Review

Clinical Cancer Research

Circulating Tumor Cells and Circulating Tumor DNA: Challenges and Opportunities on the Path to Clinical Utility

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Abstract

Recent technological advances have enabled the detection and detailed characterization of circulating tumor cells (CTC) and circulating tumor DNA (ctDNA) in blood samples from patients with cancer. Often referred to as a "liquid biopsy," CTCs and ctDNA are expected to provide real-time monitoring of tumor evolution and therapeutic efficacy, with the potential for improved cancer diagnosis and treatment. In this review, we focus on these opportunities as well as the challenges that should be addressed so that these tools may eventually be implemented into routine clinical care. *Clin Cancer Res; 21(21); 4786–800. ©2015 AACR.*

Disclosure of Potential Conflicts of Interest

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CME Staff Planners' Disclosures

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Learning Objectives

Upon completion of this activity, the participant should have a better understanding of the technologies used to detect and characterize circulating tumor cells (CTC) and circulating tumor DNA (ctDNA), different strategies of testing their clinical utility, and the potential future applications of CTCs and ctDNA in the field of precision medicine.

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Introduction

Next-generation sequencing (NGS) studies performed in bulk primary tumor specimens have demonstrated extensive interpatient (1) and, more importantly, intrapatient (2) heterogeneity. Recently, single-cell analyses of primary breast tumors have provided higher-resolution evidence of intratumor heterogeneity (3) with the finding of substantial clonal diversity and subclonal heterogeneity, such that no two individual tumor cells are genetically identical. Beyond spatial heterogeneity, solid tumors also exhibit temporal heterogeneity, evolving over time under selection pressure from treatment (4, 5). Thus, there is an increased appreciation that the management of metastatic disease should rely on analysis of contemporary tumor tissue rather than on the primary tumor diagnosed years ago (6). However, obtaining serial samples of metastatic tissue is impractical and complicated by spatial heterogeneity and sampling bias. Analysis of circulating tumor cells (CTC) and circulating tumor DNA (ctDNA) thus holds appeal and promise for noninvasive real-time assessment of tumor molecular profiles during the course of disease. Evaluation of CTCs and ctDNA may enable more sensitive monitoring of treatment efficacy and thereby guide drug selection, even potentially in the adjuvant setting where no such tools exist today.

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Table 1. CTC detection/capture methods with recently published clinical studies, 2010–2015

Enrichment methods and devices	Technology	Comments	Commercialized	Recent clinical references
Affinity-based capture (CTC surface antibody)				
Positive enrichment				
CELLSEARCH (Janssen Diagnostics)	EpCAM-coated ferrofluid nanoparticles enrichment, then IF for CK8, 18, 19; CD45; DAPI	Only FDA-approved device for patients with metastatic breast, colorectal, and prostate cancers	Yes	(23, 24, 35)
Adnatest, Adnagen	Antibody-coated magnetic beads for enrichment, then enriched cells tested by multiplex RT-PCR gene panels	Analyzes gene expression in enriched CTCs from patients with breast, prostate, colon, and ovarian cancers	Yes	(43, 127, 128)
CTC-iChip^{pos}	Combined bead and microfluidic (inertial focusing) enrichment (EpCAM^{+} mode)	Licensed by Janssen Diagnostics	In progress—Janssen Diagnostics	(129)
CytoTrack	Sample spread on glass disc that is rotated at high speed and fluorescently scanned with a laser beam	Similar recovery of rare cells as CELLSEARCH	Yes	(ref. 130—published clinical data pending)
Ephesia	Magnetic particles functionalized with EpCAM antibodies are self-assembled in a microfluidic platform	High capture specificity	No	(131)
GEDI	Microfluidic geometrically enhanced differential immunocapture (GEDI) with antibodies against specific membrane antigens such as PSMA or HER2	High capture efficiency and purity from unprocessed blood samples	In progress—Captura Diagnostics	(132, 133)
GEM chip	Geometrically enhanced mixing (GEM) chip structure increases interactions between CTCs and the surface of the antibody-coated chip	Appropriate for viable cell capture and culture	No	(134)
Graphene oxide–GO Chip	Capture using functionalized graphene oxide nanosheets on a patterned gold surface	High capture yield, even for 3–5 spiked cells/mL	No	(135)
HB-Chip	Herringbone chip structure increases interactions between CTCs and the surface of the antibody-coated chip by chaotic mixing	Although group has developed subsequent chips for CTC capture, this one was used for EMT studies in CTCs	No	(82)
ImageStream (Amnis)	Immunomagnetic sorting, followed by flow cytometry and fluorescence microscopy	Precision lower when evaluating low number of CTCs, although upgraded device available that analyzes 5,000 cells/s	Yes	(ref. 136—no published clinical data)
IsoFlux (Fluxion)	Microfluidic platform combining flow control and immunomagnetic capture	Workflow for mutational analysis of CTCs	Yes	(137)
LiquidBiopsy (Cynvenio)	Immunomagnetic capture within a microfluidic chip	Direct automated DNA profiling	Yes	(ref. 138—no published clinical data)
MACS system (Miltenyi Biotec)	Immunomagnetic CTC enrichment—by antibodies against cell surface markers or by an intracellular anti-pan cytokeratin antibody	Does not identify CK-negative CTCs but able to identify EpCAM-negative CTCs with other cell surface markers or by CD45 depletion (negative enrichment, below)	Yes	(139, 140)
Magnetic sifter	Flow-through fluidic array with magnetic pore structure for efficient separation of cells labeled with magnetic nanoparticles	Magnetically labeled target cells captured at the pore edges can then be released for culture or lysed and placed on a biosensor chip for mutational analysis	No	(141)
MagSweeper (Illumina)	Immunomagnetic capture by antibody against EpCAM or other cell surface marker	First high-throughput single-cell CTC transcriptional profiling studies in breast cancer; single-cell mutational analysis	Yes	(8, 54, 58)

(Continued on the following page)

Table 1. CTC detection/capture methods with recently published clinical studies, 2010–2015 (Cont'd)

Enrichment methods and devices	Technology	Comments	Commercialized	Recent clinical references
		in breast cancer, and single-cell whole-exome sequencing in prostate cancer		
Modular CTC sinusoidal microsystem (BioFluidica)	Three functional modules for CTC selection, counting, and phenotypic identification	Electrical sensor for counting and determining viability	Yes	(142)
OncoCEE (Biocept)	In Cell Enrichment and Extraction (CEE) microchannel, CTCs enriched with 10-antibody cocktail and analyzed by ICC and/or FISH	Able to identify CK-positive and -negative CTCs, HER2-positive CTCs, and determination of hormone receptor status	Yes	(14, 143)
Negative enrichment				
CTC-iChip^{neg}	Deterministic lateral displacement, inertial focusing, and magnetophoresis to rapidly separate CTCs from WBCs labeled with anti-CD45 and anti-CD66b Abs	Licensed by Janssen Diagnostics	In progress—Janssen Diagnostics	(144)
Microfluidic cell concentrator (MCC)	Method of concentrating pre-enriched sample into a device suitable for downstream CTC analysis	Potential for CTC analysis in multiple tumor types	No	(145)
MACS system (Miltenyi Biotec)	Immunomagnetic CTC-negative enrichment by antibodies against CD45		Yes	(146)
Quadrupole magnetic separator	Red cell lysis and immunomagnetic CD45^{+} depletion followed by IF staining	Study demonstrating rare cell heterogeneity	No	(147)
RosetteSep CTC Enrichment Cocktail; EasySep CD45 Depletion (STEMCELL Technologies)	Immunodensity negative selection cocktail for breast and lung cancers. Also have anti-CD45 immunodensity or immunomagnetic depletion	Unwanted cells are targeted for removal with Tetrameric Antibody Complexes that pellets with RBCs; also have anti-CD45 beads	Yes	(78, 148)
In vivo Ab-based capture				
CellCollector (GILUPI)	EpCAM-coated wire placed intravenously	*In vivo* detection, large blood volume screened	Yes	(149)
Label-free capture (size-based)				
Size-based microfiltration				
CellSieve (Creatv MicroTech)	Filter-based enrichment	High capture efficiency	Yes	(90, 150)
ISET (Rarecells)	Filter-based enrichment	Detection of *ALK* rearrangements on CTCs for monitoring treatment with crizotinib	Yes	(52, 151, 152)
Parylene filter (Circulogix)	Filter-based enrichment	Viable CTC capture using a 3D device	Yes	(153, 154)
ScreenCell (ScreenCell)	Filter-based enrichment	Allows downstream phenotypic analysis and cell culture	Yes	(155)
Microfluidic devices				
ClearCell FX (Clearbridge BioMedics)	Size-based separation based on Dean Flow Fractionation (inertial focusing)	Viable CTCs for downstream analysis or culture	Yes	(70, 156)
Cluster-Chip	Multiple rows of shifted triangular pillars; low shear stress	Single-cells pass through; clusters contain quiescent and proliferating cells as well as other cell types	No	(85)
Vortex	Combined use of microscale vortices and inertial focusing	Viable CTC isolation with high purity (>50%)	Yes	(157)
Label-free separation based on biophysical properties				
ApoStream (ApoCell)	Continuous flow Dielectrophoretic Field-Flow Fractionation (DEP-FFF)	Detection independent of EpCAM expression; useful for viability analysis and culture	Yes	(158)
DEPArray (Silicon Biosystems)	Moving dielectrophoretic cages	Isolation of single pure CTCs for dowstream analysis	Yes	(51, 56, 159)

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Table 1. CTC detection/capture methods with recently published clinical studies, 2010–2015 (Cont'd)

Enrichment methods and devices	Technology	Comments	Commercialized	Recent clinical references
Direct imaging				
Epic (Epic Sciences)	RBC lysis and IF for CK, CD45, and DAPI, or other markers, then high-definition imaging	Unbiased screen of all blood nucleated cells for detection of individual CTCs and clusters	Yes	(16)
FASTcell (SRI)	Fiber optic array scanning technology (FAST)	Enables the simultaneous detection of multiple tumor-specific biomarkers in a multiplexed fashion	Yes	(160)
AccuCyte–CyteFinder (RareCyte)	Density-based cell separation and automated imaging with optional single-cell picking	Dual technology platform that facilitates single-cell analysis	Yes	(161)
OncoQuick (Grenier Bio-One)	50-mL centrifugation tube with porous barrier on top of a proprietary separation medium for CTC enrichment by density centrifugation and washing	CTCs have lighter buoyant density than WBCs and RBCs, which migrate through the porous barrier, whereas CTCs remain at plasma interface	Yes	(162)
Functional assays				
EPISOT	CD45 depletion and short-term culture. IF for different markers.	Detection based on protein secretion	No	(163)
Vita-Assay (Vitatex)	Density gradient centrifugation then cells applied to collagen adhesion matrix (CAM)	Detection based on invasion properties	Yes	(164)
***In vivo* detection**				
PAFC	Photoacoustic flow cytometry	Increased sensitivity by examination of the entire blood volume *in vivo*	No	(ref. 165—preclinical models)

Abbreviations: CK, cytokeratin; DAPI, 4′,6-diamidino-2-phenylindole; ICC, immunocytochemistry; IF, immunofluorescence; RBCs, red blood cells; WBCs, white blood cells.

Circulating Tumor Cells

CTCs can be found in the bloodstream of patients with cancer as single cells or, less commonly, as cell clusters, and CTC levels have been shown to have clinical associations with survival and response to therapy (7). CTCs are presumptively shed into the vasculature from primary tumor or distant metastatic foci and are postulated to contain subpopulations of "culprit cells," which are responsible for seeding and reseeding metastases, eventually leading to patient demise (8). It is thus appealing to not only enumerate CTCs for measuring disease burden and detection of minimal residual disease but also to characterize CTCs as a means to target therapy to these putative culprit cells.

CTC enrichment and detection technologies

Several reviews have discussed the various CTC enrichment and detection technologies (7, 9–12). Table 1 presents an updated list of CTC assays that have been used to test patient samples within the past 5 years, along with their commercialization status. CTC detection or capture methods can be broadly categorized as either label dependent, using positive enrichment with cell surface markers such as epithelial cell adhesion molecule (EPCAM), also used in *in vivo* capture techniques, or label independent, enriching for CTCs based on negative selection, size, or other biophysical properties; other strategies include direct imaging of CTCs and functional assays. A significant issue in detecting and capturing CTCs by label-dependent methods is the lack of reliable immunocytochemically identifiable markers that distinguish them from normal epithelial cells. As such, because epithelial cells are rarely present in blood samples from healthy individuals and because circulating epithelial cells (CEC) in patients with cancer often carry the same genetic aberrations seen in the primary tumor (13), the common definition of CTCs has been equivalent to that of CECs: nucleated cells in the bloodstream that express epithelial cytokeratins and do not express the white blood cell surface antigen CD45. More recently, cytokeratin-negative CTCs have been identified, potentially representing tumor cells undergoing epithelial–mesenchymal transition (EMT), or alternatively, cancer stem cells that have not yet shown epithelial differentiation (14–17).

CTCs in clinical trials

Some technologies listed in Table 1 may detect distinct CTC subpopulations and therefore could be used in different clinical scenarios in the future. However, for any technology to be used in the clinic, demonstration of analytic validity (the accuracy of the test to measure the target of interest), clinical validity (the value of the test to predict the clinical outcome), and ultimately clinical utility (ability of the test to lead to improved clinical outcome when treatment choice is informed by test results) is required (9, 18). The only system currently approved by the FDA as an aid in monitoring patients with metastatic breast, colorectal, or prostate cancer is CELLSEARCH (Janssen Diagnostics; refs. 19–21). Recent data suggest that this technology can also be used for clinical trials across multiple laboratories in the nonmetastatic setting provided that continuous training and central image review is performed (22).

In April 2015, a search in the "ClinicalTrials.gov" website using the keywords "circulating tumor cell" revealed 296 studies involving CTCs. Table 2 refers to studies in multiple tumor types using CTC enumeration or characterization as an inclusion criterion. Table 3 refers to studies for the development and/or validation of CTC assays for a particular indication.

Table 2. Ongoing studies that have CTC detection or characterization as inclusion criterion

Trial	Inclusion criteria	Estimated enrollment	Study design	Primary endpoint
CirCe01 NCT01349842 Phase III (randomized)	MBC, starting third-line chemotherapy, CTCs positive	568	Early change of CT based on CTCs vs. based on clinical and radiologic criteria	OS
STIC-CTC NCT01710605 Phase III (randomized)	MBC, HR-positive, HER2-negative PT, starting first-line treatment, available CTC results	1,000	Physician vs. CTCs-driven choice for first-line treatment (ET vs. CT)	PFS, economic evaluation
DETECT-III NCT01619111 Phase III (randomized)	MBC, HER2-negative PT, ≥1 CTC HER2-positive/7.5 mL	120	(ET or CT) ± lapatinib	CTC clearance rate
Treat-CTC NCT01548677 Phase II (randomized)	EBC, HER2-negative PT, ≥1 CTC/15 mL after (neo)adjuvant chemotherapy and breast surgery	174	Adjuvant trastuzumab × 6 cycles vs. observation	CTC detection (week 18)
DETECT-IV NCT02035813 Phase II (2 cohorts)	MBC, HER2-negative PT ≥1 CTC HER2-negative/7.5 mL	520	Everolimus + ET eribulin	PFS
NCT01975142 Phase II (single arm)	MBC, HER2-negative PT, CTC HER2-amplified	480	Trastuzumab emtansine (T-DM1)	Tumor RR
VISNU-1 NCT01640405 Phase III (randomized)	MCRC, *KRAS* wild-type, no treatment for MCRC, >3 CTCs/7.5 mL	350	FOLFOX6 + bevacizumab vs. FOLFOXIRI + bevacizumab	PFS
VISNU-2 NCT01640444 Phase II (randomized)	MCRC, *KRAS* wild-type, no treatment for MCRC, <3 CTCs/7.5 mL	240	FOLFIRI + bevacizumab vs. FOLFIRI + cetuximab	PFS

NOTE: All the studies shown in this table use CELLSEARCH technology for CTC detection and/or characterization.
Abbreviations: EBC, early breast cancer; ET, endocrine treatment; FOLFIRI, chemotherapy regimen including fluorouracil, leucovorin, and irinotecan; FOLFOX6, chemotherapy regimen including fluorouracil, leucovorin, and oxaliplatin; FOLFOXIRI, chemotherapy regimen including fluorouracil, leucovorin, oxaliplatin, and irinotecan; HR, hormone receptor; MBC, metastatic breast cancer; MCRC, metastatic colorectal cancer; PFS, progression free survival; PT, primary tumor; RR, response rate.

CTC enumeration

A recent pooled analysis provided level-one evidence for the clinical validity of elevated CTC levels as a marker of poor prognosis in metastatic breast cancer (23). However, the value of CTC enumeration for treatment decision making in metastatic breast cancer was prospectively tested in the Southwest Oncology Group (SWOG) S0500 clinical trial (24). The SWOG trial evaluated the benefit of an early change in chemotherapy for patients with persistently increased CTCs at first follow-up after starting first-line chemotherapy. Of 595 evaluable patients, 123 patients with persistently elevated CTCs on day 21 of therapy were randomized to either continue the same treatment or to switch to an alternative chemotherapy of physician's choice. In this trial, an early switch to an alternative chemotherapy did not increase overall survival (OS). Although CTCs were strongly prognostic, the absence of a survival benefit from changing treatment based on elevated CTC counts suggests that earlier detection of relapse can only be important when a more effective treatment is available: Switching from one ineffective therapy to another ineffective therapy does not change outcome. Instead, changing treatment based on CTC molecular characterization might be a more promising approach to test.

In nonmetastatic breast cancer, detection of elevated CTC levels using CELLSEARCH and other platforms (25–29) is also

Table 3. Studies for the development and/or validation of CTC assays for a particular indication

Trial	Disease	Inclusion criteria	Estimated enrollment	Technology	Objective of CTC assay
COMETI Phase II NCT01701050	MBC	ER-positive/HER2-negative PT Progression after at least one line of ET	200	CELLSEARCH	To identify patients with rapid progression (within 3 mo) to a new line of ET
NCT01660776	MBC NCSLC HL DLBCL	Untreated patients	325	Multiparameter flow cytometry CELLSEARCH	To describe of blood cell types producing soluble PD-L1
NCT01830426	NSCLC	Suspected lung cancer	429	EPIC Sciences	To test CTC assay as a surrogate for diagnosis in suspected lung cancer
NCT02372448	NSCLC	Stage IIIb/IV nonsquamous NSCLC *ALK* rearrangement result by FISH analysis (gold standard method) on tumor tissue	224	ISET	To validate CTC as alternative to tumor tissue for *ALK* analysis
NCT01558349	Melanoma	Stage 4 melanoma	82	EPISPOT, CELLSEARCH	To detect circulating melanoma cells

Abbreviations: ALK, anaplastic lymphoma kinase; DLBCL, diffuse large B-cell lymphoma; ER, estrogen receptor; ET, endocrine treatment; HL, Hodgkin lymphoma; HNSCC, head and neck squamous cell carcinoma; MBC, metastatic breast cancer; NSCLC, non–small cell lung cancer; PD-L1, programmed death-ligand 1; PT, primary tumor.

associated with adverse prognosis. The ongoing Treat CTC trial (NCT01548677; Table 2) is assessing CTC dynamics as an early signal of drug activity. This trial enrolls women with primary high-risk *HER2*-nonamplified breast cancer who have detectable CTCs after completing surgery and (neo)adjuvant chemotherapy. It evaluates whether six cycles of trastuzumab (a humanized monoclonal antibody targeting the HER2 growth factor receptor) compared with observation alone will eliminate the persistent CTCs. This trial was based on several lines of evidence: (i) in preclinical models, trastuzumab appears to target the cancer stem cell population in a process that does not require *HER2* gene amplification (30); (ii) subset analyses of prospective trials demonstrate similar trastuzumab benefit for women with HER2-positive tumors by local testing but deemed HER2-negative by central pathology review (31, 32); and (iii) a single-center randomized phase II study of 75 patients with HER2-negative early breast cancer found that short-course trastuzumab can eliminate chemotherapy-resistant CK19 mRNA-positive CTCs and improve patient outcome compared with observation (33). Additional studies using CTCs in breast cancer are listed in Tables 2 and 3.

CTC enumeration has also been shown to provide prognostic information in metastatic castration-resistant prostate cancer (34, 35). In the COU-AA-301 registration trial that compared abiraterone plus prednisone with prednisone alone, the combination of CTC enumeration and lactate dehydrogenase (LDH) levels at 12 weeks posttreatment was shown to be a surrogate for OS at the individual patient level (36). Efforts are ongoing to validate this biomarker panel.

Clinical studies using CTCs in tumor types other than breast and prostate cancer are described in Tables 2 and 3.

CTC characterization

Protein expression in CTCs. Beyond CTC enumeration, characterization of protein expression on CTCs has also been used to guide treatment selection in clinical trials (Table 2). In breast cancer, HER2 protein expression on CTCs has been assessed using the CELLSEARCH technology, with demonstration that some women with HER2-negative breast cancer may have detectable HER2-positive CTCs (37, 38). However, a phase II study of single-agent lapatinib (an anti-HER2 tyrosine kinase inhibitor) did not find objective responses in patients with metastatic breast cancer with HER2-negative primary tumors and HER2-positive CTCs at study entry (39). Of 139 screened HER2-negative patients, only 96 (69%) had ≥2 CTCs, and only 7 (5%) had ≥50% HER2-positive CTCs and received treatment. One patient (1 of 139 screened patients) had durable disease stabilization, having received lapatinib as a third line of therapy, although efficacy analysis could not be done due to the low number of treated patients. Of note, of the 7 treated patients, the 6 who progressed received lapatinib as ≥fourth line therapy for advanced disease, raising the question of whether HER2 status of CTCs represents HER2 status of the bulk of metastases in very late-stage disease. An ongoing phase III trial (DETECT III, NCT01619111) is evaluating the role of adding lapatinib to chemotherapy in this same patient population (Table 2). Other investigators are using CELLSEARCH to monitor endocrine resistance in ER-positive HER2-negative metastatic breast cancer. To that end, a score based on CTC enumeration and characterization for estrogen receptor (ER), Bcl-2, HER2, and Ki67, the CTC-Endocrine Therapy Index (CTC-ETI; ref. 40), is currently being tested in the COMETI Phase II study (NCT01701050; Table 3).

CTC protein expression has also been characterized in other tumor types. In patients with metastatic colorectal cancer, thymidylate synthase expression in CTCs has been studied as a potential marker of resistance to 5-fluorouracil (41). Immunofluorescent markers have been used to study androgen receptor (AR) signaling in CTCs from patients with metastatic prostate cancer to tailor hormonal treatment approaches (42).

RNA expression in CTCs. An emerging area of investigation is transcriptional profiling of CTCs to help guide real-time drug selection. For example, a postulated reason that patients with castration-resistant prostate cancer (CRPC) may not respond to drugs that inhibit or impair AR signaling may be the presence of AR splice variants within their tumor cells. This was demonstrated by examining mRNA from CTCs collected prospectively from patients with metastatic CRPC who were enrolled in a clinical trial of abiraterone or enzalutamide treatment. When CTC mRNA was assayed for the splice variant AR-V7, a constitutively active isoform of the AR that lacks the ligand-binding domain, there was a significant association with therapeutic resistance to abiraterone and enzalutamide, drugs that indirectly (abiraterone) or directly (enzulatamide) target the AR, where this ligand-binding domain is present (43).

However, an important challenge with RNA expression analysis of CTC-enriched cell fractions is the potentially confounding signal from contaminating leukocytes. To address this challenge, multiplex PCR with CTC-specific mRNAs (44–46) and single-cell approaches are being explored. High-dimensional single-cell transcriptional profiling of CTCs purified using the MagSweeper, an immunomagnetic enrichment technology (47), revealed significant CTC heterogeneity, even within the same blood draw, suggesting a need for multidrug therapy to approach tumor molecular diversity (8). Importantly, CTCs also showed markedly different gene expression profiles compared with those in single cells from breast cancer cell lines, suggesting that CTC analysis might complement cell-line analysis in drug development. Similarly, prostate cancer also appears to be characterized by CTC heterogeneity, with distinct differences in single-cell expression of EMT-related genes between CTCs from castration-sensitive and castrate-resistant cancers (48).

DNA aberrations in CTCs. Several proof-of-concept studies have demonstrated the feasibility of detecting specific somatic mutations or other genetic alterations in pooled or single CTCs from patients with various tumor types (49–54). Moreover, nontargeted approaches are now being used to analyze whole-genome copy number aberrations in single CTCs by array comparative genomic hybridization (aCGH) and NGS techniques (55–57), including whole-exome sequencing of single CTCs (58).

A challenge when analyzing single CTCs using Sanger or NGS methods is to exclude false-positive and false-negative findings due to biases introduced by whole-genome amplification. Moreover, it remains unclear how many CTCs need to be analyzed to capture tumor heterogeneity sufficiently to predict treatment efficacy.

CTC *in vitro* and *in vivo* models for drug response

CTCs from patients have been propagated *in vitro* by multiple groups. These include short-term cultures (28 days or less) of CTCs from patients with breast, colorectal, pleural mesothelioma, prostate, urothelial, bladder, esophageal, pancreatic, gastric, and

lung cancers (59–70) and long-term cultures (6–24 months) of CTCs from patients with breast, prostate, and colorectal cancer (71–74). The purpose of such model systems would be to study drug response or, in one study, to identify multilayer clusters, which if they appear in culture by day 14, may be an early predictor of therapy resistance (70).

Several recent studies have reported the development of mouse xenografts generated directly from CTCs or from CTC cultures from patients with advanced breast, colorectal, prostate, hepatocellular, small cell lung, and gastric cancers (68, 72, 74–79). Some of these assays explore metastatic subpopulations of CTCs and others generate patient-specific models for guiding treatment.

In a xenograft assay of luminal breast cancer CTCs, it was demonstrated that, in contrast to bulk $EPCAM^+$ CTCs, an $EPCAM^+CD44^+CD47^+MET^+$ CTC subpopulation is highly enriched for metastasis-initiating cells when injected into the bone marrow of immunocompromised mice (75). In another study, a subset of EpCAM-negative CTCs taken from the peripheral blood of patients with breast cancer ($EpCAM^-$/$ALDH1^+$/$CD45^-$) was grown *in vitro*. While all EpCAM-negative CTCs grown in culture caused lung metastases after tail vein or intracardiac injection, only cells enriched for a "brain metastasis selected marker (BMSM) signature," $HER2^+$/$EGFR^+$/$HPSE^+$/$Notch1^+$, showed increased potential for both lung and brain metastasis (71).

CTCs enriched from the blood of patients with small cell lung cancer have been implanted subcutaneously into immunocompromised mice as CTC-derived explants (CDX). The CTCs were tumorigenic when there were greater than 400 CTCs in 7.5 mL of blood; generated CDX not only showed similar genomic profiles to those from the patients' CTCs but accurately reflected the donor patient's response to cisplatin/etoposide chemotherapy (78). Xenografts derived from CTC cell lines can also be used to test the efficacy of different drug combinations (68, 72). Such studies open exciting possibilities for the use of CTC genotyping and functional testing to identify rational drug combinations for clinical evaluation.

Potential limitations, however, of *in vitro* and *in vivo* models are that they are usually generated from highly aggressive tumor subclones that may not accurately reflect the spectrum of tumor cell heterogeneity, and, perhaps more importantly in the current era of burgeoning cancer immunotherapy, *in vivo* xenograft models do not recapitulate tumor–host interactions that may play a role in drug resistance. Additional work is required to optimize experimental conditions for efficient generation of these models from the majority of patients with metastatic cancer and to demonstrate that results from these models accurately reflect outcomes in the clinical setting.

CTCs and propensity for metastatic colonization

A variety of mesenchymal markers have been identified in CTCs, suggesting an EMT phenotype (8, 80–82) that may contribute to metastatic progression. Plastin3 (encoded by *PLS3* gene), an actin-binding/bundling protein, has been identified in tumors and CTCs of patients with primary and metastatic colorectal cancer, with higher expression in advanced and metastatic tumors; CTCs that express plastin 3 show an EMT phenotype and are associated with poor prognosis (83).

Although the majority of CTCs are single, CTC clusters have also been identified in blood samples from patients with metastatic cancer. Also known as circulating tumor microemboli, clusters immunomagnetically captured usng the CELLSEARCH platform have been identified in more than 30% of patients with small cell lung cancer and appear to lack apoptotic or proliferating cells, perhaps offering a survival advantage (84). The use of a new device, the Cluster-Chip, offers label-free isolation of CTC clusters with the use of triangular pillars acting as microfluidic "cluster traps" (85). This method showed that clusters were identified in 30% to 40% of patients with metastatic melanoma, breast cancer, and prostate cancer, with the majority of clusters containing 2 to 10 cells, although sometimes up to 19 cells. However, in contrast to CTC clusters isolated by CELLSEARCH in metastatic small cell lung cancer, about half the CTCs within clusters isolated by the Cluster-Chip in metastatic breast cancer were proliferating.

Similar to single migratory mesenchymal-like CTCs, CTC clusters appear to be enriched for mesenchymal markers (82, 86). It is postulated that CTC clusters may arise from oligoclonal tumor cell groups with high metastatic potential and that EMT in the CTC clusters may be mediated through TGFβ signaling by platelets attached to these clusters (refs. 82, 87, 88 ; Fig. 1).

Beyond CTCs, an increasing interest has been expressed in the role of circulating stromal cells and macrophages in metastatic progression. In mouse models, tumor cells entering the circulation together with primary tumor-derived stromal cells have a survival advantage compared with single CTCs and are more efficient in forming lung metastases (89). Similarly, circulating cancer-associated macrophage-like cells from patients with metastatic breast, prostate, and pancreatic cancers can disseminate into the circulation and interact with CTCs (90), with some observed to migrate bound to CTCs, potentially facilitating distant colonization and neovascularization. CTC clusters may express markers associated with platelet transcripts and/or tissue-derived macrophages, but not T/B/natural killer (NK) cells (85). Refinements in our ability to interrogate CTCs and associated cells may enable more rapid clinical development of targeted agents that can affect the metastatic cascade.

ctDNA

Considerations for sensitive detection of ctDNA

First recognized more than 20 years ago (91), plasma ctDNA species are identifiable by the presence of pathognomonic or previously characterized molecular alterations in corresponding tumor tissue (i.e., single nucleotide, copy number, structural, and methylation variants) and thus afford tremendous specificity (92–94). Recent advances in our understanding of the biologic properties and clinical associations of ctDNA, as well as the analytic platforms for its detection, have provided evidence that this class of biomarker may also enable a level of sensitivity suitable for noninvasive tumor monitoring.

As with CTCs, proposed clinical applications of ctDNA segregate broadly into two categories: profiling, noninvasive characterization of tumor molecular features, and quantitation, where ctDNA levels serve as a surrogate of tumor burden (92). For both categories, clinical utility will depend on reliable detection of ctDNA when it is present (analytic sensitivity), as well as the proportion of patients for whom ctDNA should be detectable (clinical sensitivity). Although ctDNA can be detected across several tumor types and generally correlates with tumor stage, absolute ctDNA levels vary widely within each subpopulation (95). Detection of ctDNA is further challenged by the high background levels of circulating wild-type DNA observed in

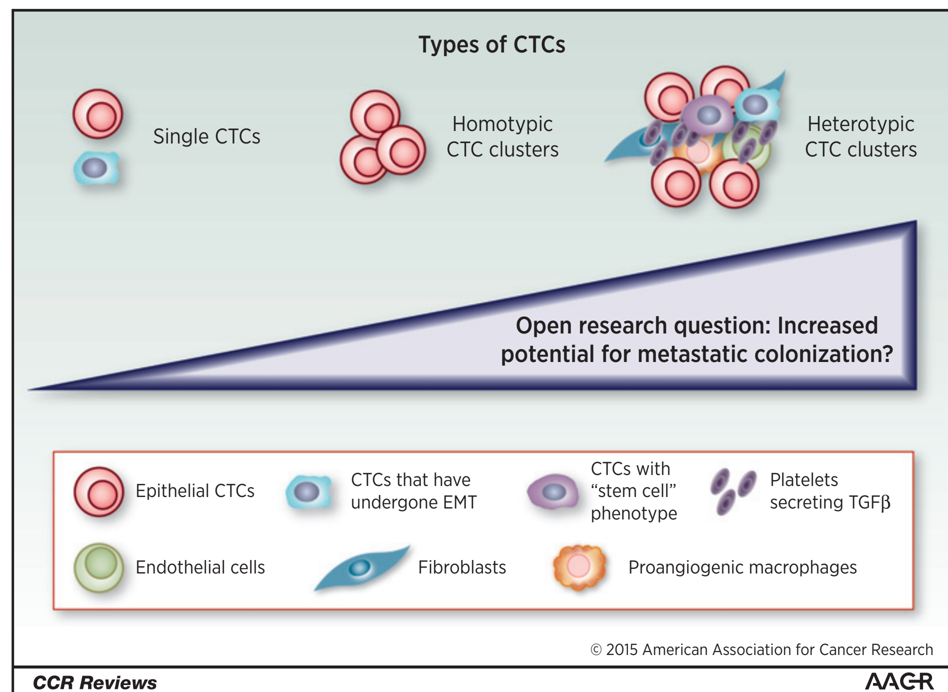

Figure 1.
Single CTCs versus different types of CTC clusters. Their potential for colonization remains an open research question.

individuals with and without cancer (96). In early-stage disease (but also in some metastatic cases), ctDNA may represent an exceedingly rare subpopulation within total cell-free DNA, at levels corresponding to one genome equivalent in 5 mL of plasma (~0.01% allele fraction), and may be undetectable in plasma volumes typically sampled (95, 97, 98). Although incompletely understood, ctDNA levels may vary according to tumor burden, anatomic proximity to vasculature, and biologic features, including apoptotic rate and metastatic potential.

Given a low signal-to-noise ratio, ctDNA detection methods must account for multiple sources of variability to have the robustness desired for clinical use. Analytic variability can arise from inefficient ctDNA recovery during sample preparation, intrinsic error rates for PCR and sequencing which exceed the lower range of ctDNA abundance, and biases in enrichment of genomic regions for analysis (97). Preanalytic variability can affect levels of background wild-type DNA due to lysis of white blood cells during plasma preparation (99), which has prompted development of standardized protocols incorporating use of specialized preservative-containing tubes (e.g., Streck Cell-Free DNA BCT; ref. 100). Detection methods should also accommodate the presence of ctDNA predominantly as 160- to 180-bp fragments, consistent with the nucleosomal pattern of DNA fragmentation arising from tumor cell apoptosis, the rate of which is likely to be the key driver of ctDNA levels; however, it has been shown that a high portion of ctDNA fragments are <100 bp and that optimal detection would then require the use of primers that target amplicons <100 bp (60 bp may be best; refs. 101–103).

The most challenging source of variability, however, comes from tumor heterogeneity. As previously discussed, tumors are characterized by marked spatial heterogeneity resulting from clonal evolution of cells harboring tumor-initiating molecular alterations (the "trunk") to subclones with additional mutations (the "branches"; ref. 4). If a "branch" mutation is selected to detect ctDNA, an absent or low-level signal may not accurately represent the overall level of ctDNA, with potential implications for clinical utility. As an example, a low level of circulating *KRAS*-mutant DNA in a patient with metastatic colorectal cancer could have a concordant result in tissue but could alternatively represent a rare subclone that would not have been detected by conventional tissue *KRAS* testing. Withholding anti-EGFR therapy for this patient might be appropriate in the former case but would be controversial in the latter. Moreover, for applications in which ctDNA is assessed longitudinally, an added challenge will be temporal heterogeneity, whereby tumor molecular profiles evolve with emergence and disappearance of dominant subclones due to the selective pressure of treatment (104–106). For broad applicability, ctDNA detection platforms should not only have high analytic sensitivity but also sufficient genomic coverage to identify a tumor with multiple molecular markers (for redundancy and inclusion of "trunk" mutations) and to anticipate molecular alterations expected with tumor evolution.

Several methods have been developed to detect ctDNA, with the predominant platforms at present based on digital PCR and NGS. Comparisons of clinical sensitivity across studies are challenging due to variability in methods, the number and type of targeted molecular alterations, tumor type, tumor stage, and preselection of patients (Table 4). With the notable exception of the studies by Bettegowda and colleagues (95) and Douillard and colleagues (107), published studies have been limited by small sample sizes. Nevertheless, a number of themes emerge. First, PCR-based approaches have very high sensitivity for ctDNA but are limited in the number of foci that can be assessed and, consequently, the addressable proportion of each population (compare the tested populations and the populations evaluable for sensitivity; Table 4). This limitation can be addressed by first identifying patient-specific molecular alterations in tumor tissue

Table 4. Selected studies of ctDNA detection in various tumor types

Study reference	Analytic platform for ctDNA	Molecular alteration	Number of patients analyzed for ctDNA	Tumor type	Stage	Sensitivity (patients with detected ctDNA/patients with marker-positive tumors)[a]
Lecomte 2002 (117)	Allele-specific PCR, methylation-specific PCR	SNV (*KRAS*) or methylation (p16)	39 (preselected)	Colorectal	I	1/3 (33%)
					II	10/13 (77%)
					III	6/9 (67%)
					IV	9/12 (75%)
Diehl 2008 (108)	BEAMing	SNV (custom assays)	18	Colorectal	II	1/1 (100%)
					III	1/1 (100%)
					IV	16/16 (100%)
Board 2010 (114)	Allele-specific PCR	SNV (*PIK3CA*)	77	Breast	Operable	0/14 (0%)
					IV	8/10 (80%)
Forshew 2012 (111)	Digital PCR, tagged amplicon sequencing	SNV (*TP53, PTEN, KRAS, BRAF, PIK3CA, EGFR*)	37	Ovarian	III, IV	21/37 (57%)
Leary 2012 (118)	Paired-end sequencing	Structural variants	10	Breast	IV	3/3 (100%)
				Colorectal	IV	7/7 (100%)
Punnoose 2012 (120)	TaqMan PCR	SNV (*KRAS, BRAF, PIK3CA, EGFR*)	25	NSCLC	IV	7/8 (88%)
Higgins 2012 (105)	BEAMing	SNV	49	Breast	IV	14/14 (100%)
Narayan 2012 (113)	Amplicon sequencing	SNV (*KRAS, BRAF, EGFR*)	30	NSCLC	III	1/1 (100%)
					IV	4/4 (100%)
Chan 2013 (166)	Bisulfite sequencing	CNV, methylation	46	Hepatocellular	BCLC A	24/26 (92%)
				Breast	Localized/IV	5/5 (100%)
				Neuroendocrine	IV	1/1 (100%)
				Sarcoma	IV	1/1 (100%)
				NSCLC	III/IV	4/4 (100%)
				Nasopharyngeal	localized/IV	6/9 (67%)
Dawson 2013 (115)	Digital PCR, tagged amplicon sequencing	SNV	30	Breast	IV	29/30 (97%)
Beaver 2014 (98)	Digital PCR	SNV (*PIK3CA*)	29	Breast	I	8/9 (89%)
					II	5/5 (100%)
Bettegowda 2014 (95)	BEAMing, tagged amplicon sequencing, PCR ligation	SNV, structural variants	640	Bladder	Localized	4/7 (57%)
					IV	3/3 (100%)
				Breast	Localized	10/19 (53%)
					IV	12/14 (86%)
				Colorectal	Localized	31/40 (78%)
					IV (Set 1)	24/24 (100%)
					IV (Set 2)	68/78 (87%)
				Endometrial	Localized	3/11 (27%)
					IV	1/1 (100%)
				Gastroesophageal	Localized	8/14 (57%)
					IV	7/7 (100%)
				Glioma	n/a	2/27 (7%)
				Head and neck	Localized	2/2 (100%)
					IV	7/10 (70%)
				Hepatocellular	localized	2/3 (67%)
					IV	1/1 (100%)
				Medulloblastoma	n/a	6/14 (43%)
				Melanoma	Localized	0/2 (0%)
					IV	15/18 (83%)
				Neuroblastoma	IV	6/9 (67%)
				NSCLC	IV	4/5 (80%)
				Ovarian	Localized	8/9 (89%)
				Pancreatic	Localized	60/121 (50%)
					IV	30/34 (88%)
				Prostate	IV	2/5 (40%)
				Renal cell	IV	2/5 (40%)
				SCLC	IV	1/1 (100%)
				Thyroid	IV	1/4 (25%)
Bidard 2013 (167)	PCR	SNV (*GNAQ/GNA11*)	26 (preselected)	Uveal melanoma	IV	22/26 (84%)
Madic 2015 (116)	Amplicon sequencing	SNV (*TP53*)	40	Breast (TNBC)	IV	21/26 (81%)
Rothé 2014 (112)	Amplicon sequencing	SNV (50 cancer genes)	17	Breast	IV	9/11 (82%)

(Continued on the following page)

Table 4. Selected studies of ctDNA detection in various tumor types (Cont'd)

Study reference	Analytic platform for ctDNA	Molecular alteration	Number of patients analyzed for ctDNA	Tumor type	Stage	Sensitivity (patients with detected ctDNA/patients with marker-positive tumors)[a]
Newman 2014 (97)	Sequencing with cancer-specific target capture	SNV, fusions	13	NSCLC	I	2/4 (50%)
					II	1/1 (100%)
					III	4/4 (100%)
					IV	4/4 (100%)
Thierry 2014 (119)	Allele-specific PCR	SNV (*KRAS, BRAF*)	95	Colorectal	IV	41/42 (98%)
Douillard 2014 (107)	Allele-specific PCR	SNV (*EGFR*)	803	NSCLC	IV	69/105 (66%)
Kidess 2014 (168)	Sequencing with sequence-specific synchronous coefficient of drag alteration (SCODA) enrichment	SNV (*KRAS, BRAF, PIK3CA, EGFR*)	38	Colorectal	I	0/2 (0%)
					II	6/8 (75%)
					III	1/2 (50%)
					IV	13/14 (93%)

Abbreviations: BCLC, Barcelona Clinic Liver Cancer; BEAMing, beads, emulsion, amplification, magnetics; CNV, copy number variations; NSCLC, non–small cell lung cancer; TNBC, triple-negative breast cancer.
[a]Detection of tumor-specific mutations in plasma (excludes cases where tumor harbored no detectable mutations).

and then developing customized ctDNA assays [e.g., PCR assays for single-nucleotide variants (SNV; refs. 95, 108) or structural variants (personalized analysis of rearranged ends, PARE (ref. 109)]. However, this approach may be limited by practicality and cost considerations. Second, newer sequencing-based platforms that account for sequencing error rate and PCR errors during library preparation [e.g. SafeSeq (ref. 110), TAm-Seq (ref. 111), CAPP-Seq (ref. 97), Ampli-Seq (ref. 112, the first published study to demonstrate the feasibility of performing deep-coverage NGS in breast cancer for the detection of mutations in hot spot regions of 50 genes in an ISO-certified laboratory), and others (113)] are achieving analytic sensitivities on par with PCR while maintaining broader genomic coverage. Finally, ctDNA detection with state-of-the-art techniques remains consistently lower for early-stage disease than for metastatic disease.

Establishing clinical utility of ctDNA

Applications based on ctDNA for noninvasive molecular profiling. The first area in which ctDNA will be proven to have clinical utility is in noninvasive profiling for the presence of actionable mutations. Several studies have now demonstrated high concordance for selected actionable mutations between paired tumor and plasma specimens, particularly for metastatic disease in breast (95, 105, 112, 114–116), colorectal (95, 108, 117–119), and non–small cell lung cancer (refs. 97, 113, 120; Table 4). Larger, prospective studies with standardized analytic methods should be conducted to validate concordance in each tumor type, enabling a more precise understanding of false-negative and false-positive rates. For metastatic disease patients who have tumors that are difficult to biopsy, who have contraindications to biopsy, or who have tumors that are traditionally challenging to diagnose by conventional means (e.g., cholangiocarcinoma), a validated ctDNA assay could have clinical utility in the near term as an "alternative to tissue biopsy."

Discordances in the molecular profiles between ctDNA and tumor tissue specimens may reflect underappreciated tumor heterogeneity within and between tumor foci. Serial ctDNA assessment may also detect the process of tumor evolution (105, 121), where the appearance of new molecular alterations on treatment may herald the emergence of resistance and potentially also predictive markers for different therapies. This phenomenon has been best described for colorectal cancer, in which ctDNA obtained after treatment of *KRAS* wild-type tumors with anti-EGFR therapies has demonstrated new molecular alterations that plausibly confer resistance, including *KRAS*, *NRAS*, *BRAF*, and *EGFR* mutations (96, 104) as well as *MET* amplification (122). Ultimately, however, prospective studies will be needed to demonstrate that treatment strategies guided by unique information provided by ctDNA yield superior clinical outcomes when compared with tissue-based approaches.

Applications based on ctDNA for noninvasive assessment of tumor load. Reliable and sensitive methods to detect and quantitate ctDNA may enable noninvasive disease monitoring in a manner analogous to *BCR-ABL* testing in chronic myeloid leukemia or HIV viral testing. Case studies in breast, colorectal, and non–small cell lung cancer have suggested that ctDNA dynamics can provide an early indicator of tumor response, which could help optimize neoadjuvant therapy or treatment of metastatic disease. Ineffective therapies could be halted with the appearance of resistance, avoiding unnecessary toxicity. Posttreatment ctDNA levels may also be useful in detecting previously unrecognized residual disease following definitive therapy. Anecdotal evidence has been reported to support this concept (7, 17, 123, 124), but large, prospective studies will be needed to demonstrate the prognostic value of residual disease detected by ctDNA. Tie and colleagues (125) have reported preliminary results of a prospective trial in stage II colon cancer evaluating the relationship of postoperative ctDNA levels with tumor recurrence. At a median follow-up of 507 days, recurrence rates were >10-fold higher in patients with detectable postoperative ctDNA (5 of 6, 88% with detectable ctDNA vs. 5 of 72, 7% without detectable ctDNA). Finally, early detection of cancer is a tantalizing application for ctDNA. At present, the relatively poor clinical sensitivity of ctDNA for early-stage disease (Table 4 and ref. 95) would result in high proportions of false negatives and, more significantly, would limit the degree of stage migration, which is critically important for screening programs to affect patient outcome. Further development of ctDNA platforms will be required before the challenges of cancer screening should be considered.

Table 5. Comparison of strengths and limitations of CTC and ctDNA liquid biopsy assays

	Strengths	Limitations
CTC	• Noninvasive • High specificity • Potentially addresses spatial and temporal tumor heterogeneity • Demonstrates colocalization of signal • Evaluates protein expression • Functional studies *ex vivo*	• Prospective collection needed to address preanalytic variation • Low signal-to-noise, especially in early-stage disease • Impact of heterogeneity on selection methods
ctDNA	• Noninvasive • High specificity • Potentially addresses spatial and temporal tumor heterogeneity • More genome equivalents per unit volume = more sensitive	• Prospective collection needed to address preanalytic variation • Low signal-to-noise, especially in early-stage disease • No colocalization • No protein expression • No functional studies

CTCs and ctDNA: Future Considerations for These Complementary Approaches

A major reason for treatment failures is our inability to monitor tumor evolution and adapt treatment accordingly. Identifying tumor recurrence at an earlier time point does not improve clinical outcome if an effective therapy is not selected or available. Liquid biopsy technologies are potentially important advances in this regard, with CTCs and ctDNA expected to play complementary roles, on the basis of their relative strengths and limitations (Table 5). Plasma ctDNA assays (disease-specific, treatment-specific, or personalized) may prove more useful for monitoring disease burden and limited molecular profiling. Once increased disease burden is recognized, then CTC analysis for comprehensive characterization of tumor DNA, RNA, and/or protein levels, including their co-localization, in known residual cancer cells may help to optimize therapy selection (92). It is also quite likely that CTCs may be particularly useful *ex vivo*, incorporated into functional studies using CTC cultures, mouse xenografts, or real-time *in vitro* assays for drug sensitivity evaluation.

Significant challenges remain, particularly with respect to analytic and clinical sensitivity. Adoption of these tools into routine clinical practice will necessitate rigorous demonstration of analytic validity, clinical validity, and, most importantly, clinical utility. One consideration in population screening is that 11% to 19% of patients with benign inflammatory conditions (e.g., Crohn disease) have small numbers of morphologically benign circulating epithelial cells detectable, which could potentially give a false-positive CTC result (126). Another risk is the detection of clinically irrelevant molecular changes due to the high sensitivity of the methods. Therefore, large annotated datasets and bioinformatic tools will be needed to distinguish potentially important genomic aberrations from noise. Moreover, only clinical studies will provide evidence about whether a genomic aberration detected in blood can predict benefit from a specific targeted agent. Although most efforts are currently focused on testing liquid biopsy in the metastatic setting, we expect that future studies will evaluate its role in the early disease setting or even as a potential tool to assist early cancer diagnosis.

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References

1. Cancer Genome Atlas Network. Comprehensive molecular portraits of human breast tumours. Nature 2012;490:61–70.
2. Nik-Zainal S, Van Loo P, Wedge DC, Alexandrov LB, Greenman CD, Lau KW, et al. The life history of 21 breast cancers. Cell 2012;149:994–1007.
3. Wang Y, Waters J, Leung ML, Unruh A, Roh W, Shi X, et al. Clonal evolution in breast cancer revealed by single nucleus genome sequencing. Nature 2014;512:155–60.
4. 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;366:883–892.
5. Hiley C, de Bruin EC, McGranahan N, Swanton C. Deciphering intratumor heterogeneity and temporal acquisition of driver events to refine precision medicine. Genome Biol 2014;15:453.
6. Lindstrom LS, Karlsson E, Wilking UM, Johansson U, Hartman J, Lidbrink EK, et al. Clinically used breast cancer markers such as estrogen receptor, progesterone receptor, and human epidermal growth factor receptor 2 are unstable throughout tumor progression. J Clin Oncol 2012;30:2601–8.
7. Krebs MG, Metcalf RL, Carter L, Brady G, Blackhall FH, Dive C. Molecular analysis of circulating tumour cells-biology and biomarkers. Nat Rev Clin Oncol 2014;11:129–44.
8. Powell AA, Talasaz AH, Zhang H, Coram MA, Reddy A, Deng G, et al. Single cell profiling of circulating tumor cells: transcriptional heterogeneity and diversity from breast cancer cell lines. PLoS One 2012;7:e33788.
9. Parkinson DR, Dracopoli N, Petty BG, Compton C, Cristofanilli M, Deisseroth A, et al. Considerations in the development of circulating tumor cell technology for clinical use. J Transl Med 2012;10:138.
10. Alix-Panabières C, Pantel K. Technologies for detection of circulating tumor cells: facts and vision. Lab Chip 2014;14:57–62.
11. Harouaka R, Kang Z, Zheng SY, Cao L. Circulating tumor cells: advances in isolation and analysis, and challenges for clinical applications. Pharmacol Ther 2014;141:209–21.
12. Jin C, McFaul SM, Duffy SP, Deng X, Tavassoli P, Black PC, et al. Technologies for label-free separation of circulating tumor cells: from historical foundations to recent developments. Lab Chip 2014;14:32–44.
13. Fehm T, Sagalowsky A, Clifford E, Beitsch P, Saboorian H, Euhus D, et al. Cytogenetic evidence that circulating epithelial cells in patients with carcinoma are malignant. Clin Cancer Res 2002;8:2073–84.
14. Mikolajczyk SD, Millar LS, Tsinberg P, Coutts SM, Zomorrodi M, Pham T, et al. Detection of EpCAM-negative and cytokeratin-negative circulating tumor cells in peripheral blood. J Oncol 2011;2011:252361.

15. Pecot CV, Bischoff FZ, Mayer JA, Wong KL, Pham T, Bottsford-Miller J, et al. A novel platform for detection of CK+ and CK- CTCs. Cancer Discov 2011;1:580–6.
16. Marrinucci D, Bethel K, Kolatkar A, Luttgen MS, Malchiodi M, Baehring F, et al. Fluid biopsy in patients with metastatic prostate, pancreatic and breast cancers. Phys Biol 2012;9:016003.
17. Serrano MJ, Ortega FG, Alvarez-Cubero MJ, Nadal R, Sanchez-Rovira P, Salido M, et al. EMT and EGFR in CTCs cytokeratin negative non-metastatic breast cancer. Oncotarget 2014;5:7486–97.
18. King JD, Casavant BP, Lang JM. Rapid translation of circulating tumor cell biomarkers into clinical practice: technology development, clinical needs and regulatory requirements. Lab Chip 2014;14:24–31.
19. Cristofanilli M, Budd GT, Ellis MJ, Stopeck A, Matera J, Miller MC, et al. Circulating tumor cells, disease progression, and survival in metastatic breast cancer. N Engl J Med 2004;351:781–91.
20. Cohen SJ, Punt CJ, Iannotti N, Saidman BH, Sabbath KD, Gabrail NY, et al. Relationship of circulating tumor cells to tumor response, progression-free survival, and overall survival in patients with metastatic colorectal cancer. J Clin Oncol 2008;26:3213–21.
21. de Bono JS, Scher HI, Montgomery RB, Parker C, Miller MC, Tissing H, et al. Circulating tumor cells predict survival benefit from treatment in metastatic castration-resistant prostate cancer. Clin Cancer Res 2008; 14:6302–9.
22. Ignatiadis M, Riethdorf S, Bidard FC, Vaucher I, Khazour M, Rothe F, et al. International study on inter-reader variability for circulating tumor cells in breast cancer. Breast Cancer Res 2014;16:R43.
23. Bidard FC, Peeters DJ, Fehm T, Nolé 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.
24. 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.
25. Ignatiadis M, Xenidis N, Perraki M, Apostolaki S, Politaki E, Kafousi M, et al. Different prognostic value of Cytokeratin-19 mRNA-positive Circulating Tumor Cells according to estrogen receptor and HER2 status in early breast cancer. J Clin Oncol 2007;25:5194–202.
26. Pierga JY, Bidard FC, Mathiot C, Brain E, Delaloge S, Giachetti S, et al. Circulating tumor cell detection predicts early metastatic relapse after neoadjuvant chemotherapy in large operable and locally advanced breast cancer in a phase II randomized trial. Clin Cancer Res 2008;14:7004–10.
27. Xenidis N, Ignatiadis M, Apostolaki S, Perraki M, Kalbakis K, Agelaki S, et al. Cytokeratin-19 mRNA-positive circulating tumor cells after adjuvant chemotherapy in patients with early breast cancer. J Clin Oncol 2009; 27:2177–84.
28. Lucci A, Hall CS, Lodhi AK, Bhattacharyya A, Anderson AE, Xiao L, et al. Circulating tumour cells in non-metastatic breast cancer: a prospective study. Lancet Oncol 2012;13:688–95.
29. 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.
30. Ithimakin S, Day KC, Malik F, Zen Q, Dawsey SJ, Bersano-Begey TF, et al. HER2 drives luminal breast cancer stem cells in the absence of HER2 amplification: implications for efficacy of adjuvant trastuzumab. Cancer Res 2013;73:1635–46.
31. Paik S, Kim C, Wolmark N. HER2 status and benefit from adjuvant trastuzumab in breast cancer. N Engl J Med 2008;358:1409–11.
32. Perez EA, Reinholz MM, Hillman DW, Tenner KS, Schroeder MJ, Davidson NE, et al. HER2 and chromosome 17 effect on patient outcome in the N9831 adjuvant trastuzumab trial. J Clin Oncol 2010;28:4307–15.
33. Georgoulias V, Bozionelou V, Agelaki S, Perraki M, Apostolaki S, Kallergi G, et al. Trastuzumab decreases the incidence of clinical relapses in patients with early breast cancer presenting chemotherapy-resistant CK-19mRNA-positive circulating tumor cells: results of a randomized phase II study. Ann Oncol 2012;23:1744–50.
34. de Bono JS, Scher HI, Montgomery RB, Parker C, Miller MC, Tissing H, et al. Circulating tumor cells predict survival benefit from treatment in metastatic castration-resistant prostate cancer. Clin Cancer Res 2008; 14:6302–9.
35. 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.
36. Scher HI, Heller G, Molina A, Attard G, Danila DC, Jia X, et al. Circulating tumor cell biomarker panel as an individual-level surrogate for survival in metastatic castration-resistant prostate cancer. J Clin Oncol 2015;33: 1348–55.
37. Ignatiadis M, Rothe F, Chaboteaux C, Durbecq V, Rouas G, Criscitiello C, et al. HER2-positive circulating tumor cells in breast cancer. PLoS One 2001;6:e15624.
38. Meng S, Tripathy D, Shete S, Ashfaq R, Haley B, Perkins S, et al. HER-2 gene amplification can be acquired as breast cancer progresses. Proc Natl Acad Sci U S A 2004;101:9393–8.
39. Pestrin M, Bessi S, Puglisi F, Minisini AM, Masci G, Battelli N, et al. Final results of a multicenter phase II clinical trial evaluating the activity of single-agent lapatinib in patients with HER2-negative metastatic breast cancer and HER2-positive circulating tumor cells. A proof-of-concept study. Breast Cancer Res Treat 2012;134:283–9.
40. Paoletti C, Muñiz 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.
41. Abdallah EA, Fanelli MF, Buim ME, Machado Netto MC, Gasparini Junior JL, Souza E, Silva V, et al. Thymidylate synthase expression in circulating tumor cells: a new tool to predict 5-fluorouracil resistance in metastatic colorectal cancer patients. Int J Cancer 2015;137:1397–405.
42. Miyamoto DT, Lee RJ, Stott SL, Ting DT, Wittner BS, Ulman M, et al. Androgen receptor signaling in circulating tumor cells as a marker of hormonally responsive prostate cancer. Cancer Discov 2012;2: 995–1003.
43. Antonarakis ES, Lu C, Wang H, Luber B, Nakazawa M, Roeser JC, et al. AR-V7 and resistance to enzalutamide and abiraterone in prostate cancer. N Engl J Med 2014;371:1028–38.
44. Ignatiadis M, Kallergi G, Ntoulia M, Perraki M, Apostolaki S, Kafousi M, et al. Prognostic value of the molecular detection of circulating tumor cells using a multimarker reverse transcription-PCR assay for cytokeratin 19, mammaglobin A, and HER2 in early breast cancer. Clin Cancer Res 2008;14:2593–600.
45. Markou A, Strati A, Malamos N, Georgoulias V, Lianidou ES. Molecular characterization of circulating tumor cells in breast cancer by a liquid bead array hybridization assay. Clin Chem 2011;57:421–30.
46. Mostert B, Sieuwerts AM, Bolt-de Vries J, Kraan J, Lalmahomed Z, van Galen A, et al. mRNA expression profiles in circulating tumor cells of metastatic colorectal cancer patients. Mol Oncol 2015;9:920–32.
47. Talasaz AH, Powell AA, Huber DE, Berbee JG, Roh KH, Yu W, et al. Isolating highly enriched populations of circulating epithelial cells and other rare cells from blood using a magnetic sweeper device. Proc Natl Acad Sci U S A 2009;106:3970–5.
48. Chen CL, Mahalingam D, Osmulski P, Jadhav RR, Wang CM, Leach RJ, et al. Single-cell analysis of circulating tumor cells identifies cumulative expression patterns of EMT-related genes in metastatic prostate cancer. Prostate 2013;73:813–26.
49. Maheswaran S, Sequist LV, Nagrath S, Ulkus L, Brannigan B, Collura CV, et al. Detection of mutations in EGFR in circulating lung-cancer cells. N Engl J Med 2008;359:366–77.
50. 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.
51. Fabbri F, Carloni S, Zoli W, Ulivi P, Gallerani G, Fici P, et al. Detection and recovery of circulating colon cancer cells using a dielectrophoresis-based device: KRAS mutation status in pure CTCs. Cancer Lett 2013;335: 225–31.
52. Pailler E, Adam J, Barthélémy A, Oulhen M, Auger N, Valent A, et al. Detection of circulating tumor cells harboring a unique ALK rearrangement in ALK-positive non-small-cell lung cancer. J Clin Oncol 2013; 31:2273–81.
53. Fernandez SV, Bingham C, Fittipaldi P, Austin L, Palazzo J, Palmer G, et al. TP53 mutations detected in circulating tumor cells present in the blood of metastatic triple negative breast cancer patients. Breast Cancer Res 2014;16:445.
54. Deng G, Krishnakumar S, Powell AA, Zhang H, Mindrinos MN, Telli ML, et al. Single cell mutational analysis of PIK3CA in circulating tumor cells

and metastases in breast cancer reveals heterogeneity, discordance, and mutation persistence in cultured disseminated tumor cells from bone marrow. BMC Cancer 2014;14:456.
55. Heitzer E, Auer M, Gasch C, Pichler M, Ulz P, Hoffmann EM, et al. Complex tumor genomes inferred from single circulating tumor cells by array-CGH and next-generation sequencing. Cancer Res 2013;73: 2965–75.
56. Polzer B, Medoro G, Pasch S, Fontana F, Zorzino L, Pestka A, et al. Molecular profiling of single circulating tumor cells with diagnostic intention. EMBO Mol Med 2014;6:1371–86.
57. Ruiz C, Li J, Luttgen MS, Kolatkar A, Kendall JT, Flores E, et al. Limited genomic heterogeneity of circulating melanoma cells in advanced stage patients. Phys Biol 2015;12:016008.
58. Lohr JG, Adalsteinsson VA, Cibulskis K, Choudhury AD, Rosenberg M, Cruz-Gordillo P, et al. Whole-exome sequencing of circulating tumor cells provides a window into metastatic prostate cancer. Nat Biotechnol 2014;32:479–84.
59. Pizon M, Zimon D, Carl S, Pachmann U, Pachmann K, Camara O. Heterogeneity of circulating epithelial tumour cells from individual patients with respect to expression profiles and clonal growth (sphere formation) in breast cancer. Ecancermedicalscience 2013; 7:343.
60. Bobek V, Kacprzak G, Rzechonek A, Kolostova K. Detection and cultivation of circulating tumor cells in malignant pleural mesothelioma. Anticancer Res 2014;34:2565–9.
61. Gallant JN, Matthew EM, Cheng H, Harouaka R, Lamparella NE, Kunkel M, et al. Predicting therapy response in live tumor cells isolated with the flexible micro spring array device. Cell Cycle 2013;12:2132–43.
62. Kolostova K, Broul M, Schraml J, Cegan M, Matkowski R, Fiutowski M, et al. Circulating tumor cells in localized prostate cancer: isolation, cultivation *in vitro* and relationship to T-stage and Gleason score. Anticancer Res 2014;34:3641–6.
63. Kolostova K, Cegan M, Bobek V. Circulating tumour cells in patients with urothelial tumours: enrichment and *in vitro* culture. Can Urol Assoc J 2014;8:E715–20.
64. Cegan M, Kolostova K, Matkowski R, Broul M, Schraml J, Fiutowski M, et al. *In vitro* culturing of viable circulating tumor cells of urinary bladder cancer. Int J Clin Exp Pathol 2014;7:7164–71.
65. Bobek V, Matkowski R, Gürlich R, Grabowski K, Szelachowska J, Lischke R, et al. Cultivation of circulating tumor cells in esophageal cancer. Folia Histochem Cytobiol 2014;52:171–7.
66. Bobek V, Gurlich R, Eliasova P, Kolostova K. Circulating tumor cells in pancreatic cancer patients: enrichment and cultivation. World J Gastroenterol 2014;20:17163–70.
67. Kolostova K, Matkowski R, Gürlich R, Grabowski K, Soter K, Lischke R, et al. Detection and cultivation of circulating tumor cells in gastric cancer. Cytotechnology 2015 Apr 11. [Epub ahead of print].
68. Yuan D, Chen L, Li M, Xia H, Zhang Y, Chen T, et al. Isolation and characterization of circulating tumor cells from human gastric cancer patients. J Cancer Res Clin Oncol 2015;141:647–60.
69. Zhang Z, Shiratsuchi H, Lin J, Chen G, Reddy RM, Azizi E, et al. Expansion of CTCs from early stage lung cancer patients using a microfluidic co-culture model. Oncotarget 2014;5:12383–97.
70. Khoo BL, Lee SC, Kumar P, Tan TZ, Warkiani ME, Ow SG, et al. Short-term expansion of breast circulating cancer cells predicts response to anticancer therapy. Oncotarget 2015;6:15578–93.
71. Zhang L, Ridgway LD, Wetzel MD, Ngo J, Yin W, Kumar D, et al. The identification and characterization of breast cancer CTCs competent for brain metastasis. Sci Transl Med 2013;5:180ra48.
72. 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.
73. Gao D, Vela I, Sboner A, Iaquinta PJ, Karthaus WR, Gopalan A, et al. Organoid cultures derived from patients with advanced prostate cancer. Cell 2014;159:176–87.
74. 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.
75. 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.
76. Rossi E, Rugge M, Facchinetti A, Pizzi M, Nardo G, Barbieri V, et al. Retaining the long-survive capacity of circulating tumor cells (CTCs) followed by xeno-transplantation: not only from metastatic cancer of the breast but also of prostate cancer patients. Oncoscience 2013;1: 49–56.
77. Sun YF, Xu Y, Yang XR, Guo W, Zhang X, Qiu SJ, et al. Circulating stem cell-like epithelial cell adhesion molecule-positive tumor cells indicate poor prognosis of hepatocellular carcinoma after curative resection. Hepatology 2013;57:1458–68.
78. 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.
79. Toyoshima K, Hayashi A, Kashiwagi M, Hayashi N, Iwatsuki M, Ishimoto T, et al. Analysis of circulating tumor cells derived from advanced gastric cancer. Int J Cancer 2015;137:991–8.
80. Lecharpentier A, Vielh P, Perez-Moreno P, Planchard D, Soria JC, Farace F. Detection of circulating tumour cells with a hybrid (epithelial/mesenchymal) phenotype in patients with metastatic non-small cell lung cancer. Br J Cancer 2011;105:1338–41.
81. Kallergi G, Papadaki MA, Politaki E, Mavroudis D, Georgoulias V, Agelaki S. Epithelial to mesenchymal transition markers expressed in circulating tumour cells of early and metastatic breast cancer patients. Breast Cancer Res 2011;13:R59.
82. Yu M, Bardia A, Wittner BS, Stott SL, Smas ME, Ting DT, et al. Circulating breast tumor cells exhibit dynamic changes in epithelial and mesenchymal composition. Science 2013;339:580–4.
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. 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.
85. Sarioglu AF, Aceto N, Kojic N, Donaldson MC, Zeinali M, Hamza B, et al. A microfluidic device for label-free, physical capture of circulating tumor cell clusters. Nat Methods 2015;12:685–91.
86. Hou JM, Krebs M, Ward T, Sloane R, Priest L, Hughes A, et al. Circulating tumor cells as a window on metastasis biology in lung cancer. Am J Pathol 2011;178:989–96.
87. Labelle M, Begum S, Hynes RO. Direct signaling between platelets and cancer cells induces an epithelial-mesenchymal-like transition and promotes metastasis. Cancer Cell 2011;20:576–90.
88. Aceto N, Bardia A, Miyamoto DT, Donaldson MC, Wittner BS, Spencer J A, et al. Circulating tumor cell clusters are oligoclonal precursors of breast cancer metastasis. Cell 2014;158:1110–22.
89. Duda DG, Duyverman AM, Kohno M, Snuderl M, Steller EJ, Fukumura D, et al. Malignant cells facilitate lung metastasis by bringing their own soil. Proc Natl Acad Sci U S A 2010;107:21677–82.
90. Adams DL, Martin SS, Alpaugh RK, Charpentier M, Tsai S, Bergan RC, et al. Circulating giant macrophages as a potential biomarker of solid tumors. Proc Natl Acad Sci U S A 2014;111:3514–9.
91. Sorenson GD, Pribish DM, Valone FH, Memoli VA, Bzik DJ, Yao SL. Soluble normal and mutated DNA sequences from single-copy genes in human blood. Cancer Epidemiol Biomarkers Prev 1994;3:67–71.
92. Kidess E, Jeffrey SS. Circulating tumor cells versus tumor-derived cell-free DNA: rivals or partners in cancer care in the era of single-cell analysis? Genome Med 2013;5:70.
93. Diaz LA Jr, Bardelli A. Liquid biopsies: genotyping circulating tumor DNA. J Clin Oncol 2014;32:579–86.
94. Ignatiadis M, Dawson SJ. Circulating tumor cells and circulating tumor DNA for precision medicine: dream or reality? Ann Oncol 2014;25: 2304–13.
95. Bettegowda C, Sausen M, Leary RJ, Kinde I, Wang Y, Agrawal N, et al. Detection of circulating tumor DNA in early- and late-stage human malignancies. Sci Transl Med 2014;6:224ra24.
96. Swarup V, Rajeswari MR. Circulating (cell-free) nucleic acids–a promising, non-invasive tool for early detection of several human diseases. FEBS Lett 2007;581:795–9.

97. Newman AM, Bratman SV, To J, Wynne JF, Eclov NC, Modlin LA, et al. An ultrasensitive method for quantitating circulating tumor DNA with broad patient coverage. Nat Med 2014;20:548–54.
98. Beaver JA, Jelovac D, Balukrishna S, Cochran R, Croessmann S, Zabransky D, et al. Detection of cancer DNA in plasma of early stage breast cancer patients. Clin Cancer Res 2014;20:2643–50.
99. El Messaoudi S, Rolet F, Mouliere F, Thierry AR. Circulating cell free DNA: Preanalytical considerations. Clin Chim Acta 2013;424:222–30.
100. Qin J, Williams TL, Fernando MR. A novel blood collection device stabilizes cell-free RNA in blood during sample shipping and storage. BMC Res Notes 2013;6:380.
101. Jahr S, Hentze H, Englisch S, Hardt D, Fackelmayer FO, Hesch RD, et al. DNA fragments in the blood plasma of cancer patients: quantitations and evidence for their origin from apoptotic and necrotic cells. Cancer Res 2001;61:1659–65.
102. Thierry AR, Mouliere F, Gongora C, Ollier J, Robert B, Ychou M, et al. Origin and quantification of circulating DNA in mice with human colorectal cancer xenografts. Nucleic Acids Res 2010;38:6159–75.
103. Mouliere F, Robert B, Arnau PE, Del Rio M, Ychou M, Molina F, et al. High fragmentation characterizes tumour-derived circulating DNA. Plos One 2011;6:e23418.
104. Diaz LA Jr, Williams RT, Wu J, Kinde I, Hecht JR, Berlin J, et al. The molecular evolution of acquired resistance to targeted EGFR blockade in colorectal cancers. Nature 2012;486:537–40.
105. Higgins MJ, Jelovac D, Barnathan E, Blair B, Slater S, Powers P, et al. Detection of tumor PIK3CA status in metastatic breast cancer using peripheral blood. Clin Cancer Res 2012;18:3462–9.
106. Gerlinger M, Swanton C. How Darwinian models inform therapeutic failure initiated by clonal heterogeneity in cancer medicine. Br J Cancer 2010;103:1139–43.
107. Douillard JY, Ostoros G, Cobo M, Ciuleanu T, Cole R, McWalter G, et al. Gefitinib treatment in EGFR mutated caucasian NSCLC: circulating-free tumor DNA as a surrogate for determination of EGFR status. J Thorac Oncol 2014;9:1345–53.
108. Diehl F, Schmidt K, Choti MA, Romans K, Goodman S, Li M, et al. Circulating mutant DNA to assess tumor dynamics. Nat Med 2008; 14:985–90.
109. Leary RJ, Kinde I, Diehl F, Schmidt K, Clouser C, Duncan C, et al. Development of personalized tumor biomarkers using massively parallel sequencing. Sci Transl Med 2010;2:20ra14.
110. Kinde I, Wu J, Papadopoulos N, Kinzler KW, Vogelstein B. Detection and quantification of rare mutations with massively parallel sequencing. Proc Natl Acad Sci U S A 2011;108:9530–5.
111. Forshew T, Murtaza M, Parkinson C, Gale D, Tsui DW, Kaper F, et al. Noninvasive identification and monitoring of cancer mutations by targeted deep sequencing of plasma DNA. Sci Transl Med 2012;4: 136ra68.
112. Rothé F, Laes J, Lambrechts D, Smeets D, Vincent D, Maetens M, et al. Plasma circulating tumor DNA as an alternative to metastatic biopsies for mutational analysis in breast cancer. Ann Oncol 2014;25: 1959–65.
113. Narayan A, Carriero NJ, Gettinger SN, Kluytenaar J, Kozak KR, Yock TI, et al. Ultrasensitive measurement of hotspot mutations in tumor DNA in blood using error-suppressed multiplexed deep sequencing. Cancer Res 2012;72:3492–8.
114. Board RE, Wardley AM, Dixon JM, Armstrong AC, Howell S, Renshaw L, et al. Detection of PIK3CA mutations in circulating free DNA in patients with breast cancer. Breast Cancer Res Treat 2010;120:461–7.
115. Dawson SJ, Tsui DW, Murtaza M, Biggs H, Rueda OM, Chin SF, et al. Analysis of circulating tumor DNA to monitor metastatic breast cancer. N Engl J Med 2013;368:1199–1209.
116. Madic J, Kiialainen A, Bidard FC, Birzele F, Ramey G, Leroy, et al. Circulating tumor DNA and circulating tumor cells in metastatic triple negative breast cancer patients. Int J Cancer 2015;136:2158–65.
117. Lecomte T, Berger A, Zinzindohoue F, Micard S, Landi B, Blons H, et al. Detection of free-circulating tumor-associated DNA in plasma of colorectal cancer patients and its association with prognosis. Int J Cancer 2002;100:542–8.
118. Leary RJ, Sausen M, Kinde I, Papadopoulos N, Carpten JD, Craig D, et al. Detection of chromosomal alterations in the circulation of cancer patients with whole-genome sequencing. Sci Transl Med 2012;4:162ra154.
119. Thierry AR, Mouliere F, ElMessaoudi S, Mollevi C, Lopez-Crapez E, Rolet F, et al. Clinical validation of the detection of KRAS and BRAF mutations from circulating tumor DNA. Nat Med 2014;20:430–5.
120. Punnoose EA, Atwal S, Liu W, Raja R, Fine BM, Hughes BG, et al. Evaluation of circulating tumor cells and circulating tumor DNA in non-small cell lung cancer: association with clinical endpoints in a phase II clinical trial of pertuzumab and erlotinib. Clin Cancer Res 2012;18: 2391–401.
121. Murtaza M, Dawson SJ, Tsui DW, Gale D, Forshew T, Piskorz AM, et al. Non-invasive analysis of acquired resistance to cancer therapy by sequencing of plasma DNA. Nature 2013;497:108–12.
122. Diaz LA Jr, Sausen M, Fisher GA, Velculescu VE. Insights into therapeutic resistance from whole-genome analyses of circulating tumor DNA. Oncotarget 2013;4:1856–7.
123. Reinert T, Schøler LV, Thomsen R, Tobiasen H, Vang S, Nordentoft I, et al. Analysis of circulating tumour DNA to monitor disease burden following colorectal cancer surgery. Gut 2015 Feb 4. [Epub ahead of print].
124. Olsson E, Winter C, George A, Chen Y, Howlin J, Tang MH, et al. Serial monitoring of circulating tumor DNA in patients with primary breast cancer for detection of occult metastatic disease. EMBO Mol Med 2015;7:1034–47.
125. Tie J, Kinde I, Wang Y, Wong H, Skinner I, Wong R, et al. Circulating tumor DNA (ctDNA) as a marker of recurrence risk in stage II colon cancer (CC). J Clin Oncol 2014;32, 5s (suppl; abstr 11015).
126. Pantel K, Denève E, Nocca D, Coffy A, Vendrell JP, Maudelonde T, Riethdorf S, Alix-Panabières C. Circulating epithelial cells in patients with benign colon diseases. Clin Chem 2012;58:936–40.
127. Andreopoulou E, Yang LY, Rangel KM, Reuben JM, Hsu L, Krishnamurthy S, et al. Comparison of assay methods for detection of circulating tumor cells in metastatic breast cancer: AdnaGen AdnaTest BreastCancer Select/Detect versus Veridex CellSearch system. Int J Cancer 2011;130:1590–7.
128. Musella V, Pietrantonio F, Di Buduo E, Iacovelli R, Martinetti A, Sottotetti E, et al. Circulating tumor cells as a longitudinal biomarker in patients with advanced chemorefractory, RAS-BRAF wild-type colorectal cancer receiving cetuximab or panitumumab. Int J Cancer 2015; 137:1467–74.
129. Ozkumur E, Shah AM, Ciciliano JC, Emmink BL, Miyamoto DT, Brachtel E, et al. Inertial focusing for tumor antigen-dependent and -independent sorting of rare circulating tumor cells. Sci Transl Med 2013;5:179ra47.
130. Hillig T, Horn P, Nygaard AB, Haugaard AS, Nejlund S, Brandslund I, et al. *In vitro* detection of circulating tumor cells compared by the CytoTrack and CellSearch methods. Tumour Biol 2015;36:4597–601.
131. Autebert J, Coudert B, Champ J, Saias L, Guneri ET, Lebofsky R, et al. High purity microfluidic sorting and analysis of circulating tumor cells: towards routine mutation detection. Lab Chip 2015;15:2090–101.
132. Kirby BJ, Jodari M, Loftus MS, Gakhar G, Pratt ED, Chanel-Vos C, et al. Functional characterization of circulating tumor cells with a prostate-cancer-specific microfluidic device. PLoS One 2012;7:e35976.
133. Galletti G, Sung MS, Vahdat LT, Shah MA, Santana SM, Altavilla G, et al. Isolation of breast cancer and gastric cancer circulating tumor cells by use of an anti HER2-based microfluidic device. Lab Chip 2014;14:147–56.
134. Sheng W, Ogunwobi OO, Chen T, Zhang J, George TJ, Liu C, et al. Capture, release and culture of circulating tumor cells from pancreatic cancer patients using an enhanced mixing chip. Lab Chip 2013;14: 89–98.
135. Yoon HJ, Kim TH, Zhang Z, Azizi E, Pham TM, Paoletti C, et al. Sensitive capture of circulating tumour cells by functionalized graphene oxide nanosheets. Nat Nanotechnol 2013;8:735–41.
136. López-Riquelme N, Minguela A, Villar-Permuy F, Ciprian D, Castillejo A, Álvarez-López MR, et al. Imaging cytometry for counting circulating tumor cells: comparative analysis of the CellSearch vs ImageStream systems. APMIS 2013;121:1139–43.
137. Harb W, Fan A, Tran T, Danila DC, Keys D, Schwartz M, et al. Mutational analysis of circulating tumor cells using a novel microfluidic collection device and qPCR assay. Transl Oncol 2013;6:528–38.
138. Winer-Jones JP, Vahidi B, Arquilevich N, Fang C, Ferguson S, Harkins D, et al. Circulating tumor cells: clinically relevant molecular access based on a novel CTC flow cell. PLoS One 2014;9:e86717.

139. Pluim D, Devriese LA, Beijnen JH, Schellens JH. Validation of a multiparameter flow cytometry method for the determination of phosphorylated extracellular-signal-regulated kinase and DNA in circulating tumor cells. Cytometry A 2012;81:664–71.
140. Chinen LT, de Carvalho FM, Rocha BM, Aguiar CM, Abdallah EA, Campanha D, et al. Cytokeratin-based CTC counting unrelated to clinical follow up. J Thorac Dis 2013;5:593–9.
141. Earhart CM, Hughes CE, Gaster RS, Ooi CC, Wilson RJ, Zhou LY, et al. Isolation and mutational analysis of circulating tumor cells from lung cancer patients with magnetic sifters and biochips. Lab Chip 2014;14: 78–88.
142. Kamande JW, Hupert ML, Witek MA, Wang H, Torphy RJ, Dharmasiri U, et al. Modular microsystem for the isolation, enumeration, and phenotyping of circulating tumor cells in patients with pancreatic cancer. Anal Chem 2013;85:9092–100.
143. Kalinsky K, Mayer JA, Xu X, Pham T, Wong KL, Villarin E, et al. Correlation of hormone receptor status between circulating tumor cells, primary tumor, and metastasis in breast cancer patients. Clin Transl Oncol 2015;17:539–46.
144. Karabacak NM, Spuhler PS, Fachin F, Lim EJ, Pai V, Ozkumur E, et al. Microfluidic, marker-free isolation of circulating tumor cells from blood samples. Nat Protoc 2014;9:694–710.
145. Casavant BP, Mosher R, Warrick JW, Maccoux LJ, Berry SM, Becker JT, et al. - A negative selection methodology using a microfluidic platform for the isolation and enumeration of circulating tumor cells. Methods 2013;64: 137–43.
146. Giordano A, Gao H, Anfossi S, Cohen E, Mego M, Lee BN, et al. Epithelial-mesenchymal transition and stem cell markers in patients with HER2-positive metastatic breast cancer. Mol Cancer Ther 2012;11:2526–34.
147. Wu Y, Deighan CJ, Miller BL, Balasubramanian P, Lustberg MB, Zborowski M, et al. Isolation and analysis of rare cells in the blood of cancer patients using a negative depletion methodology. Methods 2013;64: 169–82.
148. Liu Z, Fusi A, Klopocki E, Schmittel A, Tinhofer I, Nonnenmacher A, et al. Negative enrichment by immunomagnetic nanobeads for unbiased characterization of circulating tumor cells from peripheral blood of cancer patients. J Transl Med 2011;9:70.
149. Saucedo-Zeni N, Mewes S, Niestroj R, Gasiorowski L, Murawa D, Nowaczyk P, et al. A novel method for the *in vivo* isolation of circulating tumor cells from peripheral blood of cancer patients using a functionalized and structured medical wire. Int J Oncol 2012;41:1241–50.
150. Adams DL, Stefansson S, Haudenschild C, Martin SS, Charpentier M, Chumsri S, et al. Cytometric characterization of circulating tumor cells captured by microfiltration and their correlation to the cellsearch(®) CTC test. Cytometry A 2015;87:137–44.
151. Krebs MG, Hou JM, Sloane R, Lancashire L, Priest L, Nonaka D, et al. Analysis of circulating tumor cells in patients with non-small cell lung cancer using epithelial marker-dependent and -independent approaches. J Thorac Oncol 2012;7:306–15.
152. Ilie M, Hofman V, Long-Mira E, Selva E, Vignaud JM, Padovani B, et al. "Sentinel" circulating tumor cells allow early diagnosis of lung cancer in patients with chronic obstructive pulmonary disease. PLoS One 2014;9: e111597.
153. Zhou MD, Hao S, Williams AJ, Harouaka RA, Schrand B, Rawal S, et al. Separable bilayer microfiltration device for viable label-free enrichment of circulating tumour cells. Sci Rep 2014;4:7392.
154. Goldkorn A, Ely B, Tangen CM, Tai YC, Xu T, Li H, et al. Circulating tumor cell telomerase activity as a prognostic marker for overall survival in SWOG 0421: a phase III metastatic castration resistant prostate cancer trial. Int J Cancer 2015;136:1856–62.
155. Freidin MB, Tay A, Freydina DV, Chudasama D, Nicholson AG, Rice A, et al. An assessment of diagnostic performance of a filter-based antibody-independent peripheral blood circulating tumour cell capture paired with cytomorphologic criteria for the diagnosis of cancer. Lung Cancer 2014;85:182–5.
156. Khoo BL, Warkiani ME, Tan DS, Bhagat AA, Irwin D, Lau DP, et al. Clinical validation of an ultra high-throughput spiral microfluidics for the detection and enrichment of viable circulating tumor cells. PLoS One 2014;9: e99409.
157. Sollier E, Go DE, Che J, Gossett DR, O'Byrne S, Weaver, et al. Size-selective collection of circulating tumor cells using Vortex technology. Lab Chip 2013;14:63–77.
158. Shim S, Stemke-Hale K, Tsimberidou AM, Noshari J, Anderson TE, Gascoyne PR. Antibody-independent isolation of circulating tumor cells by continuous-flow dielectrophoresis. Biomicrofluidics 2013;7:11807.
159. Peeters DJ, De Laere B, Van den Eynden GG, Van Laere SJ, Rothe F, Ignatiadis M, et al. Semiautomated isolation and molecular characterisation of single or highly purified tumour cells from CellSearch enriched blood samples using dielectrophoretic cell sorting. Br J Cancer 2013; 108:1358–67.
160. Somlo G, Lau SK, Frankel P, Hsieh HB, Liu X, Yang L, et al. Multiple biomarker expression on circulating tumor cells in comparison to tumor tissues from primary and metastatic sites in patients with locally advanced/inflammatory, and stage IV breast cancer, using a novel detection technology. Breast Cancer Res Treat 2011;128:155–63.
161. Campton DE, Ramirez AB, Nordberg JJ, Drovetto N, Clein AC, Varshavskaya P, et al. High-recovery visual identification and single-cell retrieval of circulating tumor cells for genomic analysis using a dual-technology platform integrated with automated immunofluorescence staining. BMC Cancer 2015;15:360.
162. Königsberg R, Obermayr E, Bises G, Pfeiler G, Gneist M, Wrba F, et al. Detection of EpCAM positive and negative circulating tumor cells in metastatic breast cancer patients. Acta Oncol 2011;50:700–10.
163. 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 2013;60: 214–21.
164. Friedlander TW, Ngo VT, Dong H, Premasekharan G, Weinberg V, Doty S, et al. Detection and characterization of invasive circulating tumor cells derived from men with metastatic castration-resistant prostate cancer. Int J Cancer 2014;134:2284–93.
165. Galanzha EI, Zharov VP. Circulating tumor cell detection and capture by photoacoustic flow cytometry *in vivo* and *ex vivo*. Cancers (Basel) 2013;5:1691–738.
166. Chan KC, Jiang P, Chan CW, Sun K, Wong J, Hui EP, et al. Noninvasive detection of cancer-associated genome-wide hypomethylation and copy number aberrations by plasma DNA bisulfite sequencing. Proc Natl Acad Sci U S A 2013;110:18761–8.
167. Bidard FC, Madic J, Mariani P, Piperno-Neumann S, Rampanou A, Servois V, et al. Detection rate and prognostic value of circulating tumor cells and circulating tumor DNA in metastatic uveal melanoma. Int J Cancer 2013;134:1207–13.
168. Kidess E, Heirich K, Wiggin M, Vysotskaia V, Visser BC, Marziali A, et al. Mutation profiling of tumor DNA from plasma and tumor tissue of colorectal cancer patients with a novel, high-sensitivity multiplexed mutation detection platform. Oncotarget 2015;6:2549–61.