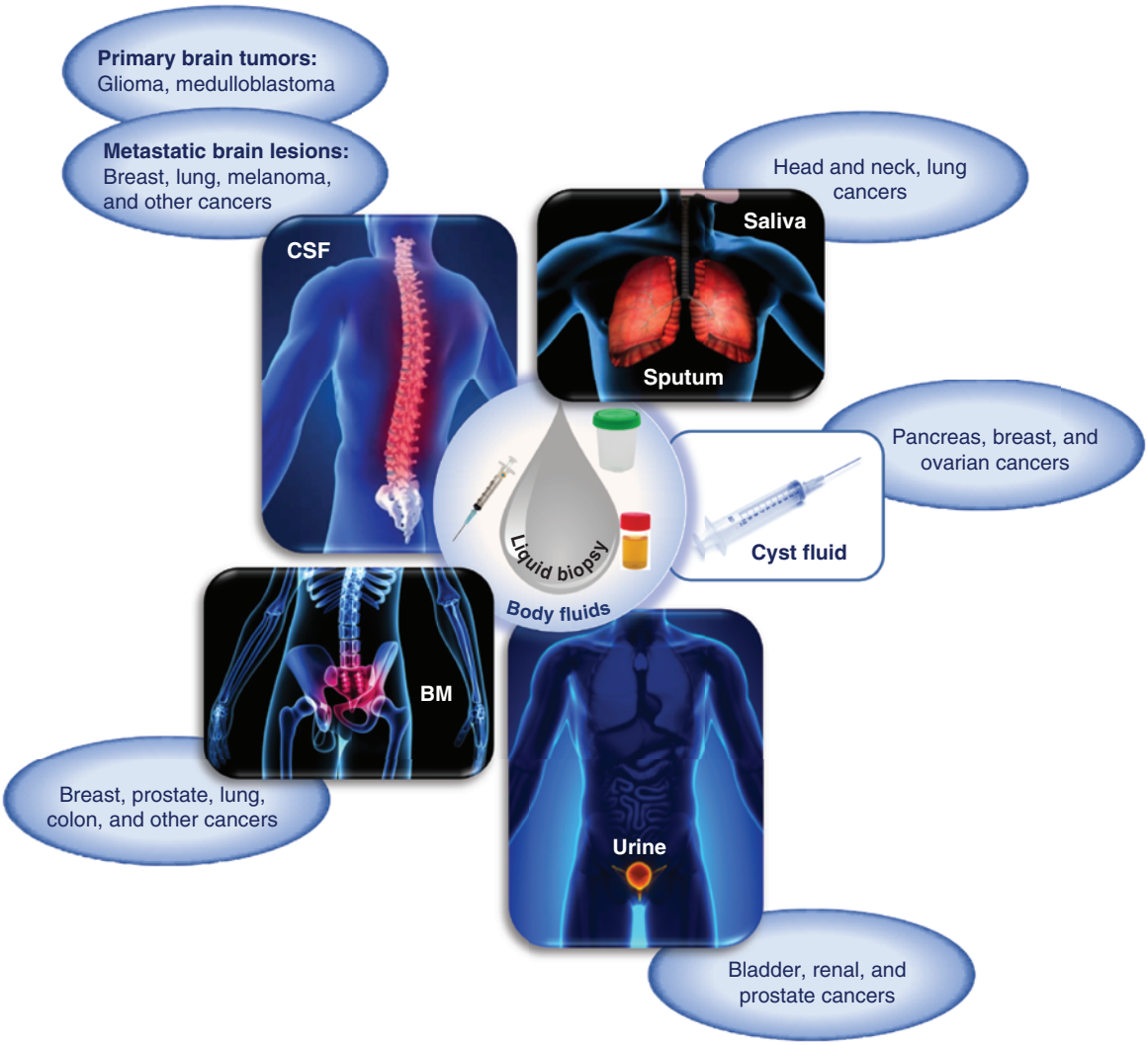

Figure 2. Liquid biopsy in body fluids (except blood) of patients with cancer. Representation of different body fluids [CSF, bone marrow (BM), saliva, sputum, cyst fluid, and urine] that are relevant for liquid biopsy and can be analyzed for CTCs, ctDNA, and other cancer biomarkers.

the future, a subsequent molecular characterization of the encapsulated CTCs will be incorporated into this innovative fluidic technology.

ctDNA

Highly sensitive and specific methods have been developed to detect ctDNA, including BEAMing Safe-Sequencing System (BEAMing Safe-SeqS), Tagged-Amplicon deep Sequencing (TamSeq), Cancer Personalized Profiling by deep Sequencing (CAPP-Seq), and digital PCR (42) to detect single-nucleotide mutations in ctDNA or whole-genome sequencing to establish copy-number changes (Fig. 3). In addition to mutation analysis, reliable tests for the assessments of epigenetic changes such as DNA methylation have been developed over the past years (43–45). For example, Luo and colleagues analyzed methylation patterns on ctDNA from multiple large cohorts of patients, including a prospective screening cohort of people at high risk of colorectal cancer (44). A methylation-based diagnostic score was identified and validated to help distinguish patients with colorectal cancer from healthy controls, as well as a prognostic score that correlated with patients' survival (44).

In principle, technologies can be divided into targeted approaches aimed to detect mutations in a set of predefined genes [e.g., mutations in the *EGFR* gene are relevant for response of patients with non–small cell lung cancer (NSCLC) to blockade by tyrosine kinase inhibitors (TKI)] or untargeted approaches (e.g., array comparative genomic hybridization, whole-genome sequencing, or exome sequencing) aimed to screen the entire genome (42). The strengths and limitations of these technologies have been recently discussed (46). Usually, targeted approaches have a higher analytic sensitivity than untargeted approaches, but strong efforts are ongoing to improve the detection limits of untargeted approaches (47, 48). Ultrasensitive technologies are now able to detect smallest amounts of ctDNA in the "sea" of normal circulating free DNA (cfDNA; ref. 49), which is a prerequisite for early detection of cancer or minimal residual disease (MRD).

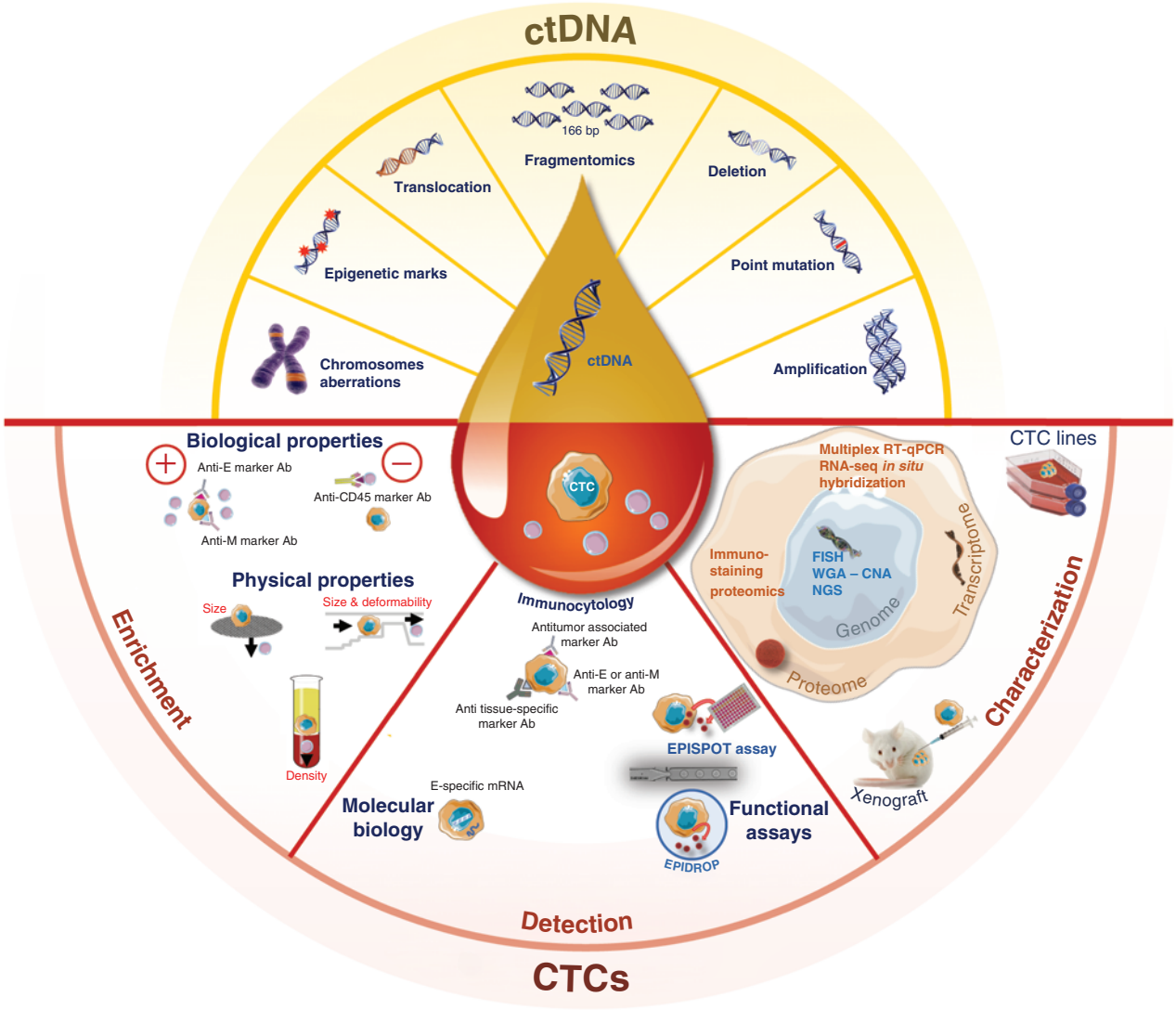

Figure 3. Technologies for CTC and ctDNA enrichment, detection, and characterization. CTCs and ctDNA can be isolated simultaneously from the same blood sample. ctDNA in the plasma: quantitative detection of ctDNA is based on the identification of various tumor-specific genetic aberrations or epigenetic marks in plasma cell-free DNA samples, primarily through DNA sequencing. cfDNA fragmentomics encompasses the study of the structural characteristics and sequence composition of plasma cfDNA (152). It includes: (i) their fragment sizes or length (number of bases), (ii) their fragment endpoints, (iii) the sequence context within the fragment (specific motif), (iv) the nucleosome footprints or position, and (v) their topologic forms (circular vs. linear). There is also emerging evidence that these cfDNA features may reveal important information of both biological and medical relevance, so that the evolving field of "fragmentomics" will likely become an integral part of cfDNA applications. CTCs in the cellular fraction: Enrichment—To discriminate CTCs from the surrounded normal immune cells in blood, they can be enriched using the (i) biological properties: CTCs can be positively selected *in vitro* or *in vivo* using antibodies to epithelial and/or mesenchymal proteins (such as EPCAM and/or cytokeratins and mesenchymal vimentin or N-cadherin) or negatively selected for through depletion of leukocytes using anti-CD45 antibodies. (ii) Physical properties: positive enrichment of CTCs can also be performed *in vitro* using assays based on CTC characteristics including size, deformability, density, and electrical charge. Detection—Following enrichment, the isolated CTCs can be identified using immunocytologic, molecular, or functional assays. (i) Immunocytology: With immunocytological platforms, CTCs are identified by membrane and/or intracytoplasmic staining with antibodies to epithelial, mesenchymal, tissue-specific, or tumor-associated markers. (ii) Molecular biology: Molecular technologies enable the identification of CTCs using RNA-based assays, such as RT-qPCR, RNA-seq, or *in situ* RNA hybridization. (iii) Functional assays: They allow the detection of viable CTCs on the basis of their biological activities; for example, the fluoro-EPISPOT assay for proteins secreted or shed by CTCs and the related EPIDROP technology that enables the detection of single functional CTCs in microdroplets. Characterization—The molecular characteristics of CTCs can be further explored at the DNA, RNA, and protein level, and the functional properties of CTCs can be investigated *in vivo* by injecting the cells into immunodeficient mice to form patient-derived xenograft models. Finally, metastasis-competent CTCs can be isolated by *in vitro* culture, allowing the establishment of CTC lines (primary or stable long-term CTC lines). CNA, copy-number alteration; DEP, dielectrophoresis; NGS, next-generation sequencing; WGA, whole-genome analysis.

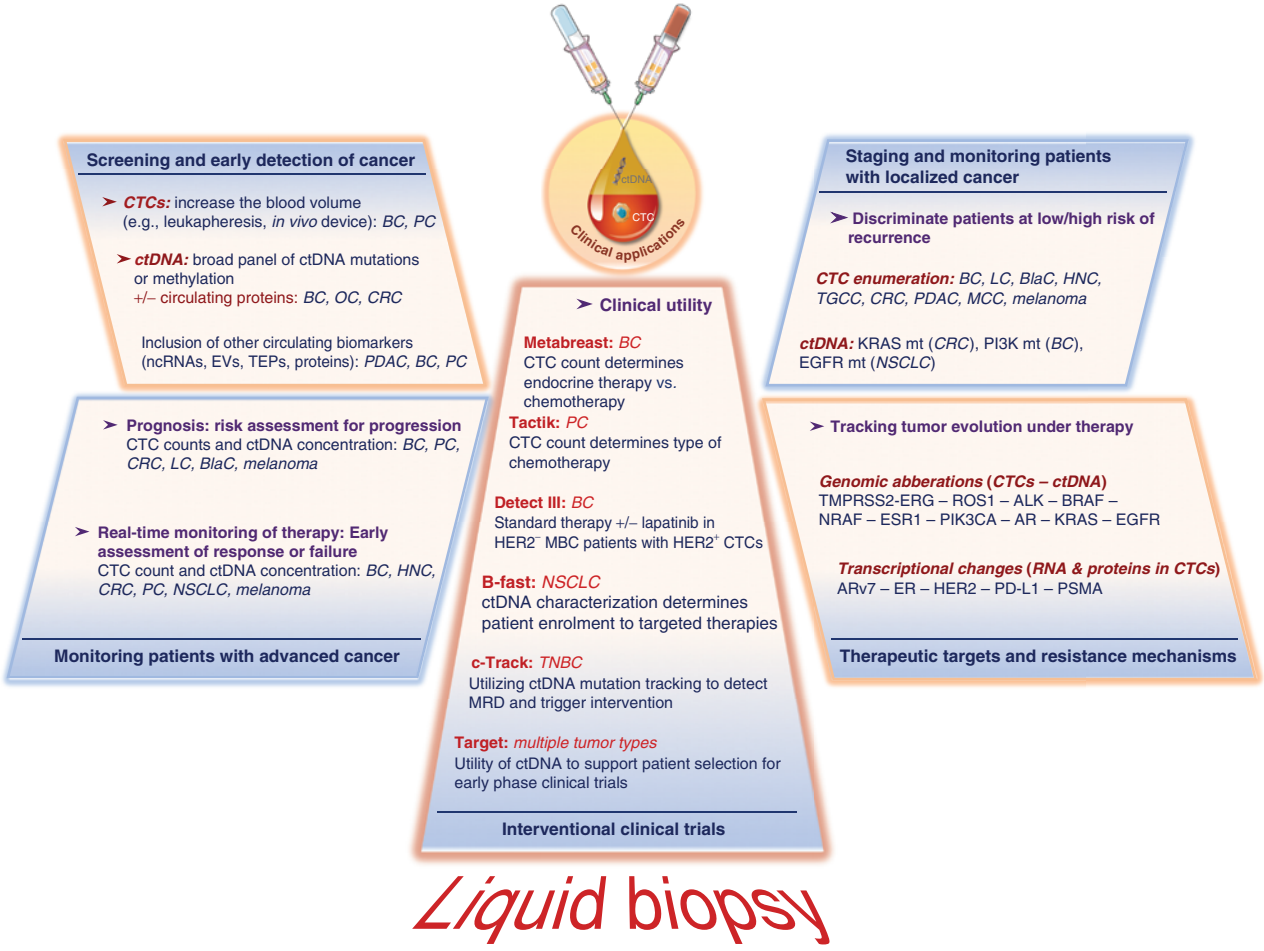

Figure 4. Clinical applications of CTCs and ctDNA as liquid biopsy for precision medicine. Noninvasive blood samples can be performed repeatedly for (i) early detection of cancer (a combination of different circulating biomarkers can increase accuracy), (ii) staging and monitoring patients with localized cancer, (iii) predicting relapse in M_0 patients or metastatic progression in patients with advanced cancer, (iv) monitoring the efficacy of therapies and discriminating early responders from nonresponders, and (v) tracking tumor evolution and identifying resistance mechanisms. Importantly, interventional clinical trials are crucial to demonstrate the clinical utility of CTCs and ctDNA. The interventional studies mentioned in this figure are examples and represent no comprehensive list of all ongoing trials. We apologize to the principal investigators of all the other excellent studies not mentioned in this figure. AR, androgen receptor; ARv7, androgen receptor-variant 7; BC, breast cancer; BlaC, bladder cancer; CRC, colorectal cancer; ER, estrogen receptor; EV, extracellular vesicle; HNC, head and neck cancer; LC, lung cancer; MCC, Merkel cell carcinoma; mt, mutation; ncRNA, noncoding RNA; OV, ovarian cancer; PC, prostate cancer; PDAC, pancreatic ductal carcinoma; PSMA, prostate-specific membrane antigen; TNBC, triple-negative breast cancer; TEP, tumor-educated platelet; TGCC, testicular germ cell cancer.

IMPORTANT AREAS OF CLINICAL APPLICATIONS OF CTCs AND ctDNA

To stimulate future investigations in the field of CTC and ctDNA research, we will discuss some of the most important questions for clinical applications. This section is based on our current knowledge and our opinion; thus, it is aimed to induce discussion among researchers rather than to represent a dogmatic view (Fig. 4).

Early Detection of Cancer

ctDNA

Early detection of cancer is one of the prime applications for ctDNA blood tests, which have gained enormous attention from academic groups, commercial vendors, and the media in recent years. The vision to detect cancer before any clinical symptoms or hints from imaging procedures used for cancer screening (e.g., mammography for breast cancer or low-dose CT for lung cancer) has led to the development of ultrasensitive assays that target mutations, copy-number aberrations, or methylation on ctDNA present at very low amounts (usually less than 0.1%). The field started with high hopes to fulfill this task in a few years because tumor-specific DNA was the ideal target for early detection, which should be more specific than the usual protein tumor markers like carcinoembryonic antigen which had failed as single biomarkers for early detection of small tumor lesions. However, despite the enormous advances in ctDNA technologies (reviewed elsewhere), it became apparent in recent years that the biology of cancer development seems to be the strongest limitation. (i) The challenge for sensitivity of ctDNA analysis is the fact that solid tumors are characterized by a plethora of genomic aberrations. Because the genetic makeup of the "occult" tumor is unknown, the ctDNA assay must encompass at least the most

frequent genomic aberrations in possible cancer genes. Even if the test is restricted to a particular application [e.g., finding lung cancer in patients with chronic obstructive pulmonary disease (COPD)], this panel is rather broad and moreover needs to be implemented into an assay that is very sensitive to detect minute amounts of ctDNA in the blood tube. To date, several technologies meet these requirements and are currently being tested for early cancer detection. In addition, research groups have added circulating proteins and used machine learning algorithms to establish a composite biomarker assay (50, 51). Such multianalyte tests also include the development of reliable ctDNA methylation assays (43) and now allow multicancer early detection (45). (ii) For cancer screening, specificity is the second key parameter. An assay with a 99% specificity being applied to 10 million individuals will lead to 100,000 false findings and considerable anxiety and cost (52). Technical advances have made ctDNA assays much more specific, but recent reports demonstrate that aging individuals show a background of mutations including cancer driver genes (53, 54). Leukocytes are a major source of this background, and those "clonal hematopoiesis of indeterminate potential" (CHIP) mutations need to be measured, and solutions for the elimination of these mutations from the total pool of ctDNA aberrations found in the individual patient are available (55). However, in addition to CHIP mutations, other sources of "background" mutations might exist, which have been recently revealed by sophisticated single-cell sequencing (53).

Researchers at GRAIL (and other sites) have therefore switched to the assessment of methylation markers on ctDNA for early detection of multiple cancers (43). Targeted methylation analysis of >50 cancer types revealed moderate sensitivities of less than 55% at a specificity of >99% with high-accuracy information on the tissue of origin (43).

Independent from the DNA target, it will be important to integrate these tests into the established imaging workflow for cancer diagnosis in large-scale cohort studies (51). For colon cancer, the Epi proColon is a blood test used for the detection of the methylated septin 9 gene (56). The test is approved by the FDA for colorectal cancer screening in people at average risk who have declined first-line screening tests.

ctDNA testing in high-risk individuals appears to be a good strategy. The LIBERATE trial under the auspices of the Princess Margaret Cancer Centre (NCT03702309) is creating a resource, including healthy carriers ("previvors") of a germline pathogenic variant in hereditary cancer predisposition genes, such as *BRCA1/2*, *NF1*, or *TP53*, and mismatch repair genes.

Interestingly, ctDNA analysis also offers transcriptional information; inference of transcription factor binding from cell-free DNA enabled tumor subtype prediction and early detection of prostate and colorectal cancer (57). Moreover, tumor cells of different origin may harbor specific methylation profiles, which may also enable the analysis of ctDNA to reveal position information (58).

CTCs

Data obtained in mouse models (59) and patients with cancer (60, 61) indicate that blood-borne dissemination of cancer cells occurs early during tumor development, which provided the rationale to explore CTCs as marker for early detection of cancer. Despite encouraging results initially published more than 10 years ago (62), the high incidences of CTCs in the peripheral blood of patients with solid tumors such as breast, lung, or prostate cancer have never been reproduced. Usually, the incidence of CTCs in patients with early-stage cancer ranges between only 10% and 30%, depending on the assay applied and the tumor type tested. Higher values have occasionally been reported, but doubts remain about the specificity of the CTC markers employed. For example, cells that lack the standard epithelial keratins and the common leukocyte marker CD45, used as positive and negative markers for CTCs worldwide, were classified as "CTCs," but it is well known that circulating endothelial cells (and probably other normal cells in blood) can have the same phenotype. Moreover, the selection of the right antibodies is crucial. Some keratin antibodies do not detect all forms of keratin proteins present in carcinoma cells, which may lead to false-negative findings. Moreover, some CD45 antibodies do not detect all isoforms of CD45, which can lead to false-positive findings. Morphologic identification of CTCs has been employed by Hofman and colleagues after enrichment of CTCs by size filtration (63). The initial results of a pilot study in patients with COPD were encouraging and generated great media attention, but the results of the larger validation study could not confirm that this approach is suitable for early detection of lung cancer (64). Taken together, the sensitivity of CTCs for early cancer diagnosis is rather low, and there is also some evidence that EPCAM-positive cells in the peripheral blood are released in noncancer patients with large bowel diseases, which might limit assay specificity (65).

Why is it so difficult to detect sufficient amounts of CTCs in the peripheral blood of patients with early-stage cancer despite the common view that tumors permanently shed high amounts of malignant cells into the circulation? This discrepancy might be explained by the high gradient of tumor cell counts in the tumor-draining vessels and the peripheral veins, as demonstrated for colorectal and lung cancers (66–68). Thus, tumor cells might be rapidly absorbed by the first organs they encounter, such as the liver in colon cancer. In later tumor stages, (micro)metastases present in regional lymph nodes or diverse distant organs contribute to the pool of CTCs in peripheral blood, which considerably increases CTC counts, as observed for all tumor types explored in CTC research. Recent strategies to increase the blood volume for CTC analysis, including the use of *in vivo* capture devices (36, 37), diagnostic leukapheresis (69), or transdermal devices (70), may overcome this limitation. However, increasing the sensitivity of CTC analyses may also lead to false-positive results in healthy controls (71) or benign inflammatory diseases (65). These challenges must be overcome to use CTC assays for early diagnosis of cancer.

Do liquid biopsy analyses also enable the localization of an occult primary or micrometastatic tumor lesion? In our opinion, this important information would probably reduce the extent and costs of the subsequent clinical workup following a positive blood test. A liquid biopsy is not a diagnostic test per se, and in the absence of an extremely specific tissue-of-origin marker, the number of erroneous follow-ups will be significant. In this context, CTCs offer the possibility to analyze transcriptional information (RNA and proteins), which might be helpful because tumor cells may express a specific

transcriptional profile depending on their organ of origin (e.g., prostate cancer cells express PSA or PSMA, and breast cancer cells express mammaglobin). However, the expression profiles can also be overlapping (e.g., keratin 19 is a good marker for breast cancer–derived CTCs and also expressed by other types of carcinoma). In addition, tumor cells show lineage plasticity and change their original differentiation markers (72). Moreover, transcriptional profiles can change when tumor cells leave hypoxic areas and jump into the well-oxygenated blood pool (73).

Staging of Early-Stage Cancer

After diagnosis of cancer, subsequent tumor staging leads to an individual risk assessment that helps the clinician to develop a personalized treatment strategy. Over the past decades, tumor staging classifications focused on the extent and differentiation grade of the primary tumor as well as the assessment of the regional lymph nodes and distant organs for the presence of metastases. CTC and ctDNA measurements now provide an opportunity to refine the current staging systems, which may help to better discriminate patients at low or high risk.

In general, the assessment of ctDNA at the time of primary diagnosis has shown less prognostic value than sequential postsurgical follow-up ctDNA analysis used for detection of early molecular relapse (see below). In contrast, CTC counts determined before neoadjuvant therapy or at the time of surgical removal of the primary tumor are closely correlated to prognosis. This difference is consistent with the view that CTC counts (in contrast to ctDNA) indicate the capacity of an individual tumor to spread via the blood circulation to distant organs. In breast cancer, clinical studies on thousands of patients have documented that CTC enumeration at initial diagnosis before (neo)adjuvant therapies will lead to a better definition of a population at higher risk of recurrence (74–77). The prognostic influence was independent from established risk factors. In patients with breast cancer undergoing neoadjuvant therapy, the prognostic influence of CTCs was also independent from the complete pathologic response of the primary tumor, indicating that the response of disseminating tumor cells to systemic chemotherapy may not always mirror the response of the primary tumor (76), which is in line with many experimental studies demonstrating a differential response of primary and disseminated tumor cells to systemic therapies. Encouraging results were also achieved for other tumor entities, including lung cancer (7, 8), bladder cancer (78, 79), head and neck cancer (40, 80, 81), testicular germ cell tumors (82), colorectal cancer (including stage II; refs. 10, 83), pancreatic cancer (84–86), Merkel cell cancer (87, 88), and melanoma (41, 89). In early-stage prostate cancer, CTC counts are very low and may require the combination of different assays to reveal significant information (37). Moreover, the clinical studies require a very long follow-up, and the value of CTCs for staging is still under investigation (90).

Early Detection of Relapse during Follow-up Examinations

Following initial staging and (neo)adjuvant therapy, CTC and ctDNA analyses can contribute to individual real-time monitoring of patients with cancer. Besides PSA monitoring in prostate cancer, there is no blood test that is specific and sensitive enough to detect early relapse before any clinical symptoms or imaging results signal the presence of overt metastases that are in most cases incurable. Recent reports on CTC and ctDNA monitoring have provided encouraging results, suggesting that early detection of relapse is feasible. In breast cancer, the detection of CTCs 2 or 5 years after initial adjuvant therapy was associated with a strong and independent risk to develop relapse (91, 92). Similar results were obtained with ctDNA as marker on smaller cohorts; here the rise in ctDNA was a good prognostic indicator, whereas the initial ctDNA value before therapy was less relevant (93). Encouraging results were also obtained in colorectal (94) and lung (95) cancers as two other major cancer types. The quantitative assessment of the dynamic changes in CTC counts and ctDNA amounts over time by sequential blood tests can also help to discriminate between indolent and aggressive MRD. We refer to "indolent MRD" when the disseminated tumor cells are not proliferating and/or proliferation is balanced by a similar rate of apoptosis, resulting in no disease progression toward overt metastasis. The interval of the blood tests depends on the tumor type and the expected speed of relapse. Depending on the tumor type and stage, molecular relapse can be detected on average 2 to 9 months before imaging shows the conventional relapse (96, 97). At present, it is unclear whether this is early enough to still achieve a cure or not. In general, attacking a lower tumor burden should have a higher chance of a cure than waiting longer, in particular if one assumes an exponential growth curve of metastatic tumor cells. The remarkable results in breast cancer have resulted in the inclusion of CTCs in the 2018 American Joint Committee on Cancer's breast cancer staging manual as a new classification, cM0(i+).

Besides quantitative information, further molecular characterization of CTCs may also help to determine the switch from indolent to aggressive MRD. CTC lines (25–27, 98) and xenografts (28, 30, 99) established from CTCs may also serve as valuable models to identify potential properties of metastasis-initiating cells.

In the next step, new types of interventional clinical studies on so-called "postadjuvant therapies" (100) need to be designed to test whether an earlier intervention based on a positive CTC/ctDNA result will lead to an improved disease-free survival or overall survival of patients with cancer. In this context, the choice of the right treatment will be also crucial: Should we use the drugs from the (neo)adjuvant therapy again, should we use the drugs usually applied in the metastatic stage in patients with advanced cancer, or should we test new drugs and combinations for this specific intermediate stage of "molecular relapse"? Molecular characterization of ctDNA and CTCs can help to shed light into the dark. ctDNA can be analyzed for druggable mutations, and CTCs can provide additional information on transcriptional plasticity, thus providing complementary information, as outlined in more detail in the next section.

Detection and Monitoring of CTCs or ctDNA in Patients with Advanced Cancer

In patients with advanced breast or prostate cancer, the sequential enumeration of CTCs in the context of systemic

therapies provides early and reliable prognostic information (101–103). In particular, the elimination of CTCs (104) or ctDNA during therapy (105) might become in the near future a well-accepted surrogate endpoint for clinical studies testing new drug.

Despite these encouraging developments, there is an urgent need for more studies focusing on the clinical utility (i.e., their capacity to help decide to adopt or to reject a therapeutic action) of ctDNA and CTC assays. Thus, interventional studies are required to demonstrate which treatment changes need to be made according to the enumeration and/or characterization of ctDNA and CTCs.

CTCs

Examples of studies based on CTC enumeration are the SWOG SO500 study (https://clinicaltrials.gov/ct2/show/NCT00382018?term=SWOG+S0500+clinical+trial&draw=2&rank=1) and METABREAST study (https://clinicaltrials.gov/ct2/show/NCT01710605?term=NCT01710605&draw=2&rank=1). The SWOG SO500 study is a randomized phase III trial. Its objective was to determine whether women with metastatic breast cancer (MBC) and elevated CTCs (≥5 per 7.5 mL of whole blood) after 3 weeks of first-line chemotherapy derive increased overall survival from changing to an alternative chemotherapy regimen (standard practice once there is clinical evidence of progressive disease) at the next course rather than waiting for clinical evidence of progressive disease before changing to an alternative chemotherapy regimen. One potential problem, however, might be that elevated CTC counts are indicators of worse prognosis, but the applied therapy may not change the course of the disease, as indicated by the results of the SWOG SO500 study (106). The reverse approach is to identify patients who may not need an aggressive treatment based on their low CTC count. In the METABREAST study (NCT01710605), patients with first-line MBC were randomized between the clinician's choice and CTC count–driven choice. In the CTC arm, patients with ≥5 CTC/7.5 mL received chemotherapy, whereas patients with <5 CTC/7.5 mL received endocrine therapy as first-line treatment (107). Among the 778 women randomized, the median age was 64 (range, 30–88) years for the 391 patients allocated to the CTC arm and 63 (range, 31–87) years for the 387 allocated to the standard arm; 138 (37%) and 103 (27%) received chemotherapy, respectively. Median progression-free survival (PFS) was 15.5 months [95% confidence interval (CI), 12.7–17.3] in the CTC arm and 13.9 months (95% CI, 12.2–16.3) in the standard arm. The primary endpoint was met, with a hazard ratio of 0.94 (90% CI, 0.81–1.09). For the first time in this field, clinical utility of CTCs has been proved. Indeed, the CTC count is a reliable biomarker method for choosing between chemotherapy and endocrine therapy as the first-line treatment in hormone receptor–positive (HR^+) $HER2^-$ MBC (107).

ctDNA

The design of ctDNA-based interventional clinical trials in oncology has been recently reviewed (108). Studies on smaller patient cohorts also indicated that ctDNA analysis might facilitate personalization of therapy. In particular, in the context of targeted therapy or immune checkpoint inhibition (ICI), detection and monitoring of ctDNA can provide helpful information relevant to treatment decisions. For example, deep sequencing (CAPP-Seq) ctDNA analysis was applied to 218 samples from 65 patients receiving chemoradiation therapy for locally advanced NSCLC, including 28 patients receiving consolidation ICI (49). Patients with undetectable ctDNA after chemoradiation therapy had excellent outcomes whether or not they received consolidation ICI. In contrast, patients with MRD after chemoradiation therapy who received consolidation ICI had significantly better outcomes than patients who did not receive consolidation ICI. In another study, blood from patients with metastatic melanomas undergoing ICI was monitored for ctDNA, and those patients who remained ctDNA-negative or became ctDNA-negative under ICI therapy had a favorable outcome as compared with those patients who were positive at baseline and remained positive during treatment (105). Next-generation sequencing (NGS) data can also be used to calculate blood-based tumor mutational burden as a potential predictor of immunotherapy response in NSCLC (109). Clinical trials exploring ctDNA-based high-throughput NGS genotyping, such as the ongoing B-FAST trial in NSCLC (NCT03178552), exemplify these concepts, and patients are enrolled to four different molecularly defined cohorts on the basis of their ctDNA result. Early changes in ctDNA dynamics upon treatment can provide information about therapeutic efficacy. In the phase III PALOMA-3 trial in advanced ER-positive breast cancer, a decline in *PIK3CA* ctDNA levels compared with baseline after 15 days of treatment with palbociclib and fulvestrant was predictive of PFS (110).

Identification of Therapy Targets and Resistance Mechanisms in Patients with Advanced Cancer

Current therapy decision-making is based on the analysis of the primary tumor resected shortly after initial diagnosis. However, it is well known that natural and therapy-induced evolution of disseminated tumor cells requires restaging of metastatic lesions. Although this has become increasingly acceptable, biopsies are invasive procedures, and some locations such as bones or brain are difficult to access. Even if one metastatic lesion can be biopsied, substantial genetic heterogeneity of metastatic lesions located at different sites in the same patient has been demonstrated (111). Finally, tracking of tumor evolution over time would require repeated biopsies in individual patients, which is doable but difficult to achieve in clinical practice. Taken together, these arguments have greatly stimulated the use of CTCs and ctDNA for the identification of therapy targets and resistance mechanisms in patients with advanced cancer over the past 10 years. In view of the enormous amount of published reports on this topic, we will focus on some key targets relevant for the response to cancer therapy (Fig. 5). In principle, diagnostic targets of liquid biopsy analysis are (i) genomic aberrations (e.g., mutations) that have been detected on ctDNA and to a lesser degree on CTCs and (ii) mRNA transcripts and proteins that are detectable only on CTCs. Real-time monitoring of these aberrations provides interesting insights into the development of resistance in patients with cancer.

Lung Cancer

NSCLC is the model tumor for the relevance of genomic aberrations (e.g., *EGFR*, *ROS1*, *ALK*, *BRAF*) relevant to cancer therapy. Work on *EGFR* mutations that affect response to

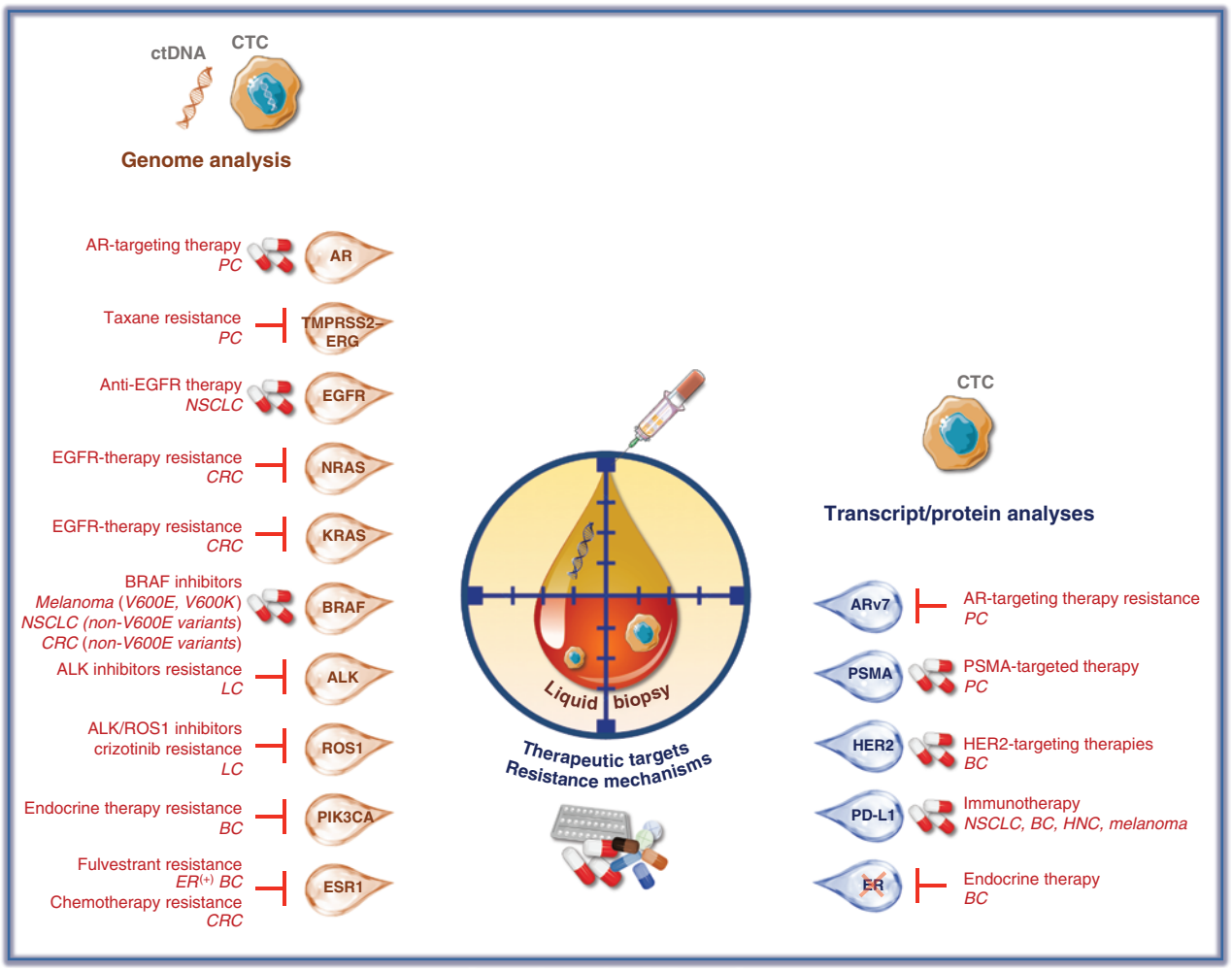

Figure 5. Therapeutic targets and resistance mechanisms. Genomic alterations in CTCs and/or ctDNA and transcriptional changes in CTCs can reveal therapeutic vulnerabilities, and this information can be integrated into the real-time monitoring of tumor evolution influenced by anticancer therapy. This figure summarizes (i) the therapeutic targets detected on CTCs and/or ctDNA to guide the clinician to give the right targeted therapies and (ii) the resistance mechanisms on the CTCs and/or ctDNA to predict the resistance and subsequently the failure to a treatment. This figure shows examples of genes in which aberrations of therapeutic relevance have been defined, predominantly in NSCLC (EGFR, ROS1, and ALK, refs. 112, 113), colorectal cancer (NRAS, refs. 89, 115, 116), breast cancer (PIK3CA, ref. 110; and ESR1, ref. 120), prostate cancer (AR and TMPRESS2–ERG fusions, refs. 132, 133), melanoma (BRAF, ref. 97), and aberrant transcription of proteins with predominant therapeutic relevance in breast cancer (ER, ref. 120; and HER2, ref. 124), prostate cancer (AR, particularly ARv7, ref. 131; and PSMA, ref. 137), and various tumor types (PD-L1; ref. 114). Although increasing levels of ctDNA revealed by sequential analyses indicate the evolution of viable tumor cells with resistance to therapy (e.g., ESR1 mutations during endocrine therapy in breast cancer; ref. 120), CTC analysis enables the detection of changes in gene transcription and protein expression that can be relevant to anticancer therapy, which is not possible with ctDNA. Thus, the drug target determines the choice of the most appropriate form of liquid biopsy analysis, and ctDNA and CTCs will provide complementary information.

anti-EGFR therapy (TKIs, antibodies) has therefore paved the road for ctDNA-based liquid biopsy, including the first FDA-cleared ctDNA assay (Cobas, Roche; ref. 112). Low-frequency mutations (e.g., in *ROS1*) or translocations (e.g., ALK) have been detected on ctDNA and CTCs (ref. 113), which allows an effective personalized treatment of an otherwise deadly disease.

Recently, ICI has become the primary option for patients with NSCLC and has now even been explored as first-line adjuvant therapy. Promising results on the use of ctDNA (e.g., assessment of tumor mutational burden) and CTCs (e.g., assessment of PD-L1 expression) have been published and reviewed in detail elsewhere (114). Recently, Moding and colleagues showed that ctDNA dynamics predict benefit from consolidation immunotherapy in locally advanced NSCLC (49).

Colorectal Cancer

The EFGR signaling pathway also plays a role in colorectal cancer, but here mutations in the EGFR gene itself are less important for response to EGFR-targeting therapies, whereas gene mutations in the gene encoding the downstream signaling molecule KRAS are known mediators of resistance. *KRAS* mutations have been detected on CTCs (115) and then became prime targets for ctDNA analyses in colorectal cancer (116). Sequential assessment of ctDNA in patients with advanced colorectal cancer receiving anti-EGFR

therapy revealed the development of resistance, which could be reversed by drug breaks (117). Recently, the genomic evolution of individual metastases during HER2 blockade was revealed by ctDNA analysis (118). Thus, real-time monitoring of ctDNA (and CTCs) can provide insights into tumor evolution with potential implications for cancer therapy. For more information, we refer you to the recent whitepaper of the NCI colon and rectal–anal task forces that outlined the current ctDNA applications and integration in colorectal cancer (119).

Breast Cancer

The ER signaling pathway is the main driver of tumor growth in 70% to 80% of breast carcinomas classified as ER$^+$. Recent work has identified mutations in the *ESR1* gene that confer resistance to endocrine therapies and can be detected on ctDNA (120). For patients who become resistant to endocrine therapy, CDK4/6 inhibitors have been recently approved for therapy, and mutations in the *PI3K* gene are known to confer resistance to this new form of therapy. These mutations have been detected first in CTCs and recently also on ctDNA with remarkable results toward the development of a predictive biomarker. As demonstrated in a retrospective analysis of samples from the phase III PALOMA-3 trial in advanced ER-positive breast cancer, a decline in *PIK3CA* ctDNA levels compared with baseline after 15 days of treatment with palbociclib and fulvestrant was predictive of PFS (110).

ER protein expression has been determined routinely for decades by immunohistochemistry. Interestingly, only 1% of primary tumor cells have to express ER to be classified as ER$^+$. Thus, it is conceivable that ER$^+$ tumors shed ER$^-$ CTCs which are the source of ER$^-$ metastases that may arise after years of ER-targeting therapies (121). Indeed, ER$^-$ CTCs were found in patients with breast cancer with ER$^+$ primary carcinomas (121). Ongoing follow-up studies will show whether these CTCs can escape ER-targeting therapies and cause relapse during or following endocrine therapy in patients with breast cancer. Endocrine therapy fails to induce a response in half of patients with HR$^+$ MBC, and almost all will eventually become refractory to endocrine therapy. To predict resistance to endocrine therapy in patients with HR$^+$ MBC, Paoletti and colleagues developed a multiparameter CTC-Endocrine Therapy Index (CTC-ETI) using the CELLSEARCH system (122). The CTC-ETI combines enumeration and CTC expression of four markers: ER, BCL2, HER2, and Ki-67. The clinical relevance of CTC-ETI is being evaluated in an ongoing clinical trial.

The *HER2* oncogene is amplified in approximately 20% of primary breast cancers, leading to overexpression of the HER2 protein on the cell surface. To date, HER2 positivity determines an own breast cancer subtype, and the HER2 protein has become a key target for therapies with antibodies and TKIs (123). There is increasing evidence that overt distant metastases and CTCs derived from these metastases have discrepant HER2 status compared with the primary tumor in up to 30% of cases (123). At present, it is not known how this discrepancy arises. HER2 expression might be truly different in the primary tumor versus the metastases (i.e., there is some kind of differential selection), or rather simply tumor heterogeneity leads to an incorrect classification because small subclones are missed. Recently, Jordan and colleagues also demonstrated transcriptional plasticity leading to HER2 expression on CTCs in patients with ER$^+$ HER2$^-$ primary breast cancers (124). Although HER2$^+$ and HER2$^-$ CTCs had comparable tumor-initiating potential, differential proliferation favors the HER2$^+$ state, whereas oxidative stress or cytotoxic chemotherapy enhances transition to the HER2$^-$ phenotype (124). Clinical trials are now under way to investigate if patients with HER2$^-$ primary tumors but HER2$^+$ CTCs will benefit from HER2-targeting therapies such as lapatinib (DETECT-III study, www.detect-studien.de and https://clinicaltrials.gov/ct2/show/NCT01619111?term=CTC+TREAT&draw=2&rank=2) or trastuzumab (CTC-TREAT study, https://clinicaltrials.gov/ct2/show/NCT01548677?term=CTC+TREAT&draw=2&rank=1).

Melanomas

TKIs targeting BRAF and MEK have shown some remarkable short-term efficacy in patients with melanoma, and the analysis of susceptibility mutations (e.g., BRAF^{V600} for BRAF inhibition) is now clinical routine. Various groups have studied these druggable mutations on ctDNA in patients with advanced melanoma with the aim of understanding the development of treatment resistance (89, 97). For example, *BRAF*^{V600E}-mutant cfDNA was detected before radiologic resistance with mean lead time reduction of 110 days (97).

Melanoma (besides NSCLC) is also the tumor type with the most impressive responses to ICI—a therapeutic concept that has revolutionized cancer therapy. Therapy with antibodies against PD-L1 and PD-1 has been rapidly implemented due to the durable long-term responses in a subset of patients with metastatic disease (125). Nevertheless, it is of utmost importance to identify up front or by serial blood monitoring those patients, and spare the remaining patients from the side effects of immunotherapy. Recently, ctDNA variation has been introduced as a tool to predict tumor response to immunotherapy in patients with metastatic melanoma receiving treatment with PD-1 inhibitors (105, 126). RECIST response was 72% (26/36) in group A (undetectable ctDNA at baseline), 77% (17/22) in group B (elevated ctDNA at baseline but undetectable within 12 weeks of therapy), and 6% (1/18) in group C (elevated ctDNA at baseline and remained elevated during treatment). Thus, patients who had persistently elevated ctDNA on therapy had a poor prognosis, and this may guide combination and sequencing of subsequent therapies. Longitudinal ctDNA profile may also contribute to the important early differentiation of pseudoprogression from true progression during immunotherapy (127). In addition, real-time monitoring of tumor evolution under immunotherapy can also be realized by CTC analysis (128), underlining once more the complementarity of both ctDNA and CTC analyses.

Prostate Cancer

The androgen receptor (AR) is the key target in prostate cancer. Most if not all tumors are hormone-sensitive at the beginning, and growth can be suppressed by hormonal therapy (90). However, this therapy will eventually lead to the development of castration-resistant prostate cancer (CRPC), which can still be treated by new generations of AR-targeting drugs such as abiraterone or enzalutamide. Recent work by Antonarakis and colleagues has shown that cells expressing

the splice variant of ARv7 lacking the ligand-binding domain can become resistant to these drugs, and ARv7 detectable on CTCs predicted clinical outcome (129, 130). Immunocytochemical detection of nuclear ARv7 expression on CTCs has been developed into a new predictive biomarker (131). Nevertheless, a marked intrapatient heterogeneity in ARv7 expression between individual CTCs has been reported by single-cell analysis (38), which may also affect clinical outcome. Besides transcriptional plasticity, amplifications of the *AR* gene locus could be detected in 30% to 38% of patients with CRPC (132, 133), and *AR* mutations were identified in CTC-enriched peripheral blood samples from patients with CRPC (134). *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 (135). Thus, both types of genomic aberrations support the growth of prostate cancer cells in patients with CRPC (134).

PSMA is a transmembrane protein that is overexpressed in most prostate cancers (136) and has gained increasing interest as a target for improved imaging (137, 138) and therapy (139). However, a high level of intrapatient heterogeneity in PSMA expression on CTCs was reported as well as discrepancies between PSMA protein expression in primary tumor tissue and corresponding CTCs (137). These results could provide an explanation for the failure of PSMA-targeted therapies to treat individual patients, as subpopulations of their metastatic tumor cells might lack PSMA expression. PSMA detection on CTCs could therefore be valuable in stratifying patients for anti-PSMA treatment. Recently, the results of a phase II trial of BIND-014 in combination with prednisone in chemotherapy-naïve metastatic CRPC were reported (139). BIND-014 is a docetaxel-containing nanoparticle targeting PSMA. The primary endpoint was radiographic PFS, but for a subset of patients, CTC counts and PSMA expression on CTCs were assessed. BIND-014 was found to be active and well tolerated, whereas serial monitoring of CTCs in blood showed that the predominant cells eliminated were those expressing PSMA (139).

CONCLUSIONS AND PERSPECTIVES

The emerging field of ctDNA and CTC research has opened new avenues for cancer diagnostics over the past 10 years with important clinical opportunities for personalized medicine in oncology.

Capture of circulating cell-free DNA (ccfDNA) from blood plasma seems to be easier than capture of CTCs, but sophisticated downstream analysis of ctDNA also requires expert skills; thus, the concordance between different technologies depends on the ccfDNA concentration (140). In early cancer stages, the rarity of the signal is due to biology, which can affect reproducibility not only between methods but also within the same method. For example, one aliquot has the ctDNA fragment, and the other aliquot of the same sample does not. Another issue for CTCs and ctDNA is the volume of blood needed (>5 mL) to accurately assess CTCs, particularly in patients with early-stage cancer. Although ctDNA has in principle a higher dynamic range than CTCs, the number of ctDNA molecules can also become a limiting factor in early cancer detection where the tumor burden is very low. The recent development of assays with high sensitivity and the ability to assess a broad spectrum of mutations on fragments of ctDNA may overcome this limitation.

Broad clinical usage will depend on standardization of both preanalytic and analytic procedures. This task requires large initiatives such as the BloodPac in the United States or the Cancer-ID in Europe (93, 141) that will be continued by the European Liquid Biopsy Society (www.elbs.eu; ref. 142). Standard operating procedures should be developed and broadly validated. Development of appropriate reference materials will also contribute to more standardized quality controls, quantification, and reporting among laboratories. Proficiency testing for standardization and insight into the performance of ctDNA assays is available through the College of American Pathology PT program (https://www.cap.org/laboratory-improvement/proficiency-testing). Applications for CTCs and ctDNA have greatly advanced in the last decade (Fig. 4). Both approaches provide overlapping information as well as unique information for specific cancers. Clinical trials are ongoing for a number of clinical applications.

Large clinical validation studies are now mandatory in order to demonstrate the clinical utility of liquid biopsy monitoring, which can be achieved by interventional trials where the biomarker result determines the treatment choice. Efforts in this direction have started using ctDNA and CTCs as liquid biomarkers. However, interventional trials link the "efficacies" of the biomarker and the chosen drug treatment in a predefined patient group, and both parts of this complex equation must work to result in a successful trial outcome. The hard lesson learned from the SWOG trial on CTCs in breast cancer is a good example (106) that a good prognostic biomarker might not be predictive if there is no effective therapy for the high-risk population who failed previous therapies. Thus, the careful design of interventional studies is crucial. The utility of ctDNA to support patient selection for early-phase clinical trials is currently being investigated (e.g., in the TARGET study; ref. 143). Additional trials that are planned or in process and will have a chance to provide data that could lead to approvals for clinical uses include the TRACERx studies in the UK, the Australian DYNAMIC studies (ACTRN12615000381583 and ACTRN12617001566325), the COBRA study (NCT-04068103) in the United States, and multiple trials in Europe (e.g., IMPROVE-IT EudraCT #2018-00070-30, MEDOCC-CrEATE, Netherlands Trial Register #NL6281/NTR6455).

ctDNA is currently the preferred biomarker for liquid biopsy analyses of druggable mutations relevant to cancer therapies. However, molecular characterization of CTCs can provide additional information. Genomic analysis of single CTCs can reveal intrapatient heterogeneity, which might contribute to treatment resistance. Furthermore, transcriptional plasticity can be an important driver for resistance to cancer therapy, and CTCs can be interrogated at the RNA and protein level. Transcriptional analysis of CTCs may also predict in the future which organ site is likely to be colonized; different organ microenvironments can select different types of tumor cells and induce different transcriptional activities as a consequence of the cross-talk between

the tumor cells and the surrounding organ cells. Single-cell CTC analysis might provide information on intrapatient heterogeneity (144), which has been shown to be an important mechanism of resistance to therapy in prostate cancer (145). Finally, cell lines or xenografts established from CTCs can be used as novel models for drug susceptibility testing, which opens a new avenue for functional analysis. Depending on the tumor types and clinical application, the combination of both CTCs and ctDNA together with other liquid biopsy markers (142), such as circulating proteins, extracellular vesicles, noncoding RNAs, and tumor-educated platelets, may augment the future diagnostic assessment of patients with cancer.

Finally, the biological processes controlling the dissemination of CTCs and DNA shedding from primary and metastatic tumors need to be further investigated, which will also help to optimize the use of these biomarkers in clinical studies. There is some indirect evidence that the release of CTCs from tumor tissue is not just a random process. Transcriptional profiling has revealed expression signatures related to blood-borne dissemination into the bone marrow (146). Experimental models revealed that tumor cells are primed for dissemination before their release into the blood circulation by special niches in the primary tumor (147, 148). The major mechanism of ctDNA release is during tumor cell apoptosis, but there is also an alternative mechanism through which ctDNA is encapsulated into exosomes (4), and this encapsulation might be regulated by specific mechanisms (149). Nucleic acids and proteins transported in exosomes from primary lesions to distant sites can be taken up by recipient cells and change tumor biology (150, 151). Moreover, the effect that different anticancer therapies might have on any of these processes has potential clinical implications and therefore needs to be explored in future studies.

Authors' Disclosures

C. Alix-Panabières reports receiving honoraria from Menarini. In addition, C. Alix-Panabières has a patent on a process to detect and/or characterize circulating tumor cells (International patent PCT No. PCT/EP2017/059209 - 18 April 2017). K. Pantel reports receiving personal fees from Menarini, Agena, and Illumina outside the submitted work; in addition, K. Pantel has a patent for Europäische Patentanmeldung (application No. 18705153.7; PCT/EP2018/054052; Method of Detecting Cancer or Cancer Cells) pending. No other disclosures were reported.

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REFERENCES

1. Pantel K, Alix-Panabieres C. Circulating tumour cells in cancer patients: challenges and perspectives. Trends Mol Med 2010;16:398–406.
2. Schwarzenbach H, Hoon DS, Pantel K. Cell-free nucleic acids as biomarkers in cancer patients. Nat Rev Cancer 2011;11:426–37.
3. Anfossi S, Babayan A, Pantel K, Calin GA. Clinical utility of circulating non-coding RNAs - an update. Nat Rev Clin Oncol 2018;15:541–63.
4. Kalluri R, LeBleu VS. The biology, function, and biomedical applications of exosomes. Science 2020;367:eaau6977.
5. Best MG, Sol N, In 't Veld S, Vancura A, Muller M, Niemeijer AN, et al. Swarm intelligence-enhanced detection of non-small-cell lung cancer using tumor-educated platelets. Cancer Cell 2017;32:238–52.
6. Scher HI, Jia X, de Bono JS, Fleisher M, Pienta KJ, Raghavan D, et al. Circulating tumour cells as prognostic markers in progressive, castration-resistant prostate cancer: a reanalysis of IMMC38 trial data. Lancet Oncol 2009;10:233–9.
7. Hou JM, Krebs MG, Lancashire L, Sloane R, Backen A, Swain RK, et al. Clinical significance and molecular characteristics of circulating tumor cells and circulating tumor microemboli in patients with small-cell lung cancer. J Clin Oncol 2012;30:525–32.
8. Krebs MG, Sloane R, Priest L, Lancashire L, Hou JM, Greystoke A, et al. Evaluation and prognostic significance of circulating tumor cells in patients with non-small-cell lung cancer. J Clin Oncol 2011;29:1556–63.
9. Aggarwal C, Meropol NJ, Punt CJ, Iannotti N, Saidman BH, Sabbath KD, et al. Relationship among circulating tumor cells, CEA and overall survival in patients with metastatic colorectal cancer. Ann Oncol 2013;24:420–8.
10. Deneve E, Riethdorf S, Ramos J, Nocca D, Coffy A, Daures JP, et al. Capture of viable circulating tumor cells in the liver of colorectal cancer patients. Clin Chem 2013;59:1384–92.
11. Bidard FC, Fehm T, Ignatiadis M, Smerage JB, Alix-Panabieres C, Janni W, et al. Clinical application of circulating tumor cells in breast cancer: overview of the current interventional trials. Cancer Metastasis Rev 2013;32:179–88.
12. Pantel K, Alix-Panabieres C. Real-time liquid biopsy in cancer patients: fact or fiction? Cancer Res 2013;73:6384–8.
13. 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.
14. Ferreira MM, Ramani VC, Jeffrey SS. Circulating tumor cell technologies. Mol Oncol 2016;10:374–94.
15. Agashe R, Kurzrock R. Circulating tumor cells: from the laboratory to the cancer clinic. Cancers 2020;12:2361.
16. Gires O, Pan M, Schinke H, Canis M, Baeuerle PA. Expression and function of epithelial cell adhesion molecule EpCAM: where are we after 40 years? Cancer Metastasis Rev 2020;39:969–87.
17. Eslami SZ, Cortes-Hernandez LE, Alix-Panabieres C. Epithelial cell adhesion molecule: an anchor to isolate clinically relevant circulating tumor cells. Cells 2020;9:1836.
18. Gonzalez-Silva L, Quevedo L, Varela I. Tumor functional heterogeneity unraveled by scRNA-seq technologies. Trends Cancer 2020;6:13–9.
19. Tellez-Gabriel M, Heymann MF, Heymann D. Circulating tumor cells as a tool for assessing tumor heterogeneity. Theranostics 2019;9:4580–94.
20. 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.
21. Aceto N, Bardia A, Miyamoto DT, Donaldson MC, Wittner BS, Spencer JA, et al. Circulating tumor cell clusters are oligoclonal precursors of breast cancer metastasis. Cell 2014;158:1110–22.
22. Lang JE, Ring A, Porras T, Kaur P, Forte VA, Mineyev N, et al. RNA-seq of circulating tumor cells in stage II-III breast cancer. Ann Surg Oncol 2018;25:2261–70.
23. Franses JW, Philipp J, Missios P, Bhan I, Liu A, Yashaswini C, et al. Pancreatic circulating tumor cell profiling identifies LIN28B as a metastasis driver and drug target. Nat Commun 2020;11:3303.

24. Alix-Panabieres C, Pantel K. Challenges in circulating tumour cell research. Nat Rev Cancer 2014;14:623–31.
25. Cayrefourcq L, Mazard T, Joosse S, Solassol J, Ramos J, Assenat E, et al. Establishment and characterization of a cell line from human circulating colon cancer cells. Cancer Res 2015;75:892–901.
26. Koch C, Kuske A, Joosse SA, Yigit G, Sflomos G, Thaler S, et al. Characterization of circulating breast cancer cells with tumorigenic and metastatic capacity. EMBO Mol Med 2020;12:e11908.
27. Yu M, Bardia A, Aceto N, Bersani F, Madden MW, Donaldson MC, et al. Cancer therapy. *Ex vivo* culture of circulating breast tumor cells for individualized testing of drug susceptibility. Science 2014;345: 216–20.
28. Baccelli I, Schneeweiss A, Riethdorf S, Stenzinger A, Schillert A, Vogel V, et al. Identification of a population of blood circulating tumor cells from breast cancer patients that initiates metastasis in a xenograft assay. Nat Biotechnol 2013;31:539–44.
29. Hodgkinson CL, Morrow CJ, Li Y, Metcalf RL, Rothwell DG, Trapani F, et al. Tumorigenicity and genetic profiling of circulating tumor cells in small-cell lung cancer. Nat Med 2014;20:897–903.
30. Faugeroux V, Pailler E, Oulhen M, Deas O, Brulle-Soumare L, Hervieu C, et al. Genetic characterization of a unique neuroendocrine transdifferentiation prostate circulating tumor cell-derived eXplant model. Nat Commun 2020;11:1884.
31. Pantel K, Alix-Panabieres C. Functional studies on viable circulating tumor cells. Clin Chem 2016;62:328–34.
32. 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.
33. Cortes-Hernandez LE, Eslami SZ, Alix-Panabieres C. Circulating tumor cell as the functional aspect of liquid biopsy to understand the metastatic cascade in solid cancer. Mol Aspects Med 2020;72: 100816.
34. 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.
35. 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.
36. Kim TH, Wang Y, Oliver CR, Thamm DH, Cooling L, Paoletti C, et al. A temporary indwelling intravascular aphaeretic system for in vivo enrichment of circulating tumor cells. Nat Commun 2019;10: 1478.
37. Kuske A, Gorges TM, Tennstedt P, Tiebel AK, Pompe R, Preisser F, et al. Improved detection of circulating tumor cells in non-metastatic high-risk prostate cancer patients. Sci Rep 2016;6:39736.
38. Koch C, Joosse SA, Schneegans S, Wilken OJW, Janning M, Loreth D, et al. Pre-analytical and analytical variables of label-independent enrichment and automated detection of circulating tumor cells in cancer patients. Cancers 2020;12:442.
39. Ramirez JM, Fehm T, Orsini M, Cayrefourcq L, Maudelonde T, Pantel K, et al. Prognostic relevance of viable circulating tumor cells detected by EPISPOT in metastatic breast cancer patients. Clin Chem 2014;60:214–21.
40. 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.
41. Cayrefourcq L, De Roeck A, Garcia C, Stoebner PE, Fichel F, Garima F, et al. S100-EPISPOT: a new tool to detect viable circulating melanoma cells. Cells 2019;8:755.
42. Heitzer E, Haque IS, Roberts CES, Speicher MR. Current and future perspectives of liquid biopsies in genomics-driven oncology. Nat Rev Genet 2019;20:71–88.
43. Liu M, Oxnard G, Klein E, Swanton C, Seiden M-V, CCGA Consortium. Sensitive and specific multi-cancer detection and localization using methylation signatures in cell-free DNA. Ann Oncol 2020; 31:745–59.
44. Luo H, Zhao Q, Wei W, Zheng L, Yi S, Li G, et al. Circulating tumor DNA methylation profiles enable early diagnosis, prognosis prediction, and screening for colorectal cancer. Sci Transl Med 2020;12:eaax7533.
45. Keller L, Belloum Y, Wikman H, Pantel K. Clinical relevance of blood-based ctDNA analysis: mutation detection and beyond. Br J Cancer 2021;124:345–58.
46. Heitzer E, Ulz P, Geigl JB. Circulating tumor DNA as a liquid biopsy for cancer. Clin Chem 2015;61:112–23.
47. Cristiano S, Leal A, Phallen J, Fiksel J, Adleff V, Bruhm DC, et al. Genome-wide cell-free DNA fragmentation in patients with cancer. Nature 2019;570:385–9.
48. Wan JCM, Heider K, Gale D, Murphy S, Fisher E, Mouliere F, et al. ctDNA monitoring using patient-specific sequencing and integration of variant reads. Sci Transl Med 2020;12:eaaz8084.
49. Moding EJ, Liu Y, Nabet BY, Chabon JJ, Chaudhuri A, Hui AB, et al. Circulating tumor DNA dynamics predict benefit from consolidation immunotherapy in locally advanced non-small-cell lung cancer. Nat Cancer 2020;1:176–83.
50. Cohen JD, Li L, Wang Y, Thoburn C, Afsari B, Danilova L, et al. Detection and localization of surgically resectable cancers with a multi-analyte blood test. Science 2018;359:926–30.
51. Lennon AM, Buchanan AH, Kinde I, Warren A, Honushefsky A, Cohain AT, et al. Feasibility of blood testing combined with PET-CT to screen for cancer and guide intervention. Science 2020; 369:eabb9601.
52. Kulasingam V, Diamandis EP. Strategies for discovering novel cancer biomarkers through utilization of emerging technologies. Nat Clin Pract Oncol 2008;5:588–99.
53. Razavi P, Li BT, Brown DN, Jung B, Hubbell E, Shen R, et al. High-intensity sequencing reveals the sources of plasma circulating cell-free DNA variants. Nat Med 2019;25:1928–37.
54. Genovese G, Kahler AK, Handsaker RE, Lindberg J, Rose SA, Bakhoum SF, et al. Clonal hematopoiesis and blood-cancer risk inferred from blood DNA sequence. N Engl J Med 2014;371:2477–87.
55. Chan H-T, Nagayama S, Chin Y-M, Otaki M, Hayashi R, Kiyotani K, et al. Clinical significance of clonal hematopoiesis in the interpretation of blood liquid biopsy. Mol Oncol 2020;14:1719–30.
56. Lamb YN, Dhillon S. Epi proColon((R)) 2.0 CE: a blood-based screening test for colorectal cancer. Mol Diagn Ther 2017;21:225–32.
57. Ulz P, Perakis S, Zhou Q, Moser T, Belic J, Lazzeri I, et al. Inference of transcription factor binding from cell-free DNA enables tumor subtype prediction and early detection. Nat Commun 2019;10:4666.
58. Liu MC, Oxnard GR, Klein EA, Swanton C, Seiden M. Response to W.C. Taylor, and C. Fiala and E.P. Diamandis. Ann Oncol 2020;31: 1268–70.
59. Rhim AD, Mirek ET, Aiello NM, Maitra A, Bailey JM, McAllister F, et al. EMT and dissemination precede pancreatic tumor formation. Cell 2012;148:349–61.
60. Husemann Y, Geigl JB, Schubert F, Musiani P, Meyer M, Burghart E, et al. Systemic spread is an early step in breast cancer. Cancer Cell 2008;13:58–68.
61. Effenberger KE, Schroeder C, Eulenburg C, Reeh M, Tachezy M, Riethdorf S, et al. Disseminated tumor cells in pancreatic cancer-an independent prognosticator of disease progression and survival. Int J Cancer 2012;131:E475–83.
62. 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.
63. Hofman V, Ilie MI, Long E, Selva E, Bonnetaud C, Molina T, et al. Detection of circulating tumor cells as a prognostic factor in patients undergoing radical surgery for non-small-cell lung carcinoma: comparison of the efficacy of the CellSearch Assay and the isolation by size of epithelial tumor cell method. Int J Cancer 2011;129: 1651–60.
64. Marquette CH, Boutros J, Benzaquen J, Ferreira M, Pastre J, Pison C, et al. Circulating tumour cells as a potential biomarker for lung cancer screening: a prospective cohort study. Lancet Respir Med 2020;8: 709–16.

65. Pantel K, Deneve E, Nocca D, Coffy A, Vendrell JP, Maudelonde T, et al. Circulating epithelial cells in patients with benign colon diseases. Clin Chem 2012;58:936–40.
66. Buscail E, Chiche L, Laurent C, Vendrely V, Denost Q, Denis J, et al. Tumor-proximal liquid biopsy to improve diagnostic and prognostic performances of circulating tumor cells. Mol Oncol 2019;13:1811–26.
67. Joosse SA, Souche FR, Babayan A, Gasch C, Kerkhoven RM, Ramos J, et al. Chromosomal aberrations associated with sequential steps of the metastatic cascade in colorectal cancer patients. Clin Chem 2018;64:1505–12.
68. Crosbie PA, Shah R, Krysiak P, Zhou C, Morris K, Tugwood J, et al. Circulating tumor cells detected in the tumor-draining pulmonary vein are associated with disease recurrence after surgical resection of NSCLC. J Thorac Oncol 2016;11:1793–7.
69. 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.
70. Galanzha EI, Viegas MG, Malinsky TI, Melerzanov AV, Juratli MA, Sarimollaoglu M, et al. In vivo acoustic and photoacoustic focusing of circulating cells. Sci Rep 2016;6:21531.
71. 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.
72. Quintanal-Villalonga A, Chan JM, Yu HA, Pe'er D, Sawyers CL, Sen T, et al. Lineage plasticity in cancer: a shared pathway of therapeutic resistance. Nat Rev Clin Oncol 2020;17:360–71.
73. Bartkowiak K, Koch C, Gartner S, Andreas A, Gorges TM, Pantel K. *In vitro* modeling of reoxygenation effects on mRNA and protein levels in hypoxic tumor cells upon entry into the bloodstream. Cells 2020;9:1316.
74. Rack B, Schindlbeck C, Juckstock J, Andergassen U, Hepp P, Zwingers T, et al. Circulating tumor cells predict survival in early average-to-high risk breast cancer patients. J Natl Cancer Inst 2014;106:dju066.
75. Janni WJ, Rack B, Terstappen LW, Pierga JY, Taran FA, Fehm T, et al. Pooled analysis of the prognostic relevance of circulating tumor cells in primary breast cancer. Clin Cancer Res 2016;22:2583–93.
76. Riethdorf S, Muller V, Loibl S, Nekljudova V, Weber K, Huober J, et al. Prognostic impact of circulating tumor cells for breast cancer patients treated in the neoadjuvant "Geparquattro" trial. Clin Cancer Res 2017;23:5384–93.
77. 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.
78. Rink M, Chun FK, Dahlem R, Soave A, Minner S, Hansen J, et al. Prognostic role and HER2 expression of circulating tumor cells in peripheral blood of patients prior to radical cystectomy: a prospective study. Eur Urol 2012;61:810–7.
79. Gazzaniga P, de Berardinis E, Raimondi C, Gradilone A, Busetto GM, De Falco E, et al. Circulating tumor cells detection has independent prognostic impact in high-risk non-muscle invasive bladder cancer. Int J Cancer 2014;135:1978–82.
80. Pantel K. Circulating tumor cells in head and neck carcinomas. Clin Chem 2019;65:1193–5.
81. Grobe A, Blessmann M, Hanken H, Friedrich RE, Schon G, Wikner J, et al. Prognostic relevance of circulating tumor cells in blood and disseminated tumor cells in bone marrow of patients with squamous cell carcinoma of the oral cavity. Clin Cancer Res 2014;20:425–33.
82. Nastaly P, Ruf C, Becker P, Bednarz-Knoll N, Stoupiec M, Kavsur R, et al. Circulating tumor cells in patients with testicular germ cell tumors. Clin Cancer Res 2014;20:3830–41.
83. Yokobori T, Iinuma H, Shimamura T, Imoto S, Sugimachi K, Ishii H, et al. Plastin3 is a novel marker for circulating tumor cells undergoing the epithelial-mesenchymal transition and is associated with colorectal cancer prognosis. Cancer Res 2013;73:2059–69.
84. Buscail E, Maulat C, Muscari F, Chiche L, Cordelier P, Dabernat S, et al. Liquid biopsy approach for pancreatic ductal adenocarcinoma. Cancers 2019;11:852.
85. Buscail E, Alix-Panabieres C, Quincy P, Cauvin T, Chauvet A, Degrandi O, et al. High clinical value of liquid biopsy to detect circulating tumor cells and tumor exosomes in pancreatic ductal adenocarcinoma patients eligible for up-front surgery. Cancers 2019;11:1656.
86. Effenberger KE, Schroeder C, Hanssen A, Wolter S, Eulenburg C, Tachezy M, et al. Improved risk stratification by circulating tumor cell counts in pancreatic cancer. Clin Cancer Res 2018;24:2844–50.
87. Boyer M, Cayrefourcq L, Dereure O, Meunier L, Becquart O, Alix-Panabieres C. Clinical relevance of liquid biopsy in melanoma and merkel cell carcinoma. Cancers 2020;12:960.
88. Riethdorf S, Hildebrandt L, Heinzerling L, Heitzer E, Fischer N, Bergmann S, et al. Detection and characterization of circulating tumor cells in patients with merkel cell carcinoma. Clin Chem 2019;65:462–72.
89. Gorges K, Wiltfang L, Gorges TM, Sartori A, Hildebrandt L, Keller L, et al. Intra-patient heterogeneity of circulating tumor cells and circulating tumor DNA in blood of melanoma patients. Cancers 2019;11:1685.
90. Pantel K, Hille C, Scher HI. Circulating tumor cells in prostate cancer: from discovery to clinical utility. Clin Chem 2019;65:87–99.
91. Trapp E, Janni W, Schindlbeck C, Juckstock J, Andergassen U, de Gregorio A, et al. Presence of circulating tumor cells in high-risk early breast cancer during follow-up and prognosis. J Natl Cancer Inst 2019;111:380–7.
92. Sparano J, O'Neill A, Alpaugh K, Wolff AC, Northfelt DW, Dang CT, et al. Association of circulating tumor cells with late recurrence of estrogen receptor-positive breast cancer: a secondary analysis of a randomized clinical trial. JAMA Oncol 2018;4:1700–6.
93. Garcia-Murillas I, Schiavon G, Weigelt B, Ng C, Hrebien S, Cutts RJ, et al. Mutation tracking in circulating tumor DNA predicts relapse in early breast cancer. Sci Transl Med 2015;7:302ra133.
94. Tie J, Wang Y, Tomasetti C, Li L, Springer S, Kinde I, et al. Circulating tumor DNA analysis detects minimal residual disease and predicts recurrence in patients with stage II colon cancer. Sci Transl Med 2016;8:346ra92.
95. Abbosh C, Birkbak NJ, Swanton C. Early stage NSCLC - challenges to implementing ctDNA-based screening and MRD detection. Nat Rev Clin Oncol 2018;15:577–86.
96. Chaudhuri AA, Chabon JJ, Lovejoy AF, Newman AM, Stehr H, Azad TD, et al. Early detection of molecular residual disease in localized lung cancer by circulating tumor DNA profiling. Cancer Discov 2017;7:1394–403.
97. Haselmann V, Gebhardt C, Brechtel I, Duda A, Czerwinski C, Sucker A, et al. Liquid profiling of circulating tumor DNA in plasma of melanoma patients for companion diagnostics and monitoring of BRAF inhibitor therapy. Clin Chem 2018;64:830–42.
98. Soler A, Cayrefourcq L, Mazard T, Babayan A, Lamy PJ, Assou S, et al. Autologous cell lines from circulating colon cancer cells captured from sequential liquid biopsies as model to study therapy-driven tumor changes. Sci Rep 2018;8:15931.
99. Kita K, Fukuda K, Takahashi H, Tanimoto A, Nishiyama A, Arai S, et al. Patient-derived xenograft models of non-small cell lung cancer for evaluating targeted drug sensitivity and resistance. Cancer Sci 2019;110:3215–24.
100. Pantel K, Hayes DF. Disseminated breast tumour cells: biological and clinical meaning. Nat Rev Clin Oncol 2018;15:129–31.
101. Bidard FC, Peeters DJ, Fehm T, Nole F, Gisbert-Criado R, Mavroudis D, et al. Clinical validity of circulating tumour cells in patients with metastatic breast cancer: a pooled analysis of individual patient data. Lancet Oncol 2014;15:406–14.
102. Cristofanilli M, Pierga JY, Reuben J, Rademaker A, Davis AA, Peeters DJ, et al. The clinical use of circulating tumor cells (CTCs) enumeration for staging of metastatic breast cancer (MBC): international expert consensus paper. Crit Rev Oncol Hematol 2019;134:39–45.
103. Lorente D, Olmos D, Mateo J, Bianchini D, Seed G, Fleisher M, et al. Decline in circulating tumor cell count and treatment outcome in advanced prostate cancer. Eur Urol 2016;70:985–92.
104. Heller G, McCormack R, Kheoh T, Molina A, Smith MR, Dreicer R, et al. Circulating tumor cell number as a response measure of

prolonged survival for metastatic castration-resistant prostate cancer: a comparison with prostate-specific antigen across five randomized phase III clinical trials. J Clin Oncol 2018;36:572–80.
105. Lee JH, Long GV, Boyd S, Lo S, Menzies AM, Tembe V, et al. Circulating tumour DNA predicts response to anti-PD1 antibodies in metastatic melanoma. Ann Oncol 2017;28:1130–6.
106. Smerage JB, Barlow WE, Hortobagyi GN, Winer EP, Leyland-Jones B, Srkalovic G, et al. Circulating tumor cells and response to chemotherapy in metastatic breast cancer: SWOG S0500. J Clin Oncol 2014;32:3483–9.
107. Bidard FC, Jacot W, Kiavue N, Dureau S, Kadi A, Brain E, et al. Efficacy of circulating tumor cell count-driven vs clinician-driven first-line therapy choice in hormone receptor-positive, ERBB2-negative metastatic breast cancer: the STIC CTC Randomized Clinical Trial. JAMA Oncol 2021;7:34–41.
108. Araujo DV, Bratman SV, Siu LL. Designing circulating tumor DNA-based interventional clinical trials in oncology. Genome Med 2019;11:22.
109. Gandara DR, Paul SM, Kowanetz M, Schleifman E, Zou W, Li Y, et al. Blood-based tumor mutational burden as a predictor of clinical benefit in non-small-cell lung cancer patients treated with atezolizumab. Nat Med 2018;24:1441–8.
110. O'Leary B, Hrebien S, Morden JP, Beaney M, Fribbens C, Huang X, et al. Early circulating tumor DNA dynamics and clonal selection with palbociclib and fulvestrant for breast cancer. Nat Commun 2018;9:896.
111. Gerlinger M, Rowan AJ, Horswell S, Larkin J, Endesfelder D, Gronroos E, et al. Intratumor heterogeneity and branched evolution revealed by multiregion sequencing. N Engl J Med 2012;366:883–92.
112. Douillard JY, Ostoros G, Cobo M, Ciuleanu T, McCormack R, Webster A, et al. First-line gefitinib in Caucasian EGFR mutation-positive NSCLC patients: a phase-IV, open-label, single-arm study. Br J Cancer 2014;110:55–62.
113. Berger LA, Janning M, Velthaus JL, Ben-Batalla I, Schatz S, Falk M, et al. Identification of a high-level MET amplification in CTCs and cfTNA of an ALK-positive NSCLC patient developing evasive resistance to crizotinib. J Thorac Oncol 2018;13:e243–e6.
114. Hofman P, Heeke S, Alix-Panabieres C, Pantel K. Liquid biopsy in the era of immuno-oncology: is it ready for prime-time use for cancer patients? Ann Oncol 2019;30:1448–59.
115. 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.
116. Siravegna G, Mussolin B, Buscarino M, Corti G, Cassingena A, Crisafulli G, et al. Clonal evolution and resistance to EGFR blockade in the blood of colorectal cancer patients. Nat Med 2015;21:795–801.
117. Van Emburgh BO, Arena S, Siravegna G, Lazzari L, Crisafulli G, Corti G, et al. Acquired RAS or EGFR mutations and duration of response to EGFR blockade in colorectal cancer. Nat Commun 2016;7:13665.
118. Siravegna G, Lazzari L, Crisafulli G, Sartore-Bianchi A, Mussolin B, Cassingena A, et al. Radiologic and genomic evolution of individual metastases during HER2 blockade in colorectal cancer. Cancer Cell 2018;34:148–62.
119. Dasari A, Morris VK, Allegra CJ, Atreya C, Benson AB III, Boland P, et al. ctDNA applications and integration in colorectal cancer: an NCI Colon and Rectal-Anal Task Forces whitepaper. Nat Rev Clin Oncol 2020;17:757–70.
120. Schiavon G, Hrebien S, Garcia-Murillas I, Cutts RJ, Pearson A, Tarazona N, et al. Analysis of ESR1 mutation in circulating tumor DNA demonstrates evolution during therapy for metastatic breast cancer. Sci Transl Med 2015;7:313ra182.
121. Babayan A, Hannemann J, Spotter J, Muller V, Pantel K, Joosse SA. Heterogeneity of estrogen receptor expression in circulating tumor cells from metastatic breast cancer patients. PLoS One 2013;8:e75038.
122. Paoletti C, Muniz MC, Thomas DG, Griffith KA, Kidwell KM, Tokudome N, et al. Development of circulating tumor cell-endocrine therapy index in patients with hormone receptor-positive breast cancer. Clin Cancer Res 2015;21:2487–98.
123. Fehm T, Muller V, Aktas B, Janni W, Schneeweiss A, Stickeler E, et al. HER2 status of circulating tumor cells in patients with metastatic breast cancer: a prospective, multicenter trial. Breast Cancer Res Treat 2010;124:403–12.
124. 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.
125. Zimmer L, Livingstone E, Hassel JC, Fluck M, Eigentler T, Loquai C, et al. Adjuvant nivolumab plus ipilimumab or nivolumab monotherapy versus placebo in patients with resected stage IV melanoma with no evidence of disease (IMMUNED): a randomised, double-blind, placebo-controlled, phase 2 trial. Lancet 2020;395:1558–68.
126. Seremet T, Planken S, Schreuer M, Jansen Y, Delaunoy M, El Housni H, et al. Illustrative cases for monitoring by quantitative analysis of BRAF/NRAS ctDNA mutations in liquid biopsies of metastatic melanoma patients who gained clinical benefits from anti-PD1 antibody therapy. Melanoma Res 2018;28:65–70.
127. Lee JH, Long GV, Menzies AM, Lo S, Guminski A, Whitbourne K, et al. Association between circulating tumor DNA and pseudoprogression in patients with metastatic melanoma treated with anti-programmed cell death 1 antibodies. JAMA Oncol 2018;4:717–21.
128. Hong X, Sullivan RJ, Kalinich M, Kwan TT, Giobbie-Hurder A, Pan S, et al. Molecular signatures of circulating melanoma cells for monitoring early response to immune checkpoint therapy. Proc Natl Acad Sci U S A 2018;115:2467–72.
129. Antonarakis ES, Tagawa ST, Galletti G, Worroll D, Ballman K, Vanhuyse M, et al. Randomized, noncomparative, phase II trial of early switch from docetaxel to cabazitaxel or vice versa, with integrated biomarker analysis, in men with chemotherapy-naive, metastatic, castration-resistant prostate cancer. J Clin Oncol 2017;35:3181–8.
130. Rao A, Antonarakis ES. Circulating tumor cell-based or tissue biopsy-based AR-V7 detection: which provides the greatest clinical utility? Ann Transl Med 2019;7:S354.
131. Scher HI, Graf RP, Schreiber NA, McLaughlin B, Lu D, Louw J, et al. Nuclear-specific AR-V7 protein localization is necessary to guide treatment selection in metastatic castration-resistant prostate cancer. Eur Urol 2017;71:874–82.
132. Shaffer DR, Leversha MA, Danila DC, Lin O, Gonzalez-Espinoza R, Gu B, et al. Circulating tumor cell analysis in patients with progressive castration-resistant prostate cancer. Clin Cancer Res 2007;13:2023–9.
133. Leversha MA, Han J, Asgari Z, Danila DC, Lin O, Gonzalez-Espinoza R, et al. Fluorescence in situ hybridization analysis of circulating tumor cells in metastatic prostate cancer. Clin Cancer Res 2009;15:2091–7.
134. Jiang Y, Palma JF, Agus DB, Wang Y, Gross ME. Detection of androgen receptor mutations in circulating tumor cells in castration-resistant prostate cancer. Clin Chem 2010;56:1492–5.
135. Sridhar SS, Freedland SJ, Gleave ME, Higano C, Mulders P, Parker C, et al. Castration-resistant prostate cancer: from new pathophysiology to new treatment. Eur Urol 2014;65:289–99.
136. Ristau BT, O'Keefe DS, Bacich DJ. The prostate-specific membrane antigen: lessons and current clinical implications from 20 years of research. Urol Oncol 2014;32:272–9.
137. Gorges TM, Riethdorf S, von Ahsen O, Nastal YP, Rock K, Boede M, et al. Heterogeneous PSMA expression on circulating tumor cells: a potential basis for stratification and monitoring of PSMA-directed therapies in prostate cancer. Oncotarget 2016;7:34930–41.
138. Maurer T, Schwamborn K, Schottelius M, Wester HJ, Schwaiger M, Gschwend JE, et al. PSMA theranostics using PET and subsequent radioguided surgery in recurrent prostate cancer. Clin Genitourin Cancer 2016;14:e549–e52.
139. Autio KA, Dreicer R, Anderson J, Garcia JA, Alva A, Hart LL, et al. Safety and efficacy of BIND-014, a docetaxel nanoparticle targeting prostate-specific membrane antigen for patients with metastatic castration-resistant prostate cancer: a phase 2 clinical trial. JAMA Oncol 2018;4:1344–51.
140. Lampignano R, Neumann MHD, Weber S, Kloten V, Herdean A, Voss T, et al. Multicenter evaluation of circulating cell-free DNA extraction and downstream analyses for the development of standardized (Pre)analytical work flows. Clin Chem 2020;66:149–60.

141. O'Leary B, Cutts RJ, Liu Y, Hrebien S, Huang X, Fenwick K, et al. The genetic landscape and clonal evolution of breast cancer resistance to palbociclib plus fulvestrant in the PALOMA-3 trial. Cancer Discov 2018;8:1390–403.
142. Alix-Panabieres C. The future of liquid biopsy. Nature 2020;579:S9.
143. Rothwell DG, Ayub M, Cook N, Thistlethwaite F, Carter L, Dean E, et al. Utility of ctDNA to support patient selection for early phase clinical trials: the TARGET study. Nat Med 2019;25:738–43.
144. 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.
145. 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.
146. Werner S, Brors B, Eick J, Marques E, Pogenberg V, Parret A, et al. Suppression of early hematogenous dissemination of human breast cancer cells to bone marrow by retinoic acid-induced 2. Cancer Discov 2015;5:506–19.
147. Liu Y, Cao X. Characteristics and significance of the pre-metastatic niche. Cancer Cell 2016;30:668–81.
148. Ganesh K, Massague J. Targeting metastatic cancer. Nat Med 2021;27:34–44.
149. Yokoi A, Villar-Prados A, Oliphint PA, Zhang J, Song X, De Hoff P, et al. Mechanisms of nuclear content loading to exosomes. Sci Adv 2019;5:eaax8849.
150. Hoshino A, Costa-Silva B, Shen TL, Rodrigues G, Hashimoto A, Tesic Mark M, et al. Tumour exosome integrins determine organotropic metastasis. Nature 2015;527:329–35.
151. Wortzel I, Dror S, Kenific CM, Lyden D. Exosome-mediated metastasis: communication from a distance. Dev Cell 2019;49:347–60.
152. Chiu RWK, Heitzer E, Lo YMD, Mouliere F, Tsui DWY. Cell-free DNA fragmentomics: the new "Omics" on the block. Clin Chem 2020;66:1480–4.

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