INVITED REVIEW

Liquid biopsies: Potential and challenges

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Funding information
Deutsche Forschungsgemeinschaft, Grant/Award Number: SPP2084 Bone; Deutsche Krebshilfe, Grant/Award Number: 70112504; H2020 European Research Council, Grant/Award Number: Advanced Investigator Grant INJURMET/ 834974; H2020 Marie Skłodowska-Curie Actions, Grant/Award Number: 765492; Innovative Medicines Initiative, Grant/Award Number: 115749-CANCER-ID

Abstract

The analysis of tumor cells or tumor cell products obtained from blood or other body fluids ("liquid biopsy" [LB]) provides a broad range of opportunities in the field of oncology. Clinical application areas include early detection of cancer or tumor recurrence, individual risk assessment and therapy monitoring. LB allows to portray the entire disease as tumor cells or tumor cell products are released from all metastatic or primary tumor sites, providing comprehensive and real-time information on tumor cell evolution, therapeutic targets and mechanisms of resistance to therapy. Here, we focus on the most prominent LB markers, circulating tumor cells (CTCs) and circulating tumor-derived DNA (ctDNA), in the blood of patients with breast, prostate, lung and colorectal cancer, as the four most frequent tumor types in Europe. After a brief introduction of key technologies used to detect CTCs and ctDNA, we discuss recent clinical studies on these biomarkers for early detection and prognostication of cancer as well as prediction and monitoring of cancer therapies. We also point out current methodological and biological limitations that still hamper the implementation of LB into clinical practice.

KEYWORDS
blood, carcinomas, circulating tumor cells, circulating tumor DNA, liquid biopsy

Abbreviation: ADT, androgen deprivation therapy; AJCC, American Joint Committee on Cancer; AR, androgen receptor; ARs, AR signaling; ARv7, AR splice variant 7; AS-NEPB-PCR, allele-specific, nonextendable primer blocker PCR; AS-PCR, allele-specific PCR; AUC, area under the curve; BCSS, breast cancer-specific survival; BI-RADS, Breast Imaging Reporting and Data System; bp, base pair; BPH, benign prostatic hyperplasia; BRPC, biochemically recurrent prostate cancer; CAPP-Seq, CAncer Personalized Profiling by deepSequencing; CAPRA, Cancer of the Prostate Risk Assessment; ctDNA, cell-free DNA; cfRNA, cell-free RNA; ctDNA, cell-free tumor nucleic acid; CHIP, clonal hematopoiesis of indeterminate potential; CI, confidence interval; COLD-PCR, coamplification at lower denaturation temperature; CRC, colorectal cancer; CRPC, castrate-resistant prostate cancer; CSC, cancer stem cell; CSF, cerebrospinal fluid; CTC, circulating tumor cell; ctDNA, circulating tumor-derived DNA; CCAM, all cells that are (a) nonapoptotic, (b) have a nucleus, (c) are CD45− and (d) have cytomorphometric features consistent with CTCs (size, shape, nuclear-to-cytoplasm ratio, etc.); DDFS, distant disease-free survival; ddPCR, droplet digital PCR; DEP, detectrophoresis; DFS, disease-free survival; DM, digital mammography; DRE, digital rectal examination; DSM, disease-specific mortality; EGFR, epidermal growth factor receptor; EMT, epithelial-to-mesenchymal transition; EPISPOT, epithelial immunospot; ER, estrogen receptor; ESR1, estrogen receptor 1; EV, extracellular vesicle; FDA, Food and Drug Administration; FDR, false discovery rate; FV, first void; HER2, human epidermal growth factor receptor 2; HR, hazard ratio; LB, liquid biopsy; LBD, ligand-binding domain; LC, lung cancer; LRFS, locoregional relapse-free survival; mCSPC, metastatic castration-resistant prostate cancer; mCSPC, metastatic castration-sensitive prostate cancer; mFAST-SeqS, modified Fast Aneuploidy Screening Test-Sequencing System; MRD, minimal residual disease; mRNA, messenger RNA; MS-APCR, methylation-specific PCR; NAT, neoadjuvant therapy; NCDB, National Cancer Database; NGS, next-generation sequencing; NR, not reached; NSCLC, nonsmall-cell lung cancer; OR, odds ratio; OS, overall survival; PC, prostate cancer; PCR, polymerase chain reaction; PFS, progression-free survival; PMP, prostate microparticles; PNA-LNA, peptide nucleic acid-locked nucleic acid; PSA, prostate-specific antigen; PSMA, prostate-specific membrane antigens; PSMA-PET, PSMA-directed positron emission tomography; PUR, prostate urine risk; RT-PCR, reverse transcription polymerase chain reaction; rPFS, radiographic progression-free survival; RT, radiotherapy; SCLC, small cell lung cancer; Tam Seq, targeted amplicon deep sequencing; TKI, tyrosine kinase inhibitor; TNBC, triple-negative breast cancer; TR, time ratio; TWIST1, Twist-related protein 1; WES, whole exome sequencing; WGA, whole genome amplification; WGS, whole genome sequencing.

Isabel Heidrich, Lucija Ačkar and Parinaz Mossahebi Mohammadi contributed equally to our work.

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1 | INTRODUCTION

Despite significant advances in the diagnosis and treatment of solid tumors, distant metastases remain the main cause of cancer-related deaths. However, mere analysis of the resected primary tumor alone, current standard practice in oncology, can provide misleading information regarding the characteristics of metastases. Metastases can develop unique genomic characteristics that might not be detected when examining the primary tumor. In addition, metastases are often present in different organs at the same time and have one strongly organ-dependent heterogeneity.

This diagnostic dilemma led to the development of the “liquid biopsy” (LB) concept. LB is the real-time analysis of tumor cells or tumor cell products (eg, cell-free circulating nucleic acids [ctDNA, cfRNA], extracellular vesicles or proteins) released into the blood or other body fluids by primary or metastatic tumor lesions. LB enables the development of new methods for early detection of primary cancer or disease relapse, monitoring the efficacy of cancer therapies and determining therapeutic targets and resistance mechanisms to adapt therapy to the specific needs of an individual. The database ClinicalTrials.gov (www.clinicaltrials.gov) currently lists 701 clinical trials using circulating tumor cells (CTCs) and 359 trials using circulating tumor-derived DNA (ctDNA) in the four major tumor entities (breast, lung, prostate and colorectal cancer), which documents the high interest in CTCs and ctDNA as biomarkers. Considerable advances have been made in the development of technologies to detect blood-based, tumor-specific biomarkers, such as CTCs and ctDNA, and in the development of downstream analyses of CTCs and ctDNA to obtain novel information on natural or therapy-selected tumor evolution in cancer patients. In addition, new members of the LB marker family include extracellular vesicles, microRNAs and tumor-educated platelets.

In this review, we will focus on CTCs and ctDNA as the most prominent LB markers, with emphasis on studies in patients with breast, prostate, lung and colorectal cancer as the most frequent solid tumors worldwide. After a brief introduction into the methodology, we will discuss the current clinical applications of CTCs and ctDNA. We analyzed scientific work published within the past 3 years and dealt with the importance of the use of CTCs and ctDNA for early detection, risk assessment and monitoring of cancer therapies.

1.1 | CTCs: methodology and technical challenges

Efficient positive enrichment of CTCs can be achieved by approaches that exploit differences between tumor cells and normal blood cells, including the differential expression of tumor-associated cell surface proteins (eg, EpCAM, mucin-1, HER2 or EGFR) or distinct physical properties (eg, larger size or reduced deformability) of the tumor cells (Figure 1A). In contrast, CTCs can also be enriched by negative selection, that is, normal blood cells are removed by antibodies against CD45 or other antigens expressed on leukocytes or circulating endothelial cells. Label-independent technologies based on size exclusion include microfiltration technologies, which involve passing blood through filters with small pores or microfluidic steps that are calibrated to capture CTCs. Other technologies use inertial focus strategies to separate CTCs from other blood components as Hydro-Seq, which is a scalable hydrodynamic scRNA-seq barcoding technique, for high-throughput CTC analysis or dielectrophoresis (DEP), which isolates CTCs based on the different electric charges of tumor and blood cells. Most enriched CTCs are single isolated cells but enrichment of CTC clusters is possible and allows now deeper insights into the exciting biology of CTC clusters.

After enrichment, reliable methods are used to identify individual CTCs with specific tumor-associated biomarkers at the protein level or to a lesser degree at the messenger RNA (mRNA) level (Figure 1A). For patients with epithelial tumors (carcinomas), keratins have been well established as CTC markers. However, these epithelial markers can be downregulated during epithelial-to-mesenchymal transition (EMT), which might lead to false-negative findings. CTCs might frequently undergo EMT, indicating the need for assays based on new EMT markers. Besides EMT, metabolic markers and clusters also seem to define the biological potential of CTCs.

Downstream analysis of individual CTCs or clusters at the DNA, RNA or protein level have become possible over the past decade. Isolation of single CTCs can be achieved by micromanipulation or DEP-Array technology, but it usually requires sufficiently high starting CTC concentrations. For DNA analysis of single cells, whole genome amplification (WGA) methods need to be employed to generate sufficient quantities of DNA for subsequent sequencing analyses. WGA may induce bias and therefore new WGA-free approaches are currently being developed. Besides RNA sequencing, multiplex reverse transcription polymerase chain reaction (RT-PCR) can provide already some insights into the heterogeneity of CTCs. Analysis at the protein level usually employ immunostaining, but new approaches of multiplex proteomic approaches are on the horizon. Besides descriptive approaches, functional CTC assays exist (eg, the Epithelial Immunospot (EPISPOT) that is based on the measurement of secreted protein by viable CTCs after short-term culture). In patients with extremely high CTC counts (usually >100 per mL blood), the functional properties of CTCs can be further investigated by the establishment of long-term cell culture/cell lines or the development of xenograft models (Figure 1A). However, the establishment of these models requires many months and the efficacy rates are very low, which makes them unsuitable as tool for clinical trials or decision-making in individual patients. Nevertheless, CTC lines and xenografts provide unique first insights into the largely unknown functional properties of CTCs.

1.2 | ctDNA: methodology and technical challenges

Cell-free DNA (cfDNA) circulating in the peripheral blood is mostly released through necrosis and apoptosis but potentially also by secretion through extracellular vesicles. CfDNA consists mostly of 166 bp, which is consistent with the length of a DNA fragment.
wrapped around a nucleosome. Since DNA is degraded to these mononucleosomal units during apoptosis, this size indicates that programmed cell death is the primary way of release. In cancer patients, only a small portion of cfDNA (usually 0.01%-5%) is shed into the blood by tumor cells. Functional assays can be used to detect viable CTCs based on their biological activities (e.g., the fluoro-epithelial ImmunoSPOT (EPISPOT) assay for certain proteins secreted or shed by CTCs and the related EPISPOT in a drop (EPIDROP) technology that enables the detection of single CTCs in microdroplets). The functional properties of CTCs can also be investigated in vivo by the establishment of CTC-derived xenografts. B, ctDNA detection technologies. ctDNA analysis is based on the identification of tumor-specific aberrations or epigenetic marks in cfDNA samples. Ultrasensitive targeted approaches like droplet digital PCR (ddPCR) or BEAMing and NGS technologies (Tam-Seq, Safe-SeqS, CAPP-Seq) are able to detect pre-specified cancer-associated mutations at high sensitivity. Refined real-time PCR methods, like allele-specific PCR (AS-PCR), allele-specific nonextendable primer blocker PCR (AS-NEPB-PCR), coamplification at lower denaturation temperature (COLD-PCR) or peptide nucleic acid-locked nucleic acid (PNA-LNA) PCR clamp allow fast, cheap and sensitive detection of mutations. Untargeted approaches like whole genome sequencing, whole exome sequencing or FastSeqS allow the unbiased detection of genomic aberrations without requiring prespecified information about the mutation pattern of the respective primary tumor (Color figure can be viewed at wileyonlinelibrary.com)

FIGURE 1 A, Technologies for enrichment, detection and characterization of circulating tumor cells (CTCs). CTCs isolated from blood samples can be enriched using marker-dependent techniques: CTCs can be positively selected using antibodies to epithelial or tumor-associated proteins (e.g., EpCAM) or negatively selected by depletion of leukocytes using anti-CD45 antibodies. Positive enrichment of CTCs can also be performed using assays based on physical CTC characteristics, including size, deformability, density and electrical charge. After enrichment, the isolated CTCs can be identified using immunocytochemical assays like membrane and/or cytoplasmic staining with antibodies to epithelial, mesenchymal, tissue-specific or tumor-associated markers (e.g., keratins). Molecular assays enable the identification of CTCs at the DNA, RNA and protein level. Functional assays can be used to detect viable CTCs based on their biological activities (e.g., the fluoro-epithelial ImmunoSPOT (EPISPOT) assay for certain proteins secreted or shed by CTCs and the related EPISPOT in a drop (EPIDROP) technology that enables the detection of single CTCs in microdroplets). The functional properties of CTCs can also be investigated in vivo by the establishment of CTC-derived xenografts. B, ctDNA detection technologies. ctDNA analysis is based on the identification of tumor-specific aberrations or epigenetic marks in cfDNA samples. Ultrasensitive targeted approaches like droplet digital PCR (ddPCR) or BEAMing and NGS technologies (Tam-Seq, Safe-SeqS, CAPP-Seq) are able to detect pre-specified cancer-associated mutations at high sensitivity. Refined real-time PCR methods, like allele-specific PCR (AS-PCR), allele-specific nonextendable primer blocker PCR (AS-NEPB-PCR), coamplification at lower denaturation temperature (COLD-PCR) or peptide nucleic acid-locked nucleic acid (PNA-LNA) PCR clamp allow fast, cheap and sensitive detection of mutations. Untargeted approaches like whole genome sequencing, whole exome sequencing or FastSeqS allow the unbiased detection of genomic aberrations without requiring prespecified information about the mutation pattern of the respective primary tumor (Color figure can be viewed at wileyonlinelibrary.com)
(2.1 million new cases of breast cancer and 627 000 deaths worldwide in 2018, WHO World Cancer Report, 2020).^{11}

1.3.1 | Circulating tumor cells

Many patients with primary breast cancer relapse even more than 20 years after primary tumor resection.^{26} The sequential analysis of CTCs before, during, and after therapy has enabled real-time monitoring of tumor evolution in individual patients with metastatic breast cancer.^{37} In early-stage patients, CTC status at baseline has prognostic relevance in patients receiving adjuvant^{38} or neoadjuvant^{39,40} therapy (Table 1). In addition, sequential follow-up evaluation after primary therapy might be able to detect minimal residual disease (MRD).^{6,36} CTC counts 2 or 5 years after completion of adjuvant chemotherapy predicted an unfavorable outcome^{43,44} (Table 1). Thus, CTCs might enrich a high-risk group that can profit more from radiotherapy than patients without CTCs.^{42}

The molecular characterization of CTCs can provide information relevant to cancer therapy. For example, monitoring of CTC-Top1 expression can identify patients with overall survival (OS) benefits from treatment with topoisomerase-1 inhibitor etirinotecan pegol.^{45} Recent investigations have also shown a role of the androgen receptor (AR) in breast cancer. De Kruijff et al in 2019 assessed AR-mRNA and found a discordance between the AR-mRNA status in CTC samples and the corresponding primary tumors^{49} with potential implications for the selection of breast cancer patients for AR inhibitor therapy.

The assessment of CTC-RNA signatures can be also helpful. Kwan et al in 2018 used 17 markers (AGR2, CXCL13, CXCL14, FAT1, FAT2, MGP, MUC16, PGR, etc.)^{50} in breast cancer patients to show that an increased CTC value was associated with clinical outcome (Table 1). Magbanua et al in 2018 analyzed the DNA and mRNA profiles of EPCAM-positive CTCs by microfluidic-based multiplex QPCR array^{41} and discordant expression signatures between CTCs and matching primary tumors (Table 1). Furthermore, patients with Ki-67 (proliferation marker)-positive CTCs had significantly reduced progression-free survival (PFS) and OS compared to patients with low proliferative CTCs.^{41}

Endocrine therapy is the hallmark in patients with hormone receptor-positive patients (~70% of all breast cancers) but resistance to therapy occurs in a substantial fraction of patients. Estrogen receptor-1 (ESR1) mutations can result in a transcriptional profile that favors tumor progression.^{51} In estrogen receptor (ER)-positive patients, failure to suppress ER signaling in CTCs predicted early progression after 3 weeks of endocrine therapy.^{46} The drug-refractory ER signal transmission within CTCs only partially overlapped with the presence of ESR1 mutations, which indicates additional mechanisms of acquired endocrine drug resistance.^{46}

Molecular characterization of CTCs opens new avenues for a better understanding of cancer biology with potential implications for the design of new therapies. CTCs with EMT-associated (TWIST1) and cancer-stem-cell (CSC) transcripts (CD24, CD44, ALDH1) had an unfavorable survival.^{47} Furthermore, chemotherapy increased the incidence of CSC+/partial EMT + CTCs that may represent a chemoresistant subpopulation of CTCs^{46} (Table 1). In contrast, Ebright et al in 2020 showed that CTCs expressing epithelial markers (together with proliferation markers) were indicative of poor clinical outcome.^{51} Moreover, overexpression of RPL15, which encodes a component of the large ribosomal subunit, increased metastatic growth in multiple organs and selectively improved translation of other ribosomal proteins and cell cycle regulators. Tumor suppressor genes of the TP53 gene family—MDM2 and MDMX—promote the release and/or survival of CTCs in triple-negative breast cancer.^{52}

The interaction between tumor cells and blood cells is an important field for CTC research. Szczesna et al in 2019 discovered cell-cell junction and cytokine receptor pairs that define CTC-neutrophil clusters.^{53} Glountela et al in 2019 also presented the DNA methylation landscape of CTC clusters and showed that binding sites for CSC and proliferation-associated transcripts (OCT4, NANOG, SOX2 and SIN3A) in CTC clusters are specifically activated by hypomethylation.^{13} Targeting CTC clusters^{53} might be a new strategy to prevent blood-borne tumor cell dissemination.

1.3.2 | Circulating tumor-derived DNA

In primary breast cancer, early detection of relapse is a prime diagnostic target. Sequential patient-specific ctDNA analysis every 6 months for up to 4 years was able to predict relapse with a lead time of up to 2 years (median: 8.9 months)^{54} (Table 2). Besides mutations, DNA methylation is relevant in cancer development and progression. The application of methylation-specific PCR probing five cancer-relevant genes (KLK10, SOX17, WNT5A, MSH2, GATA3) on ctDNA^{55} predicted clinical outcome in various subtypes of nonmetastatic breast cancer.

The multiplicity of new therapies for breast cancer presents a challenge for treatment selection. Monitoring of ctDNA during endocrine therapy has shown that mutations in the ER genes (ESR1) are being selected during therapy^{56} (Table 2). Thus, there is a need for new therapeutic approaches overcoming resistance. CDK4/6 inhibition has improved survival in advanced ER-positive breast cancer and monitoring of ctDNA levels might predict early response.^{57} In the PALOMA-3 study, palbociclib plus fulvestrant induced a lower PIK3CA-ctDNA ratio (mutated copies/ml) compared to fulvestrant plus placebo, and the PIK3CA-ctDNA assessment anticipated the improved PFS seen with palbociclib (Table 2). Combining endocrine therapy with PI3K-mTOR inhibition has also shown promise in ER-positive breast cancer in the POSEIDON study. PFS of metastatic breast cancer patients receiving everolimus plus exemestane varied depending on the properties of ctDNA; patients with low or no ctDNA exposure and with less than three mutations on ctDNA showed longer PFS and OS.^{58} In patients receiving immune checkpoint inhibitors, tumor heterogeneity determined on ctDNA predicted response to treatment.^{55} Overall, these are promising steps toward the use of ctDNA for monitoring tumor burden in breast cancer patients receiving new forms of therapy.
### TABLE 1: Clinical studies on prognostic relevance of CTCs in breast cancer

| Study (year) | Inclusion criteria | N pats. | Detection method | Prognostic relevance | Reference |
|--------------|--------------------|---------|------------------|----------------------|-----------|
| Magbanua et al (2018) | Metastatic breast cancer (MBC) | 105 | EPCAM-based immunomagnetic enrichment /FACS/ microfluid-based multiplex QPCR | Patients with CTCs with high proliferation status (MKI67)—had a significantly reduced PFS ($P = .0011$) and OS ($P = .0095$) compared to patients with low MKI67 status | 41 |
| Goodman et al (2018) | Stage pT1-pT2 and pN0-pN1 breast cancer (NCDB and SUCCESS trial cohorts, various subtypes) | 3213 | ICC (CellSearch) | RT was associated with longer OS in patients with CTCs (TR, 4.37; 95% CI, 2.71-7.05; $P < .001$). CTC-positive patients treated with RT exhibited longer LRFS (TR 2.73; 95% CI, 1.62-4.80; $P < .001$), DFS (TR 3.03; 95% CI 2.22-4.13; $P < .001$) and OS (TR 1.83; 95% CI 1.23-2.72; $P = .003$) | 42 |
| Trapp et al (2019) | Phase III SUCCESS A trial; early-stage, high-risk breast cancer patients | 1087 | ICC (CellSearch) | CTC status 2 years after chemotherapy had statistically significant prognostic relevance for OS (HR 3.91; 95% CI 2.04-7.52; $P < .001$) and DFS (HR 2.31; 95% CI 1.50-3.55; $P < .001$) | 43 |
| Sparano et al (2018) | Human epidermal growth factor receptor 2-negative stage II-III breast cancer without clinical evidence of recurrence between 4.5 and 7.5 years after primary surgical treatment and followed by adjuvant systemic therapy | 353 | ICC (CellSearch) | Positive CTC test result associated with a 13.1-fold higher risk of recurrence (HR 13.1; 95% CI 4.7-36.3) | 44 |
| Rugo et al (2018) | Metastatic breast cancer (MBC) | 656 | ApoStream/multiplex immunofluorescence assay | EP-treated patients with low C2D1Top1+ CTCs had improved OS compared to those with higher positivity (14.1 months vs 11.0 months, respectively; HR 0.7; $P = .02$) | 45 |
| Bidard et al (2018) | Stages I to III breast cancer (various subtypes) treated using NAT | 1574 | ICC (CellSearch) | Number of CTCs detected before NAT correlated to OS ($P < .001$), DFS ($P < .001$) and LRFS ($P < .001$) but not to pathological complete response | 40 |
| Janni et al (2016) | Stages I to III breast cancer | 3173 | ICC (CellSearch) | CTC detection at the time of diagnosis was independently prognostic of DFS (HR 1.82; 95% CI 1.47-2.26), DFS (HR 1.89; 95% CI 1.49-2.40), BCSS (HR 2.04; 95% CI 1.52-2.75) and OS (HR 1.97; 95% CI 1.51-2.59) | 38 |
| Riethdorf et al (2017) | Stages I to III breast cancer | 213 | ICC (CellSearch) | Detection of ≥1 CTCs or ≥2 CTCs per 7.5 mL of blood collected before NAT was associated with reduced DFS ($P = .031$ and $P < .0001$, respectively) and OS ($P = .0057$ and $P < .0001$, respectively), whereas CTCs detected after NAT were not | 39 |
| Kwan et al (2018) | Stages I to IV breast cancer (various subtypes) | 110 | Microfluidic CTC-iChip/ddPCR | In a second prospective cohort with metastatic breast cancer ($n = 30$), the baseline CTC correlated with OS ($P = .02$), as did the sustained CTC signal after 4 weeks of treatment ($P = .01$) | 46 |
| Strati et al (2019) | Stages I and II breast cancer b | 119 | Quadruplex RT-qPCR/single RT-qPCR | Patients with CTCs overexpressing TWIST1 had poorer OS compared to TWIST-negative patients (83.6 months vs 115.8 months; $P = .019$); ER/PR-negative | 47 |
CtDNA measurements also hold promise for early detection in breast cancer. In a pioneering study, blood samples were screened for mutations within 58 cancer genes and detection rates varied between 46% and 67% in Stages I to III breast cancer (Table 2). To encompass tumor heterogeneity, a complex blood test based on the detection of more than 1000 mutations in 16 cancer genes was combined with the measurement of eight tumor-associated blood plasma proteins. Despite a high test specificity, overall sensitivity was only 33% in breast cancer. In clinical practice, ctDNA measurements need to be combined with standard-of-care approaches. In breast cancer, the Breast Imaging Reporting and Data System (BI-RADS) is used for the radiological assessment of a suspicious breast lesion. ctDNA results combined with BI-RADS scores has shown to predict the presence of cancer with a sensitivity of 74% and specificity of 92%, with less favorable values in earlier stages (Table 2). Besides mutations, several groups currently investigate whether the analysis of DNA methylation (or other epigenetic signatures) on ctDNA might provide better alternative with reduced risk of false-positive events.

### 1.4 Prostate cancer: clinical applications

Prostate cancer (PC) is the second most common cancer in men and represents a very heterogeneous disease with aggressive and clinically indolent courses.

#### 1.4.1 Circulating tumor cells

The prostate-specific antigen (PSA) screening of blood in men is conducted for many years. After elevated PSA levels, prostate needle biopsies are necessary to allow histological assessment. Low accuracy of PSA as a biomarker and difficulties in distinguishing between indolent and aggressive PCs lead to unnecessary biopsies and overtreatment of patients. An underinvestigated side effect of prostate biopsies is the potential spread of tumor cells into the blood circulation, recently suggested by an increase in CTCs after biopsy in patients with PC. Moreover, potential tumor spread was also investigated in nonmetastatic high-risk PC patients before and after radiotherapy. Application of three different CTC enumeration technologies (CellSearch, fluoro-EPISPOT assay and CellCollector) disproved the hypothesis that radiotherapy leads to a release of tumor cells into the circulation.

CTC analysis has in particular driven biomarker development in patients with metastatic castration-resistant prostate cancer (mCRPC). Here, large-scale studies have shown that the CTC count is a reliable biomarker for treatment response and prognosis in patients receiving chemotherapy or AR-targeting therapies. Despite improvement in the field of androgen deprivation therapy (ADT) with enzalutamide (direct AR antagonist) or abiraterone acetate (CYP 17 inhibitor), 20% to 40% of patients still show resistance to these next-generation ADT drugs. Response to these ADT drugs is reduced if the AR splice variant (ARv7) is expressed in tumor cells, while response to taxanes is maintained. However, mCRPC patients eligible for taxane therapy after failure of ADT might face taxane resistance, as recently shown by molecular characterization of CTCs.

ARv7 detection on CTCs has been used as predictive marker. Detection of nuclear ARv7 immunostaining in CTCs, feasible with the Oncotype DX Arv7 Nucleus DetectTest, has shown that CRPC patients with ARv7-positive CTCs benefit from taxane therapy rather than from AR signaling (ARS) inhibition. Additionally, the amount of ARv7-positive CTCs increased during AR-targeted therapy, indicating that this therapy promotes the emergence of resistance by selecting tumor cells expressing ARv7. However, a small subset of patients resistant to ARS inhibition showed ARv7-positive CTCs prior to therapy.
| Study (year)                  | Inclusion criteria                                      | N patients | Detection method                        | Prognostic relevance                                                                                                                                                                                                                                                                                                                                 |
|------------------------------|---------------------------------------------------------|------------|-----------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Coombes et al (2019)         | Stages I to III breast cancer                           | 45         | Ultra-deep NGS                         | ctDNA was detected with a sensitivity of 89% before the clinical or radiological relapse. Relapse was detected 8.9 months (median) before imaging                                                                                                                                                                                                                           |
| Ma et al (2020)               | HER2-positive breast cancer patients in a metastatic state and under treatment with pyrotinib | 47         | HiSeq3000 sequencing system            | Patients with high tumor heterogeneity had worse PFS (30.0 weeks) compared to patients with low tumor heterogeneity (60.0 weeks) (HR 2.9; P = .02)                                                                                                                                                                                                                 |
| Schiavon et al (2016)         | Advanced breast cancer (stage not specified)            | 171        | Ultrahigh sensitivity multiplexed digital PCR assay | Patients with ESR1 mutations had a substantially shorter PFS on subsequent AI-based therapy (HR 3.1; 95% CI 1.9-23.1; log-rank P = .0041)                                                                                                                                                                                                                      |
| O’Leary et al (2018)          | Advanced (metastatic breast cancer) ER-positive, HER2-negative breast cancer randomized phase III PALOMA-3 study; CDK4/6 inhibitor palbociclib and fulvestrant | 455        | TaqMan Copy Number Reference Assay/multiplex ddPCR                      | Relative change in PIK3CA ctDNA level after 15 days treatment predicted PFS in patients on palbociclib and fulvestrant (HR 3.94; log-rank P = .0013)                                                                                                                                                                                                                   |
| Kruger et al (2019)           | Postmenopausal, metastatic state, ER-positive, HER2-negative breast cancer | 164        | Ion Torrent S5XL NGS system            | Patients with low or no ctDNA levels had longer mean PFS of 5.7 months (P = .006) and OS of 124.8 months (P = .008) in contrast to patients with high ctDNA levels (4.4 months and 107.7 months). Patients with <3 specific mutations had longer PFS (median: 5.4 months) compared to patients with ≥3 mutations (3.4 months; P < .001)                                                                                                      |
| Early detection               |                                                         |            |                                         |                                                                                                                                                                                                                                                                                                                                                       |
| Cristiano et al (2019)        | Stages I to IV breast cancer                            | 54         | DELFI (DNA evaluation of fragments for early interception)                | DELFI performance for breast cancer detection: sensitivity: 70% (CI 56%-82%); specificity: 57% (CI 43%-71%)                                                                                                                                                                                                                                           |
| Cohen et al (2018)            | Stages I to III breast cancer                           | 418        | AMPure and sequenced on an Illuma MiSeq or 2 HiSeq 4000 instrument        | CancerSEEK performance for breast cancer: sensitivity: 33%; specificity: >99%                                                                                                                                                                                                                                                                               |
| Zhang et al (2019)            | Stages I and II breast cancer                           | 152        | NGS of 102 cancer-related genes      | ctDNA combined with BI-RADS scores: sensitivity: 74.2%; specificity: 92.0%                                                                                                                                                                                                                                                                              |

Abbreviations: BI-RADS, Breast Imaging Reporting and Data System; CI, confidence-interval; CTC, circulating tumor cell; ctDNA, circulating cell-free tumor DNA; ddPCR, droplet digital PCR; OS, overall survival; PFS, progression-free survival.
| Study (year) | Inclusion criteria | N pat. | Detection method | Prognostic relevance | Reference |
|-------------|--------------------|--------|-----------------|----------------------|-----------|
| Markou et al (2018) | High-risk primary PC (PSA ≥ 20 ng/mL and/or Gleason score on biopsy ≥8 and/or clinical tumor stage ≥2c), before and after surgery or RT | 108 | Antibody-based assays (CellSearch, CellCollector, EPISPOT Assay) | No difference in CTC rates after surgery (74.1% vs 66.6%). Change in marker expression before and after therapy: epithelial markers, 48.1% vs 7.4%; EMT markers, 7.4% vs 63.0%; stem cell markers, 33.3% vs 0% | 17 |
| Satelli and Batth et al (2017) | Metastatic hormone-sensitive and castration-resistant PC | 48 | ICC (CellSearch, MACS) | CSV CTC count and PSA level were correlated to castrate resistance (both P < .001). AUC-ROC for CSV CTC quantification in discrimination of patients with stable and progressive disease was 0.9556 (P = .00054) (sensitivity: 93.3%; specificity: 94.4%). For CellSearch CTC counts 0.8009 (P < .0001), (sensitivity: 83.3%, specificity: 33.3%) | 18 |
| McDaniel et al (2017) | Metastatic castration-resistant PC | 41 | ICC (EPIC platform, CellSearch) | Counts of all CTC types except CTC clusters were associated with high lactate dehydrogenase (P ≤ .0001) and low albumin (<0.05), all CTC and apoptotic CTC counts with the presence of visceral metastasis (P = .02 and P = .049, respectively), and number of nontraditional CTCs with OS (P = .03) | 20 |
| Chen et al (2018) | Metastatic and early PC (all stages) | 54 | ICC (CanPatrol platform, multi-RNAISH) | Positive rate of PGK1+/G6PD+ CTCs was higher in metastatic patients than in nonmetastatic patients (51.7% vs 8.0% [P = .002]). Hybrid CTCs were associated with Gleason score, tumor stage, tPSA level and cancer metastasis (P < .05). PGK1+/G6PD+ CTC number was correlated with the number of hybrid CTCs (r = 0.807; P < .001) | 19 |
| Carlsson et al (2016) | BRPC, mCSPC and mCRPC | 141 | ICC and NGS on tumor cells | For mCRPC, the presence of tumor cells in blood or BMA was associated with shorter PFS (median 151 vs 335 days, P < .001 or 140 vs 347 days, P = .003, respectively) and OS (median 415 days vs NR, P = .002 or 438 vs NR, P < .001). Patients with high proportion of tumor cell clusters in BMA showed shortest PFS (high 106 days vs low 246, P = .002) and OS (high 338 days vs low NR, P = .03), patients with tumor cells simultaneously in blood and BMA had shorter PFS (111 vs 182 days, P = .02) | 21 |
| Lorente et al (2016) | mCRPC with baseline CTC ≥5 cells/7.5 mL | 486 | ICC (CellSearch) | 30% CTC decline was associated with better survival at 4 weeks (14.4 vs 7.9 months; HR 0.45, 95% CI 0.36-0.56; P < .001), 8 weeks (15.4 vs 7.9 months; HR 0.41, 95% CI 0.33-0.53; P < .001) and 12 weeks (16.1 vs 9.7 months; HR 0.39, 95% CI 0.33-0.5; P < .001). PSA response was significantly associated with 30% CTC decline at 4 weeks (OR 14.8; P < .001), 8 weeks (OR 18; P < .001) and 12 weeks (OR 13.6; P < .001) | 69 |
| Climent et al (2017) | mCRPC with ECOG-PS 0-2, previous DTX treatment | 70 | ICC (CellSearch) | 30% CTC decline after 4 weeks of treatment showed favorable association with response based on PSA (77% vs 53%; P = .267), clinical benefit (68% vs 31%; P = .07), median OS (15.8 months vs 7.2 months) (P = .175) and median PSA-PFS (7.8 months vs 3.1 months; P = .004) | 70 |
| Study (year) | Inclusion criteria | N pats. | Detection method | Prognostic relevance | Reference |
|--------------|--------------------|---------|-----------------|----------------------|-----------|
| Budna-Tukan and Swierczewska et al (2019) | High-risk PC (PSA ≥20 ng/mL and/or Gleason score on biopsy ≥8 and/or clinical tumor stage ≥2c), before and after RT | 68 | Antibody-based assays (CellSearch, CellCollector, EPISPOT Assay) | No difference in matched-pair analysis of CTC counts before and after RT for all three assays (CellSearch P = .28, dual fluoro-EPISPOT P = .27, CellCollector P = .36) | 16 |
| Annala et al (2018) | mCRPC eligible for treatment with abiraterone and enzalutamide | 202 | Whole-exome and/or deep targeted sequencing on cfDNA | No differential efficacy between abiraterone and enzalutamide within any genomic or clinical subgroup. BRCA2 or ATM and TP53 were significantly associated with shorter TTP (P < .001). High AR gain in 43% of patients with primary resistance vs 18% of patients responded to therapy for >12 weeks (P = .01) | 71 |
| Scher et al (2016) | Progressive mCRPC undergoing a change in treatment | 161 | ICC (EPIC platform) | Frequency of ARv7+ CTCs increased by line of therapy; 3% prior to first-, 18% prior to second- and 31% prior to third or subsequent lines of therapy (P < .001). More favorable survival times with taxanes relative to ARS inhibitors for AR-V7+ patients (HR 0.24; 95% CI 0.10-0.57; P = .035). Pre-ARS inhibitor samples with AR-V7+ CTCs were associated with worse rPFS (median 2.3 vs 14.5 months; P < .001), time on therapy (median 2.1 vs 6.8 months; P < .001) and OS (median, 4.6 months vs not reached; P < .001). Difference in OS for pretaxane AR-V7+ vs AR-V7− samples (median, 8.9 vs 19.8 months; P < .001) | 72 |
| Singhal et al (2018) | mCRPC | 41 | ICC (EpCam microbeads) multiplex qPCR | Increased expression of WNT5a (HR 3.62; 95% CI 1.63-8.05; P = .002), AURKA (HR 5.56; 95% CI 1.79-17.20; P = .003) and BMP7 (HR 3.86; 95% CI 1.60-9.32; P = .003) were associated with earlier mortality (FDR < 10%). WNT5b, KLK2 and PSA were significantly associated with OS | 73 |
| Mateos et al (2017) | mCRPC with ECOG ≤2 and OS < 3 months, receiving taxane therapy | 29 | ICC (CellSearch) RT-qPCR | Significant lower OS in patients with CTC levels ≥5 CTCs/7.5 mL. High levels of AR, CYP19 and GDF15 were associated with poor PFS rates. AR, GDF15 and BIRC5 were predictors of OS. A logistic model using KLK3 and BIRC5 showed high specificity and sensitivity compared to CellSearch to discriminate patients with more aggressive evolution | 74 |
| Annala et al (2017) | mCRPC | 319 | Targeted Sequencing | Median time from ADT initiation to castration resistance was 11.8 months (95% CI 5.1-18.4) vs 19 months (95% CI 15.4-22.6; log-rank P = .031) in germline mutation-negative patients. Median PSA PFS on first-line AR-targeted therapy was 3.3 months (95% CI 2.7-3.9) vs 6.2 months (95% CI 5.1-7.3; log-rank P = .01) for patients without germline mutations, for patients receiving chemotherapy as initial therapy for mCRPC 7.2 months (95% CI 5.6-8.7) vs 8.0 months (95% CI 7.1-9.1; log-rank P = .127) for patients without germline mutations | 75 |

Abbreviations: ARS, AR signaling; BRPC, biochemically recurrent prostate cancer; CI, confidence interval; CSV, cell-surface vimentin; DSM, disease-specific mortality; FDR, false discovery rate; HR, hazard ratio; ICC, immunocytochemistry; mCRPC, metastatic castration-resistant prostate cancer; mCSPC, metastatic castration-sensitive prostate cancer; NR, not reached; OR, odds ratio; OS, overall survival; PC, prostate cancer; rPFS, radiographic progression-free survival; RT, radiotherapy; TTP, time to progression.
suggesting the rare occurrence of primary resistance, which may play a role if AR-targeted therapy is applied in earlier stages of PC.\textsuperscript{72} Besides immunostaining of the ARv7 protein, other assays have focused on the detection of ARv7 mRNA using RT-PCR\textsuperscript{22,79} or in situ hybridization with padlock probes,\textsuperscript{80} demonstrating substantial intrapatient heterogeneity of ARv7 expression on CTCs. A large proportion of patients not responding to next-generation ADT are ARv7 negative but harbor other AR variants (in particular ARv3) that might also confer resistance.\textsuperscript{74} Moreover, analysis of CTCs revealed a role of the proliferative Wnt signaling pathway, a downstream mediator of ARS, in androgen resistance, as it was significantly upregulated in the majority of patients.\textsuperscript{73} Finally, there is some preliminary evidence that CTC clusters might affect AR-targeted therapy\textsuperscript{21} (Table 3).

CTC characteristics might guide new forms of patient management such as prostate-specific membrane antigen (PSMA)-directed diagnostics and therapies.\textsuperscript{81} PSMA-directed positron emission tomography (PSMA-PET) has become a valuable tool in PC diagnostics\textsuperscript{82}; however, CTC analysis has revealed a striking intrapatient and interpatient heterogeneity of PSMA expression on CTCs of PC patients.\textsuperscript{81} Thus, future studies can show if CTC-PSMA analysis can help to guide PSMA-PET imaging.

1.4.2 | Circulating tumor-derived DNA

A comparative analysis of 45 mCRPC patients showed that ctDNA exhibits all driver DNA mutations present in matched metastatic tissue and also revealed exclusive mutations.\textsuperscript{83} Due to the genomic heterogeneity and the frequent occurrence of structural gene rearrangements in PC, mutations have a limited value as PC markers. In contrast, DNA methylation is one of the epigenetic changes that is involved in early tumorigenesis and occurs with high frequency in PC. Hence, different panels of DNA methylation markers have been tested as an LB test for early detection of PC from blood or urine DNA\textsuperscript{84-88} with high accuracy, targeting hypermethylation of ST6GALNAC3, CCDC181 and GSTP1me and promoter methylation of APCme, FOXA1me, GSTP1me, HOXD3me, RAR\textit{\textgamma}2me, RASSF1Ame, SEP-T9me and SOX17me (Table 4).

ctDNA is also a valuable target for genomic aberrations of the AR gene including mutations and amplifications or splice variants that can convey resistance to ADT; investigation of these biomarkers could identify patients that might benefit more from other therapeutic approaches such as taxane chemotherapy, poly-ADP-ribose polymerase inhibition or bone tropic radioisotopes.\textsuperscript{71,79} For example, the high number of AR amplifications (+8 copies) on ctDNA was associated with primary resistance to the ADT. Also, mutations in the AR ligand-binding domain tended to correlate with shorter time to progression.\textsuperscript{71} Primary resistance was also associated with genomic structural rearrangements resulting in truncated AR genes that encode AR proteins with intact N-terminal and DNA-binding domain but lack the ligand-binding domain.\textsuperscript{71} Patients resistant to ADT might profit from therapy with poly-ADP-ribose polymerase inhibitors such as olaparib, and ctDNA measurement can facilitate patient selection and monitoring of therapy.\textsuperscript{75}

1.5 | Lung cancer: clinical applications

Lung cancer (LC) is the most common cancer in the world with the highest mortality rate compared to other tumor entities\textsuperscript{89} (1.8 million deaths in 2018, WHO World Cancer Report). Nonsmall cell lung cancer (NSCLC) accounts for approximately 85% of all diagnosed LCs, where the most frequently diagnosed histological subtypes are adenocarcinoma (ADC, 40%-50% of diagnosis) and squamous cell carcinoma (SCC, 20-30% of diagnosis).\textsuperscript{90} Small cell lung cancer (SCLC) accounts for approximately 15% of all diagnosed LCs, where the patients are often heavy smokers and tumor cells express neuroendocrine markers.\textsuperscript{91}

1.5.1 | Circulating tumor-derived DNA

Lung cancer is frequently diagnosed too late to achieve curative resection, which points to the urgent need for improvement of early detection. The imaging methods nowadays used in some countries for LC screening is low-dosed computed tomography (LDCT). Even though with definition for a positive screen by LDCT given by United States-based National Lung Screening Trial, mortality was reduced, this method suffers from a high number of false-positive findings.\textsuperscript{89} Thus, several ongoing studies try to find out if ctDNA (or other LB analytes) can be used for early detection and complement or replace LDCT. For example, Yang et al included NSCLC and SCLC of Stages I to IV and mutations were detected in TP53, EGFR, BRAF, CTNNB1, ARID1A, ERBB2, PDGFR and KRAS genes with similar detection rates in earlier (Stages I and II) and later (III and IV) stages.\textsuperscript{92} Sensitive detection in early stages is crucial for an LC screening test. Although the mutations in tumor biopsies and plasma usually overlap well, ctDNA assessment appears to represent a wider spectrum of mutations, which may represent intrapatient tumor heterogeneity better than a tissue biopsy.\textsuperscript{93} Moreover, mutations in driver genes (such as EGFR, KRAS, ALK, BRAF, ERBB2, ROS1 and RET) were more frequently observed than nondriver mutations.\textsuperscript{93} Besides mutations, DNA methylation is also explored as ctDNA marker in lung cancer. Real-time PCR analysis of plasma from NSCLC and SCLC patients (Stages I to IV) showed that PTGER4/SHOX2 methylation could discriminate patients with and without malignant lung disease with high specificity and sensitivity.\textsuperscript{99}

Besides blood, cerebrospinal fluid (CSF) has been assessed for LB in patients with brain/central nervous system involvement in ADC\textsuperscript{96}; EGFR-activating mutations were consistent with those in primary tumors, while higher detection rates were found in CSF than plasma, indicating a blockade of free ctDNA release due to the blood-brain barrier. Interestingly, the majority of copy number variations that were detected in CSF ctDNA were unique and not identified in primary tissue,\textsuperscript{94} suggesting that brain metastases might undergo a unique selection of viable tumor clones.

cDNA assessment can be also used for prognostication (Table 5). A higher mutation load in the plasma ctDNA is correlated with survival in NSCLC.\textsuperscript{92} For example, high level of EGFR mutations in plasma of NSCLC patients prior to treatment with EGFR-tyrosine kinase
inhibitor (TKI) are correlated with increased tumor burden and poor prognosis.\textsuperscript{95} In SCLC, mutations in ARTX, SETBP1, PBRM1 and EP300 on cfDNA correlated with unfavorable OS and ATM, PBRM1 and SETBP1 with reduced PFS.\textsuperscript{91} TKIs targeting EGF, EML4-ALK fusion and ROS1 translocations are commonly used to treat NSCLC patients with the appropriate molecular tumor composition.\textsuperscript{100} EGF mutations (in particular EGF T790M), EGF amplifications, EML4-ALK fusion and ALK copy number gain on cfDNA in blood occured in NSCLC patients who developed resistance to TKIs.\textsuperscript{94,95,101-104} Besides EGF-T790M, rarer mutations also confer resistance to osimertinib including EGF G719A, EML4-ALK fusion, ERBB2 R143Q and EGFR C797S.\textsuperscript{101,102} At the moment, the only FDA-approved test for clinical utility is AS-PCR cobas EGFR Mutation Test v2, for identification of patients with metastatic NSCLC eligible for treatment with erlotinib.\textsuperscript{28,29} Furthermore, by cfDNA analysis, MET amplification and copy number gain were observed after development of resistance to TKI and after development of resistance to chemotherapy or immunotherapy.\textsuperscript{94,101} A recent case study of an ALK-positive patients has shown an 8-fold MET amplification in CTCs and an approximately 7-fold MET amplification in cfDNA after development of resistance to alectinib or crizotinib.\textsuperscript{105} In SCLC, quantitative changes in ctDNA levels correlated with responses to platinum-based chemotherapy.\textsuperscript{106} The signal-to-noise ratio was much higher in comparison with NSCLC cases, supporting future ctDNA monitoring in SCLC.\textsuperscript{106}

### 1.5.2 | Circulating tumor cells

The detection of CTCs from NSCLC patients is challenging, while SCLC patients exhibit on average more than 10 times higher CTC counts.\textsuperscript{107,108} Interestingly, CTCs are more common in pulmonary
| Study (year) | Inclusion criteria | N pats. | Detection method | Prognostic relevance | Reference |
|-------------|-------------------|---------|-----------------|---------------------|-----------|
| Yang et al (2017) | Stages I to IV of diverse cancers, mainly LC | 177 (103 of LC) | NGS (Guardant360 ctDNA test covering 73 cancer-related genes) | >3 mutations in plasma cfDNA is correlated with poor survival ($P = .0012$) | 92 |
| Tsui et al (2018) | Stage IV NSCLC | 50 | Gene panel for TAm-Seq PCR; digital PCR for quantification of exon 19 deletion, T790M and L858R EGFR mutations; shallow WGS | TKI-naïve patients with low levels of EGFR-activating mutation in pretreatment plasma tended to have better PFS and OS ($P = .06$ for both; patients with EGFR and TP53 mutations tended to have worse prognosis for PFS $P = .109$ and for OS $P = .035$) | 95 |
| Du et al (2018) | Stages I to IV SCLC | 24 | WGS and panel for targeted sequencing (127 genes across 12 tumor types) | Unfavorable OS is correlated with mutations in SETBP1, PBRM1, ATRX, EP300 Poor PFS is correlated with mutations in PIK3CG Favorable OS is correlated with mutations in PIK3CG Significant predictors in multivariate analysis: mutations in SETBP1, PBRM1, ATRX, EP300 and ATM (OS/PFS, $P = .0185/ .0294$; HR 2.1476/1.8998; 95% CI 1.1369-4.0564/1.0662-3.3849) | 91 |
| Hanssen et al (2018) | Patients with oligobrain metastasis and/or additional distant metastasis | 87 | ICC (CellSearch) | Brain metastatic CTC-positive patients have shorter OS, compared to CTC-negative patients ($P = .001$). CTC-positive patients with lung metastases have shorter OS ($P < .0001$). | 96 |
| Boffa et al (2017) | Patients with stages I to IV NSCLC | 112 | Not specified → blood was collected in Streck tubes and shipped to Epic Sciences | Patients with >1.1 PD-L1(+) CCAM/mL experienced a worse median survival (16.1 months vs not reached) and worse 2-year survival than those with ≤1.1 PD-L1(+) CCAM/mL (31.2% vs 78.8%, $P = .00159$). In multivariable Cox model adjusting for AJCC staging, expression of >1.1 PD-L1(+) CCAM/mL was an independent predictor of mortality risk (HR 3.85; 95% CI 1.64-9.09; $P = .002$). | 97 |
| Guibert et al (2018) | Patients with advanced NSCLC | 96 | ISET technology | Patients with high CTC count (43/89) had worse outcomes compared to those with a low CTC count (PFS: HR 2.44 [1.46-4.07], $P = .0004$; OS: HR 2.37 [1.22-4.60], $P = .0088$). PD-L1+ CTCs and PD-L1 expression in tissue had no significant impact on PFS or OS | 98 |
| De Wit et al (2019) | Stages IIIb and IV NSCLC | 97 | mFAST-SeqS assay, deep-Seq and Safe SeqS of BRAF, EGFR, KRAS and NRAS genes of cfDNA ICC (Cell Search) for EpCam$^{\text{high}}$ CTCs Microsieves (VyCap) for EpCam$^{\text{low}}$ CTCs | EpCam$^{\text{high}}$ CTCs (HR 2.1; 95% CI 1.2-3.7; $P = .014$), ctDNA (HR 1.9; 95% CI 1.1-3.4; $P = .032$) associated with poor OS EpCam$^{\text{low}}$ CTCs did not associate with OS | 99 |

**Abbreviations:** AJCC, American Joint Committee on Cancer; CCAM—all cells that are (a) nonapoptotic, (b) have a nucleus, (c) are CD45(−) and (d) have cytomorphometric features consistent with CTCs (size, shape, nuclear-to-cytoplasm ratio, etc.); CI, confidence interval; cfDNA, cell-free DNA; ctDNA, circulating tumor DNA; CTC, circulating tumor cell; HR, hazard ratio; ICC, immunocytochemistry; LC, lung cancer; mFAST-SeqS, modified Fast Aneuploidy Screening Test-Sequencing System; NGS, next-generation sequencing; NSCLC, nonsmall cell lung cancer; OS, overall survival; PFS, progression-free survival; SCLC, small cell lung cancer; Tam-Seq, tagged-amplicon deep sequencing; TKI, tyrosine kinase inhibitor; WGS, whole genome sequencing.
vein than in peripheral vein (43% vs 22%, respectively), suggesting that CTCs are easily trapped on their way from the primary lesion to distant sites. In addition, CTCs in NSCLC are more prone to undergo EMT leading to reduced EpCAM expression, which underlines the need for additional markers such as MET or combination of EGFR and HER3. PIK3CA and ALDH1. Illie et al observed a strong correlation between MET status on tumor tissue and CTCs; moreover, CTCs isolated with the label-independent ISET filters expressed more frequently MET (72% of CTCs) as compared to the EpCAM-based CellSearch system (11% of CTCs). The ongoing discovery of CTC biomarkers might also lead to the identification of CTCs with varying metastatic potential.

Several studies have shown the prognostic relevance of CTC counts in NSCLC and SCLC (Table 5). For example, patients with detectable circulating tumor micro-emboli or ≥2 CTCs in peripheral blood had 8-fold increased risk of disease recurrence and 7-fold increased risk of death. Patients with brain metastases have reduced frequency of CTCs in NSCLC but even the presence of these CTC was associated with shorter OS.

Further characterization of CTCs may provide better discrimination of the prognostic value of CTCs. Although some studies on NSCLC showed a correlation between PD-L1 expression on CTCs with poor outcome, others could not find such a correlation. Moreover, EpCAM$^{low}$ CTCs were prognostic in Stage IIIb/IV NSCLC, but EpCAM$^{low}$ CTCs were not significantly correlated to outcome, consistent with the experimental findings that tumor cells with complete EMT are not able to form metastasis.

CTC analysis is not restricted to mutations but can also reveal mechanisms of resistance to therapy that are based on transcriptional plasticity. Regarding the success of immune checkpoint inhibition in NSCLC therapy, the assessment of PD-L1 on CTCs has received attention over the past 5 years. Interestingly, two recent studies suggest that CTCs might provide additional information compared to analysis of tumor biopsies. Besides technical issues such as sampling errors, this discordance might also reflect the biology of NSCLC and suggests that CTCs are selected cells with a particular phenotype different from the bulk of the primary tumor. Interestingly, patients with PD-L1-positive CTCs at baseline were more frequently nonresponders to nivolumab compared to patients who had PD-L1-negative CTCs, and after developing resistance to nivolumab all patients had PD-L1-positive CTCs. Nivolumab is an antibody that binds to PD1 on T cells and inhibits the interaction with PD-L1 on tumor cells, resulting in T-cell activation and tumor cell lysis. Future studies have to explore why the emergence of PD-L1-positive CTCs is associated with the resistance to immunotherapy.

1.6 | Colorectal cancer: clinical applications

Colorectal cancer (CRC) is the third-most common cancer in both sexes worldwide and ranks second in terms of mortality (880,000 deaths in 2018; WHO World Cancer Report, 2020). In nonmetastatic CRC, the preoperative CTC detection is an independent prognostic marker. Consistent with the high rates of liver metastasis in CRC, comparative evaluation of mesenteric and peripheral blood showed that CTCs are trapped in the liver. In Stage III patients undergoing curative resection CTCs followed by mFOLFOX chemotherapy, CTC counts predicted relapse. Thus, CTC detection might help to identify high-risk CRC patients. Interventional studies are now needed to assess whether CRC patients with CTCs will profit from chemotherapy.

In advanced CRC, CTC enumeration before and during treatment predicts PFS and OS and provides additional information beyond CT imaging. Moreover, molecular characterization of CTCs helps to get information for targeted therapy, including KRAS, BRAF and PIK3CA mutations. For example, the effectiveness of anti-EGFR antibody therapy in patients with CRC is negatively affected by mutations in KRAS, which encodes a key GTPase that orchestrates signaling downstream of EGFR. An in-depth analysis of individual CTCs from patients with CRC demonstrated striking levels of intrapatient and interpatient heterogeneity in KRAS status. The occurrence and concordance of these mutations in metastatic colorectal cancer may vary among primary tumors, CTCs and metastatic tumors and during treatment. Thus, CTCs might help to understand tumor evolution in CRC.

A recent prospective trial on more than 600 patients also indicated the potential of CTCs for early detection of CRC; Tsai et al showed a significant association between CTC counts and stages of adenoma-carcinoma progression (likelihood ratio $P$ value of $<.0001$).

1.6.2 | Circulating tumor-derived DNA

cDNA analysis has also contributed to a better understanding of tumor evolution and response to therapy in patients with CRC. Despite a high level of concordance between the mutational status of KRAS in tumor tissue and cDNA, cDNA can sometimes harbor KRAS mutations that are not detected in primary lesion. The Idylla ctKRAS mutation test and the OncoBEAM RAS CRC IVD Kit are qPCR and dPCR assays, respectively, for KRAS mutation detection. Sequential cDNA analysis during EGFR inhibition has revealed that KRAS and NRAS mutations can rapidly emerge as a result of the selective pressure exerted by targeted therapy. Interestingly, the emergent population of KRAS-mutant subclones could decline upon withdrawal of anti-EGFR therapy, suggesting the potential to guide "cyclical therapy" characterized by sequential withdrawal and reintroduction of EGFR inhibitors on the basis of cDNA analyses. Patient-specific cDNA assays can be developed by mutation analysis of primary tumors. Furthermore, the cDNA analyses also assisted in distinguishing recurrent CRC from a second primary cancer.

The evaluation of early changes in cDNA concentration as a marker of therapeutic efficacy is another important goal; by...
analyzing the evolution of the ctDNA concentration at inclusion and before second or third chemotherapy cycle, patients could be clearly classified in good or bad ctDNA responder.\textsuperscript{138} In a prospective analysis of 1046 plasma samples from 230 patients with Stage II colon cancer using NGS-based assays,\textsuperscript{139} ctDNA was detected after surgery in 7.9\% of patients who received no adjuvant chemotherapy. After a median follow-up duration of 27 months, the recurrence rate was higher in the ctDNA-positive patients than in the ctDNA-negative patients (78.7\% vs 9.8\%; HR 18.0, 95\% CI 7.9-40.0; \(P < .001\)).\textsuperscript{139} CtDNA detection after completion of adjuvant chemotherapy was also associated with shorter relapse-free survival (HR 11.0, 95\% CI 1.8-68; \(P = .001\)). Taken together, these results indicate that postoperative ctDNA detection provides evidence of MRD that is relevant to the clinical outcomes of CRC patients. The average lead time to imaging in the pioneering study by Scholer et al\textsuperscript{140} was 9.4 months compared to CT-based detection of CRC recurrence. Future interventional studies will show whether this lead time is sufficient for early therapeutic intervention to prevent metastatic relapse.

Methylation analysis of ctDNA has potential as diagnostic and prognostic biomarker in CRC. For example, Luo et al have recently shown that a single ctDNA methylation marker, cg10673833, yielded sensitivity of 89.7\% and specificity of 86.8\% for detection of CRC and precancerous lesions in a high-risk population of 1493 participants in a prospective cohort study.\textsuperscript{141} The prognostic prediction model also effectively predicted the prognosis and survival of patients with CRC (\(P < .001\)).

2 | CONCLUSIONS

The presented studies support the clinical validity of both ctDNA and CTC for improved risk assessment (staging), monitoring of cancer therapies and early detection of relapse in cancer patients. For cancer screening, ctDNA has the advantage of higher concentrations of bioanalytes compared to the very low CTC counts in early-stage patients. However, it should be noted that the concentration of ctDNA is also low in early-stage cancer patients, which has stimulated the recent development of ultrasensitive ctDNA assays. Applying these assays has revealed a background of cancer-associated mutations in normal white blood cells, which may contaminate the ctDNA fraction.\textsuperscript{23}

Tumor heterogeneity is a hallmark of solid tumors and has an impact on the classification, diagnosis and future treatment of cancer. Assessment of ctDNA and in particular CTCs can be also used to encompass intrapatient and interpatient tumor heterogeneity in cancer patients.\textsuperscript{3,142} The degree of tumor heterogeneity in individual patients is an underinvestigated mechanism of resistance, which can only be targeted by combinatorial therapies. Addressing the challenge of low CTC numbers, apheresis can highly increase the number of CTCs, allowing better downstream analysis of intrapatient heterogeneity within research studies on selected patients.\textsuperscript{143}

Both ctDNA and CTCs have advantages and disadvantages as LB markers. Although isolating ctDNA from blood plasma is easy, capturing CTCs from whole blood is more demanding. The efforts needed for the subsequent downstream analyses depend on the desired read-out and the stage of disease. Detecting single mutations or CTCs in patients with advanced disease is less demanding than assessing the broad panel of mutations in early-stage patients with low amounts of ctDNA or CTCs.\textsuperscript{3,6} The concentration of ctDNA and CTCs depends also on the localization of the tumor tissue (eg, primary or metastatic brain lesions are difficult to assess by blood analyses).

For detection of druggable mutations (eg, in EGFR gene), ctDNA analyses have been well accepted. In addition, expression signatures derived from CTCs might enable real-time monitoring of therapy-induced resistance pathways and this information can be complementary to the analysis of mutations (eg, the combination of tumor mutational burden assessed on ctDNA and PD-L1 expression on CTCs in patients receiving immune checkpoint inhibition therapies).

ctDNA and CTC assays need more standardization; international consortia like the European Liquid Biopsy Society (ELBS, www.elbs.eu) can play an important role in this effort.\textsuperscript{35} Additional LB biomarkers are on the horizon, including extracellular vesicles,\textsuperscript{8} tumor-educated platelets\textsuperscript{10} or circulating microRNAs.\textsuperscript{144} Composite biomarker panels need to be tested in clinical studies with well-established endpoints to demonstrate clinical validity and utility, which will be key to introduce LB into clinical practice. Furthermore, experimental studies must gain more knowledge on the biology of LB markers, which can then in turn be retranslated to the bedside to improve the clinical use of LB analytes.

ACKNOWLEDGMENTS
Klaus Pantel received funding from European Union’s Horizon 2020 research and innovation program under the Marie Skłodowska-Curie grant agreement No. 765492, European IMI research project CANCER-ID (115749-CANCER-ID), Deutsche Krebshilfe (Nr. 70112504), Deutsche Forschungsgemeinschaft (DFG) SPP2084 μBone and ERC Advanced Investigator Grant INJURMET (Nr. 834974). Open access funding enabled and organized by Projekt DEAL.

CONFLICT OF INTEREST
KP has ongoing patent applications related to CTCs. KP has received honoraria from Menarini, Agena, Novartis, Roche, and Sanofi and research funding from European Federation of Pharmaceutical Industries and Associations (EFPIA) partners (Angle, Menarini and Servier) of the CANCER-ID program of the European Union-EFPIA Innovative Medicines Initiative. The other authors declare no conflict of interest.

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