



Liquid Biopsy for Prognosis and Treatment in Metastatic Colorectal Cancer: Circulating Tumor Cells vs Circulating Tumor DNA

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Abstract

Liquid biopsy recently gained widespread attention as a noninvasive alternative/complementary technique to tissue biopsy in patients with cancer. As technological advances have improved both feasibility and turnaround time, liquid biopsy has expanded tumor molecular analysis with acknowledgement of both spatial and temporal heterogeneity, overcoming many limitations of traditional tissue biopsy. Because of its diagnostic, prognostic, and predictive value, liquid biopsy has been extensively studied also in metastatic colorectal cancer. Indeed, as personalized medicine establishes its role in cancer treatment, genetic biomarkers unveiling the emergence of early resistance are needed. Among the wide variety of tumor analytes amenable to collection, circulating DNA and circulating tumor cells are the most adopted approaches, and both carry clinical relevance in colorectal cancer. However, few studies focused on comparing feasibility between these two approaches. In this review, we discuss the potential implications of liquid biopsy in metastatic colorectal cancer, assessing the advantages and drawbacks of circulating DNA and circulating tumor cells, and highlighting the most relevant trials for clinical practice.

1 Introduction

Colorectal cancer (CRC) is one of the most common and most lethal cancers, representing 10.2% of new cases and 9.2% of cancer-related deaths, with a 5-year relative overall survival (OS) ranging from 90% in localized disease to 14% in metastatic CRC (mCRC) [1, 2]. In current clinical practice, therapeutic choices are driven by tumor biopsy-derived molecular profiles, contemplating the status of *RAS* and *BRAF* genes, and the DNA mismatch repair (MMR) system. Implementation of next-generation sequencing (NGS) broadened the molecular analysis with the aim of expanding targeted therapies also in mCRC [3, 4]. In this scenario, liquid biopsy (LB) gained widespread attention as a noninvasive alternative/complementary technique to tissue biopsy. The term LB refers to the analysis of tumor-derived

Key Points

Circulating tumor cells and circulating tumor DNA have been extensively studied in metastatic colorectal cancer with regard to their diagnostic, prognostic, and predictive impact; however, data of direct comparison between these two techniques are lacking.

Circulating tumor DNA yields higher sensitivity and suitability than circulating tumor cells, thus being the main plasma biomarker employed in clinical trials and closer to reach clinical practice for metastatic colorectal cancer. However, circulating tumor cells boast the unique potential to serve as a platform for ex vivo culture and xenografting.

Randomized clinical trials are needed to establish how to integrate liquid biopsy to improve the prognosis of patients affected by metastatic colorectal cancer.

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biomarkers, most frequently in the blood, including circulating DNA and circulating tumor cells (CTCs) [5]. Liquid biopsy has been identified as a diagnostic, prognostic, and predictive biomarker, allowing early cancer detection, molecular profiling, estimation of relapse risk, selection of anticancer drugs, monitoring of treatment response, and identification of drug resistance mechanisms [6]. Advantages over tissue biopsy, including minimal invasiveness and fast turnaround, allow feasibility of close repeated testing and extensive molecular characterization by depicting both spatial heterogeneity (intra-tumoral and between different tumor sites) and temporal heterogeneity (mainly caused by anticancer treatments over time) [7, 8]. In particular, mCRC was proven highly heterogeneous, with continuous clonal evolution especially under the selective pressure of anti-cancer agents. Thus, routine monitoring of real-time tumor-associated genomic changes may appoint LB as a mainstay for treatment selection for the continuum of care in mCRC; furthermore, spatial omni-comprehensiveness of LB may overcome tissue biopsy as a more accurate tool for high-burden tumors [9–11]. In this review, we discuss the potential implications of LB in mCRC focusing on comparison between CTCs and circulating DNA.

2 Definition and Techniques Adopted for LB

2.1 CTCs

First discovered through LB in the late nineteenth century, CTCs are intact cancer cells originating from both the primary tumor and metastases, allowing an understanding of cancer spreading and metastasis [12]. Circulating tumor cells present with different proportions in various tumors, usually with a low concentration due to a short half-life (1–10 cells per 10 mL of blood as compared to 10^7 – 10^8 leukocytes) [13]. Heterogeneity hampers the definition of both sensitive and specific markers for collection. Circulating tumor cells are mainly separated by means of certain physical (i.e., filtration) and biological properties (i.e., cell surface expression), generally requiring an enrichment step (to maximize collection) and a detection step [14]. To date, the CellSearch system is the only US Food and Drug Administration-approved platform for the clinical detection of CTCs in patients with cancer, demonstrating a reproducible CTC count and prognostic value also in mCRC. It uses positive enrichment through epithelial cell adhesion molecule antibodies and then detection by immunostaining for cytokeratins and DAPI as markers for intact epithelial cells, together with absent CD45 as an exclusion marker labeling leukocytes [15]. In CRC, CTCs were detectable through CellSearch with a 36.2% sensitivity, correlating with clinical staging (up to 60.7% in mCRC) [16]. As an alternative, the

AdnaTest integrates epithelial cell adhesion molecule-based enrichment with detection of cancer-specific genes through polymerase chain reaction (PCR), overcoming the expression loss of surface proteins due to epithelial-mesenchymal transition [17, 18]. Moreover, the combination of the CellSearch assay with the AdnaTest further improved the CTC detection rate in mCRC (CellSearch positivity rate 33%, AdnaTest 30%, combined 50%) [19]. The advent of microfluidic platforms such as the ‘CTC-chip’ further increased detection rates, efficiently collecting viable CRC-CTCs in microposts coated with a mixture of antibodies, under laminar flow [20]. When comparing CellSearch with IsoFlux, a microfluidic system with immunomagnetic implementation, the CRC-CTC yield was eight times higher with the latter [21]. Enrichment through leukapheresis could also substantially increase the CRC-CTC detection when combined with CellSearch [22]. The EPISPOT test and photoacoustic flow cytometry, the latter allowing *in vivo* CTC detection through the skin, are the most cutting-edge technologies in development [23, 24]. Overall, technological advances have led to dozens of new platforms with implemented capture efficiency, throughput rates, purity, and capability of molecular analysis [25, 26].

2.2 Circulating DNA

Circulating free DNA (cfDNA) was first detected in the mid-Twentieth century, consisting of fragments of non-encapsulated DNA in the bloodstream of healthy individuals [27]. In the late 1970s, comparable circulating tumor DNA (ctDNA) from malignant cells was discovered in patients with cancer [28]. As a result of increased cell turnover, patients with cancer usually have higher cfDNA levels than healthy subjects, with a variable ctDNA-to-cfDNA ratio of 0.01–90% depending on the tumor type, biological behavior, and cancer stage [29]. Among various malignancies, mCRC harbors the highest ctDNA amount [30]. Apart from genetic alterations, epigenetic modifications can be captured on ctDNA, most commonly in the form of DNA methylation. Difficulties in ctDNA detection include low-frequency aberrations and non-neoplastic age-dependent alterations in common driver genes. Hence, ctDNA detection requires highly sensitive and specific approaches [31]. Targeted methods such as quantitative and digital PCR (dPCR) mainly differ in their limit of ctDNA detection, reaching rates of 0.1% and 0.005%, respectively [32, 33]. Further high-resolution PCR-based technologies such as BEAMing, ARMS, and UltraSEEK further enhance the sensitivity to 0.001% and allele specificity up to a single base difference with the detection of multiple minor variants in a single reaction [34–36]. While PCR remains confined to the analysis of few loci, NGS provides simultaneous targeted characterization of multiple genomic alterations focusing

on regions of interest for mutations, but also copy number variations and chromosomal rearrangements [37, 38]. Untargeted whole genome sequencing further expands the genomic analysis, with potential applicability to extensive clonal tracking due to tumor progression or treatments, at the expense of higher costs and lower sensitivity especially for low-frequency ctDNA. However, fragment size analysis and selective sequencing of specific fragment sizes boosted ctDNA detection for whole genome sequencing in plasma samples from patients with mCRC [39]. Furthermore, a new whole genome sequencing method named Plasma-Seq overcame this issue by providing sequencing in less than 48 h [40, 41]. Finally, circulating methylated DNA (cmDNA) is similarly evaluated through targeted or untargeted interrogation. Apart from conventional direct bisulfite sequencing or pyrosequencing, novel methods such as methylation-specific PRC and methyl-BEAMing were found capable of sensitive detection of cmDNA [42, 43]. A summary of circulating DNA and CTCs with regard to advantages and drawbacks of both methods is available in Fig. 1, while the main features with regard to mCRC are listed in Table 1.

2.3 Exosomes

Exosomes are small extracellularly secreted vesicles (50–150 nm), containing evaluable biomarkers such as nucleic acids [44]. Given the preservation of RNA from RNAase enzymatic activity, exosomes may provide a source of messenger RNA and microRNA (miRNA), the latter being small non-coding RNA molecules implied in RNA silencing and carcinogenesis [45, 46]. Exosomes can be isolated from peripheral blood using biophysical properties such as centrifugation or precipitation, and immunoaffinity capture. After extraction procedures, targeted or untargeted methods are employed for RNA detection [47]. Despite limited research in this field for CRC, serum exosomal miRNA such as miRNA21, miRNA23, miRNA19a, and miRNA92a were identified as the most increased in this disease, some of them correlating with tumor prognosis, recurrence, and sensitivity to anti-cancer agents [48–52]. However, an in-depth analysis of exosomes in mCRC is beyond the scope of this review.

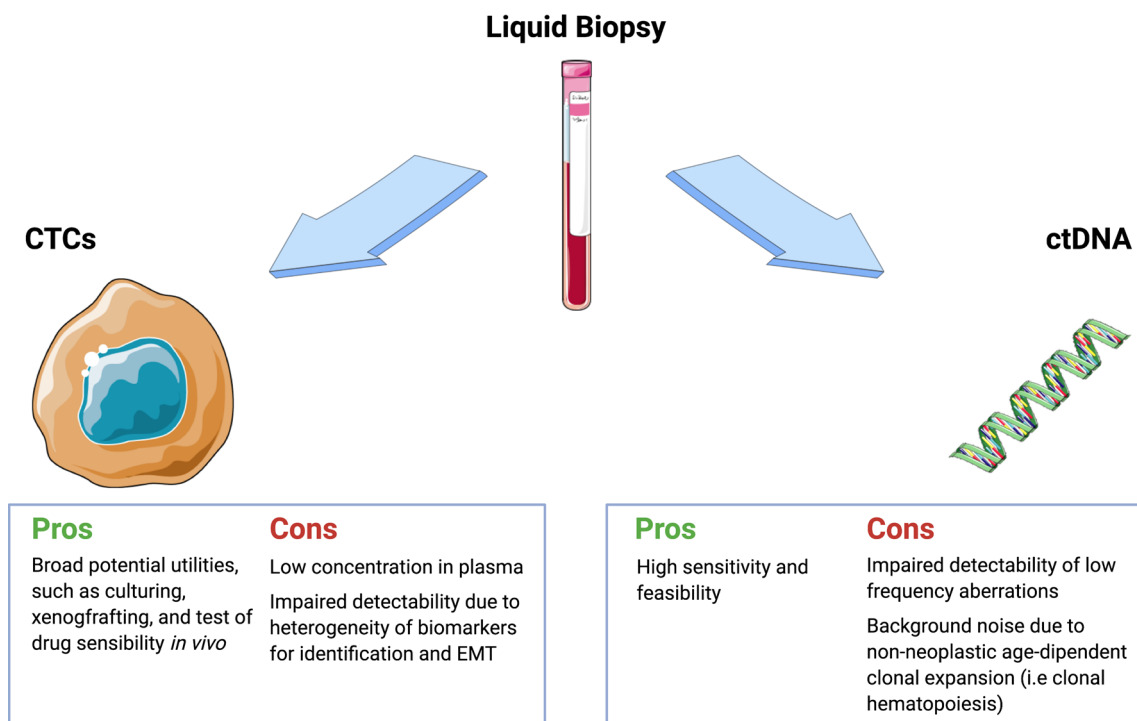




Fig. 1 Advantages and disadvantages of circulating tumor cells (CTCs) and circulating tumor DNA (ctDNA) in metastatic colorectal cancer (mCRC). Collection of CTCs is mainly impaired by their low abundance in plasma (median 2 CTCs/7.5 mL of peripheral blood in mCRC). Furthermore, high heterogeneity of cell surface expression and loss of epithelial markers (due to epithelial-mesenchymal transition [EMT]) also contribute to complexity in CTC isolation; their use allows cell culturing and xenografting, hence bridging the clinical

and preclinical scenarios. On the contrary, ctDNA is easily detectable in peripheral blood and its feasibility allows intensive clonal tracking and monitoring of emerging resistance mechanisms during active treatment in mCRC; however, a low frequency of certain genomic aberrations and interference of non-neoplastic clonal expansion may compromise sensitivity and specificity. Created with smart.servier.com

Table 1 Main features of CTCs and ctDNA focusing on mCRC. Created with smart.servier.com

	CTCs	ctDNA
		
Origin	Viable and apoptotic cells	Mainly apoptotic cells
Components	DNA, RNA, proteins, metabolites	DNA
Suitable analyses	Genomics (mutations, copy number alterations, epigenetic alterations, fusion genes); transcriptomics (mRNA, including splice variants); proteomics; single-cell level analysis	Mutations, copy number alterations, epigenetic alterations, fusion genes
Culturing and xenografting	Yes	No
Sensitivity and specificity	Low sensitivity due to low abundance in plasma (especially for mCRC), heterogeneity of biomarkers for identification and EMT; around 50% sensitivity when combining CellSearch assay and the AdnaTest; variable specificity according to detection methods	High sensitivity due to large abundance in plasma (especially for mCRC); detection of mutant alleles with a fractional abundance up to 0.001% with dPCR; improved sensitivity and specificity with emerging tumor-informed techniques; impaired specificity due to background noise from non-neoplastic age-dependent alterations (i.e., clonal hematopoiesis)
Applications in the continuum of care for patients with mCRC	Prognosis, prediction of treatment response, molecular profiling, clonal evolution tracking and early identification of resistance mechanisms, treatment response monitoring, early detection of recurrence and MRD, in vivo tests of drug sensitivity	Prognosis, prediction of treatment response, molecular profiling, clonal evolution tracking and early identification of resistance mechanisms, treatment response monitoring, early detection of recurrence, and MRD

CTCs circulating tumor cells, ctDNA circulating tumor DNA, dPCR digital polymerase chain reaction, EMT epithelial-mesenchymal transition, mCRC metastatic colorectal cancer, MRD minimal residual disease, mRNA messenger RNA

3 Prognostic and Predictive Value of LB

As treatment options expanded in mCRC, imaging studies remain the main discriminating factor for progression to anticancer therapy. Blood biomarkers are needed to predict tumor response and determine *a priori* reliable prognostic groups of patients with mCRC.

3.1 CTCs

Several studies demonstrated that CTC levels are associated with shorter survival in mCRC, as they positively correlate with tumor burden and aggressiveness. Cohen and colleagues proved feasibility of CTC isolation in 50 patients with mCRC, with 77% presenting with ≥ 1 CTC/7.5 mL of blood and a median yield of 2 CTCs/7.5 mL, dramatically less than other malignancies [53, 54]. In a following study on 430 patients with mCRC, ≥ 3 CTCs/7.5 mL was associated with shorter progression-free survival (PFS) and OS (PFS 4.5 vs 7.9 months, $p = 0.0002$; OS 9.4 vs 18.5 months, $p < 0.0001$). Conversion of baseline CTC count under cytotoxic chemotherapy from ≥ 3 to < 3 within 5 weeks of treatment was associated with longer PFS and OS (PFS 6.2 vs 1.6 months; $p = 0.02$; OS 11.0 vs 3.7 months, p

$= 0.0002$) [55]. The combination of CTCs and carcinoembryonic antigen (CEA) was capable of finer stratification in patients with baseline CEA ≥ 25 ng/mL, as those with < 3 baseline CTCs had longer OS (OS 20.8 vs 11.7 months, $p = 0.001$) and, among patients with a similar CEA reduction after treatment, the three CTC cut-off at 6–12 weeks distinguished two opposite prognostic groups (OS 19.8 vs 5 months, $p = 0.0001$) [56]. These results were corroborated by several studies, including ancillary studies of the CAIRO2 and the MACRO TTD trials, and a meta-analysis of 2388 patients with CRC [57–59]. Association of the CTC count with poorer PFS and OS was also confirmed through a meta-analysis of 264 patients with resectable colorectal liver metastases (CRLM) [OS hazard ratio (HR) 2.47, $p < 0.0001$; PFS HR 2.07, $p < 0.0001$] [60]. High-CTC levels were related with non-resectability and impaired survival after metastasectomy, suggesting CTCs as a tool for decision making before liver resection. Furthermore, patients with ≥ 2 CTCs/7.5 mL experienced reduced PFS and OS when analyzing all patients and resectable patients only ($p < 0.001$) [61]. Given the proven prognostic value, some authors suggest that CTCs could help identify those patients benefiting the most from treatment intensification, avoiding high-toxicity regimens in low-CTC mCRC [62]. High-quality,

well-designed, large-scale, multicenter prospective interventional studies are needed to assess if CTC-based therapeutic decisions could improve prognosis in mCRC. To date, the main effort to employ CTCs as a prognostic tool for treatment intensification or de-escalation in patients with mCRC comes from the VISNU-1 and VISNU-2 trials. In the phase III VISNU-1 trial, patients with mCRC with three or more baseline CTCs/7.5 mL (poor prognostic factor) were randomized to receive first-line bevacizumab with either FOLFOXIRI or FOLFOX, with superior PFS for the triplet arm [63]. Conversely, low-risk (< 3 CTCs/7.5 mL) patients with *RAS* wild-type (WT) mCRC were treated with doublet FOLFIRI with bevacizumab or cetuximab in the phase II VISNU-2 trial, to assess the influence of *BRAF* and *PI3K* mutational status on treatment efficacy [64]. Because a high-CTC count was established as a negative prognostic factor, this non-invasive biomarker may guide treatment selection in the mCRC first-line setting [65].

3.2 Circulating DNA

As with CTCs, circulating DNA was found to be prognostic in mCRC. Spindler and colleagues measured cfDNA in 55 patients with mCRC, achieving a wide range distribution (22–3922 ng/mL blood) and a mean value of 1157 ng/mL, with cfDNA levels > 1000 ng/mL correlating with shorter survival ($p = 0.02$) [29]. Similarly, a retrospective post hoc study of the NORDIC-VII trial found that high cfDNA levels before chemotherapy were associated with an impaired outcome (OS HR 1.83, $p < 0.001$), then confirmed by a meta-analysis of 1076 patients with mCRC (OS HR = 2.39, $p < 0.0001$) [66, 67]. A retrospective analysis of the CORRECT trial showed that clinical benefit from regorafenib was associated with *KRAS* and *PIK3CA* WT ctDNA status (*KRAS* WT vs mutant-pinteraction 0.74; *PIK3CA* WT vs mutant-pinteraction 0.85) as well as low cfDNA concentration (low vs high cfDNA-pinteraction 0.601) [68]. Furthermore, combined analysis of cfDNA and ctDNA added a supplementary prognostic impact [69]. Early changes in ctDNA levels were suggested as a biomarker for treatment response and outcomes in patients with mCRC. In a study of 29 patients with mCRC assessing ctDNA levels at baseline and 8 weeks after chemotherapy, ctDNA halved the predicted radiological response and resulted in longer PFS and OS (PFS HR 0.33, $p = 0.04$; OS HR 0.30, $p = 0.08$) [70]. Similarly, serial ctDNA analysis was able to identify a subsequent increase in ctDNA in initial responders to cytotoxic agents, effectively anticipating tumor progression as proven by shorter PFS and OS [71]. These results are consistent with those from the 82 patients with mCRC in the PLACOL study, monitoring ctDNA or hypermethylated alleles by dPCR [72]. The prognostic role of ctDNA was confirmed also in 41 patients with CRLM, where a decreased ctDNA/b-globin

ratio after liver surgery predicted longer disease-free survival (366 vs 102 days, $p < 0.001$) [73]. In another cohort of 18 patients with CRLM, ctDNA was more sensitive than CEA in detecting minimal residual disease (ctDNA 100% vs CEA 56%, $p = 0.008$) and predicting postoperative recurrence ($p = 0.003$) [74]. In the PREDATOR trial, sensitivity for minimal residual disease was improved by personalized tumor-informed ctDNA detection, assessing plasma mutations according to erstwhile tumor tissue profiling in 113 oligometastatic patients with CRC (HR 4.59, $p < 0.001$) [75]. Finally, cmDNA due to epigenetic changes was employed to circumvent the absence of cancer-specific mutations in cfDNA. In a study assessing five methylated loci, 87% of 182 patients with mCRC showed positivity in one or more loci, dynamically correlating with overall response rate and PFS in those receiving anticancer therapy [76]. Amatu et al. confirmed baseline and dynamic cmDNA as prognostic and predictive in patients with mCRC treated with regorafenib [77]. Validation of circulating DNA in prospective clinical trials is warranted. The ongoing trial NCT03844620 is currently evaluating ctDNA capability to serve as a guide for precocious interruption of regorafenib or TAS-102 independently from radiological progression [78].

4 Molecular Profiling and Monitoring of Resistance Mechanisms Through LB

In the era of personalized medicine, the administration of targeted therapies is still relatively impeded by difficulties in tissue biopsy, which is not as feasible as necessary for extended molecular characterization and assessment of evolving molecular targets. LB is currently under investigation as a tool for genomic profiling in mCRC. Figure 2 shows a potential algorithm for LB as a guide for treatment selection and monitoring according to clonal tracking in patients with mCRC receiving active treatment.

4.1 CTCs

Cayrefourcq and colleagues were the first to report the successful establishment of a stable mCRC-derived CTC line. However, only one long-term CTC line was achieved from 71 unique blood samples (1.4%). Noteworthy, this required a CTC count over 300/10 mL, available only for two samples. Despite the low success rate of CTC line generation, culturing allowed NGS-based genomic analysis, followed by single-cell transcriptome, proteome, and secretome analysis, and xenotransplantation. Importantly, *KRAS* and *BRAF* concordance was proven between primary tumor, metastases, cell line, and xenografts [79]. Moreover, CTC genotyping allowed the identification of tissue-undetected mutations and secondary mechanisms of resistance to anti-epidermal

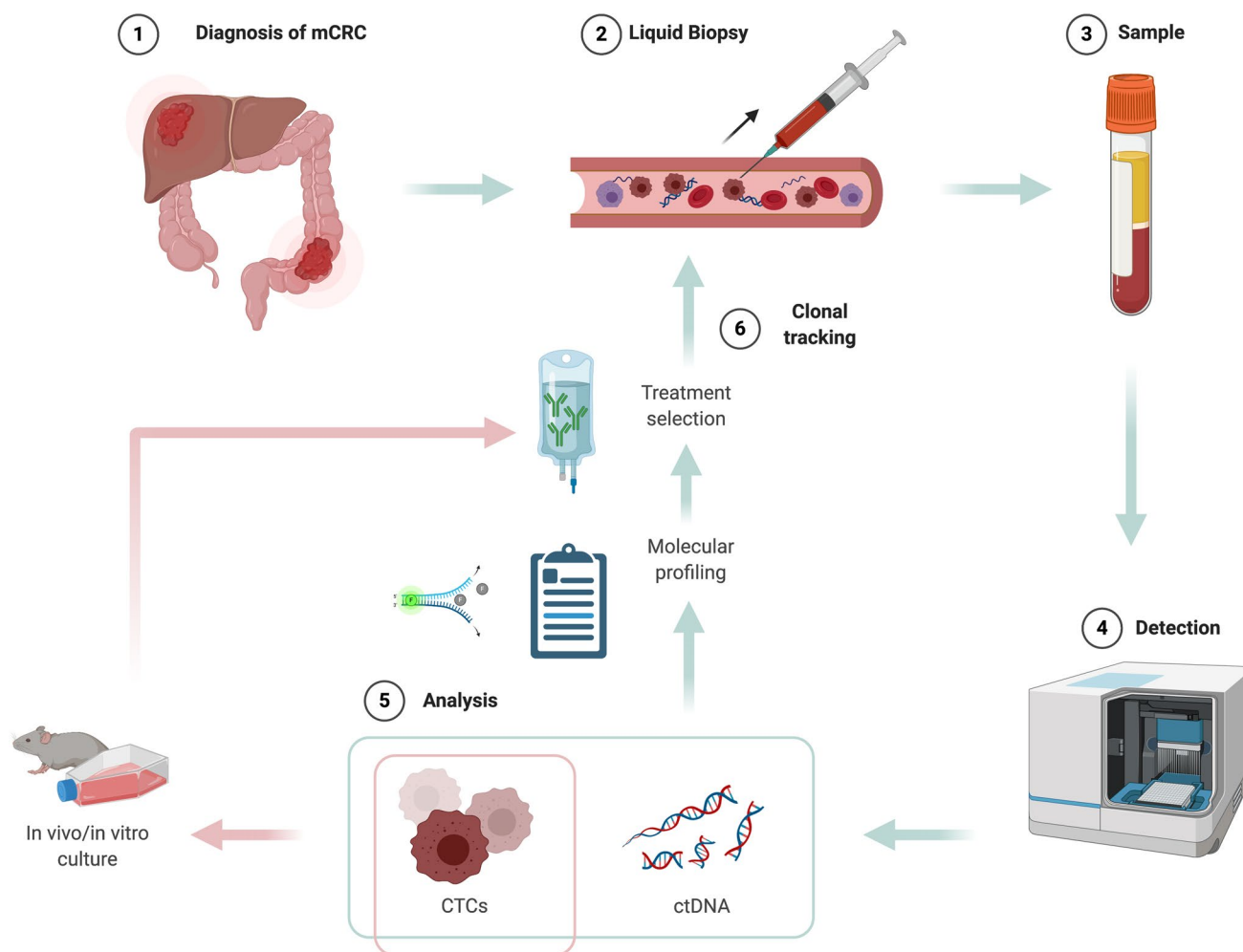


Fig. 2 Clonal tracking with liquid biopsy (LB) in patients with metastatic colorectal cancer (mCRC) receiving active treatment. After mCRC diagnosis (1), the collection of blood samples for LB (2,3) allows the detection of circulating tumor DNA (ctDNA) and circulating tumor cells (CTCs) (4). Their analysis provides useful information for molecular profiling and treatment selection; additionally,

CTCs can be employed for culturing and xenografting, thus adding relevant data of tumor sensitivity to the selected treatment (5). Given the high feasibility of LB, new blood samples are then collected during active treatment, to monitor the emergence of resistance through clonal tracking (6). Created with BioRender.com

growth factor receptor (EGFR) drugs [80, 81]. Altogether, the rarity of CTCs and strain from CTC line establishment are the major drawbacks in this setting [82].

4.2 Circulating DNA

The tumor mutational landscape is reflected by ctDNA, and high concordance with tissue analysis (> 80%) has been reported regarding main genomic alterations in mCRC [10, 30, 70]. The ongoing COLOMATE (NCT03765736) and TARGET trials are screening ctDNA genetic alterations in mCRC for enrolment onto targeted therapy-based/early-phase clinical trials [83]. Here, follows the main fields of interest for ctDNA profiling in mCRC.

4.2.1 Resistance to Anti-EGFR MoAb

Mutant status of *RAS* or *BRAF* is known to confer primary resistance to anti-EGFR monoclonal antibodies (MoAb) [84, 85]. With the advent of LB, many studies focused on the detection of *RAS* ctDNA in patients with mCRC, proving > 90% concordance with tissue analysis [10, 86–89]. The absence of liver metastases and primary tumor resection were the main clinical factors associated with unsuccessful ctDNA detection in the AGEO RASANC prospective study [89]. In the CAPRI-GOIM trial, first-line cetuximab-based therapy yielded consistent PFS and OS dichotomy between patients with *RAS* MT and WT mCRC, when retrospectively assessed by both LB and tissue biopsy [90]. Discordant

cases were attributed to high spatial heterogeneity, as tissue biopsy cannot fully reflect clonality in mCRC. Indeed, a higher frequency of ctDNA *KRAS*, *NRAS*, and *BRAF* mutations were found as compared with tissue (59% vs 44%, 11.8% vs 8.8%, and 14.4% vs 7.2%, respectively) [91]. Even if previously sensitive to an anti-EGFR MoAb, most *RAS* WT mCRC eventually acquire secondary resistance. Siravegna et al. tracked clonal evolution through ctDNA during treatment with anti-EGFR MoAb and found emerging alterations in *RAS*, *ERBB2*, *FLT3*, *EGFR*, and other genes [10]. A prospective LB-based trial monitored patients with *KRAS* WT mCRC receiving first-line FOLFIRI-cetuximab and found that persistent *RAS*, *BRAF*, and *PIK3CA* WT circulating status correlated with prolonged response, while an increase in mutant cfDNA predicted acquired resistance [92]. Besides, LB drastically anticipated radiographic progression as ctDNA *KRAS* MT alleles were detectable > 10 months beforehand [11]. Siena et al. reported results from a prospective analysis in patients with mCRC, confirming that serial plasma biopsies are more inclusive than tissue biopsies for evaluating *RAS* acquired mutations under selective pressure of anti-EGFR treatment. In a series of 15 patients with paired tumor tissue biopsy and plasma samples, BEAMing on LB showed a far higher *RAS* MT emergence rate than tissue analysis by BEAMing and NGS (57.1% vs 7.1% and 9.5%, respectively, $p = 0.008$), though without an impact on PFS. However, only *RAS* mutations were investigated by LB in this study, leaving aside other known molecular mechanisms of resistance [93]. A similar prospective investigation expanded the analysis to *ERBB2* and *MET* amplifications, with high heterogeneity [94]. Eventually, LB could help detect those preexisting tissue biopsy-concealed resistant subclones proliferating and expanding under positive pressure of anti-EGFR treatments, until conferring resistance to therapy [95].

4.2.2 Resistance to HER2-Targeted Therapies

Several actionable genomic alterations are emerging as resistance mechanisms and potential therapeutic targets in mCRC. Approximately 5% of advanced *KRAS* WT mCRC were found harboring *ERBB2* amplification/HER2 overexpression (HER2+). Recently, *ERBB2* amplification was found responsible for both primary and secondary resistance to anti-EGFR MoAb [96]. In this population of mCRC, anti-HER2 dual blockade regimens were successfully tested in the HERACLES (trastuzumab-lapatinib) and MyPathway (trastuzumab-pertuzumab) trials with a similar ORR of around 30%, and the most recent MOUNTAINEER (trastuzumab-tucatinib) with a 55% ORR. Data from the DESTINY-CRC01 trial using the antibody drug-conjugate trastuzumab-deruxtecan have been released at ASCO 2020 with promising results also in anti-HER2 pretreated mCRC

[97–100]. Liquid biopsy was recently explored as a method of assessment for HER2 status in a cohort of 18 patients with cetuximab-resistant mCRC, with 4/18 (22%) classified as ctDNA HER2+, concordantly with tissue samples at re-biopsy [101]. In a larger cohort of 344 patients with advanced gastrointestinal tumors (74% of which mCRC), ctDNA analysis found 5% had ctDNA HER2+ [102]. Siravegna and coworkers assessed concordance between LB and tissue analysis in a retrospective study from the HERACLES trial, with ctDNA correctly identifying 96.6% of samples as HER2+ and predicting benefit from HER2-targeted therapy [103]. Furthermore, an NGS analysis on ctDNA identified primary resistance mechanisms, undetectable with tissue biopsy, revealing that interventional LB could prevent ineffective employment of anti-HER2 treatment in > 85% of cases [104].

4.2.3 Other Biomarkers for Targeted Therapy

Around 10% of CRC harbor *BRAF* mutations, mostly V600E, conferring poor prognosis and representing an unmet medical need. The BEACON trial recently established *BRAF* as a therapeutic target in mCRC [105]. In an earlier study of 85 tissue-selected *BRAF* V600E MT mCRC, cfDNA BEAMing analysis revealed that 83.5% had detectable-*BRAF* mutations in plasma at baseline, dynamically predicting treatment response to anti-*BRAF* therapy [106]. Immunotherapy is a well-established therapeutic option for MMR-deficient mCRC [3, 4]. While few studies reported >98% accuracy of ctDNA MMR-deficient noninvasive identification, an ongoing prospective trial (NCT03594448) is investigating serial LB as a detection method for MMR-deficient mCRC [107, 108].

5 LB-Driven Retreatment Strategies with Anti-EGFR MoAb

Within the continuum of care, retreatment with anti-EGFR MoAb is a valid therapeutic strategy, especially in the way of a rechallenge after a wash-out period from previous progression [109, 110]. Liquid biopsy is the most suitable approach to determine restored sensitivity to anti-EGFR MoAb before a rechallenge. The CRICKET trial, a proof-of-concept study of third-line rechallenge with cetuximab-irinotecan, prospectively collected ctDNA in 28 patients with tissue-proven *RAS/BRAF* WT mCRC, revealing that only patients with *RAS* WT ctDNA at the time of the rechallenge were achieving partial response and positive correlation with PFS (PFS 4.0 vs 1.9 months, $p = 0.03$) [111]. These results, together with the post-hoc analyses of JACCRO CC-08, JACCRO CC-09, and E-Rechallenge trials, suggest that ctDNA could guide the selection of patients benefiting from

an anti-EGFR rechallenge, by excluding those with detectable resistant clones [112, 113]. The ongoing biomarker-driven CHRONOS trial (NCT03227926) will strengthen these findings by adopting ctDNA analysis as an inclusion criterion. This proof-of-concept prospective study enrolls patients with *RAS/BRAF* WT mCRC and prior sensitivity to anti-EGFR treatment, who then developed secondary resistance and were treated with one or more following lines of therapy. Candidates are only eligible for panitumumab rechallenge when absence/> 50% decrease in ctDNA *RAS* fractional mutation is proven after progression on first-line anti-EGFR, together with the absence of ctDNA *EGFR* ectodomain mutations. Similarly, the phase II NCT03087071 trial involves LB for *RAS*, *BRAF*, and *EGFR* ectodomains before rechallenge with panitumumab: patients without any *RAS* and *BRAF* mutations receive panitumumab as a single agent, whereas others receive a combination of panitumumab and trametinib. Martinelli and colleagues recently announced a proof-of-concept LB-driven prospective study of sequential treatments in patients with *RAS/BRAF* WT mCRC (CAPRI 2-GOIM trial), where treatment choices will be defined by ctDNA results. According to the study design, LB is collected after first-line anti-EGFR treatment to determine whether progression is on account of secondary resistance to anti-EGFR or other independent mechanisms, offering two sequence strategies: a continuum treatment with cetuximab beyond first line with a chemotherapy regimen switch in patients with ctDNA *RAS* WT; or a second-line treatment with FOLFOX-bevacizumab in patients with ctDNA *RAS* MT mCRC and then, if the patient reverts back to ctDNA *RAS* WT, rechallenge with cetuximab-irinotecan in the third line or, conversely, a standard-of-care third-line treatment in the case of persistent ctDNA *RAS* MT [114]. Finally, ctDNA could guide the correct sequence of anti-EGFR MoAb. When secondary resistance is due to certain mutations of EGFR ectodomain identified as S492R, K467, or R451C mutants, the tumor ceases to be sensitive to cetuximab but not to panitumumab, thus allowing switching of anti-EGFR moAb [115]. According to a recent study, up to 16% of S492R EGFR mutations were detected in 239 post-cetuximab plasma samples of patients with mCRC [116].

6 Direct Comparison between CTCs and Circulating DNA

Betgeowda and colleagues used dPCR to identify rearrangements in CTC-DNA and ctDNA of nine patients with CRC, of which five had mCRC. In 100% of cases, ctDNA could be measured, whereas CTC-DNA was detectable in three out of five (60%), those with the most abundant

ctDNA levels. The average number of ctDNA fragments was > 50-fold higher than analogous levels of CTCs [30]. Similarly, Germano et al. performed a parallel evaluation of CTCs and ctDNA in a cohort of 20 patients with mCRC. A 100% ctDNA detectability was assessed by droplet dPCR (ddPCR), whilst CTCs were isolated in 37% of patients, with >1 CTC/10 mL of blood in only two patients, again in those with a higher amount of ctDNA ($p < 0.005$). Tissue-LB concordance for ctDNA *RAS*, *BRAF*, and *ERBB2* was 85%, while CTCs were too limited for this analysis [117]. In the prospective PRODIGE-14 trial, levels of CTCs and *KRAS* ctDNA were investigated in a cohort of 153 patients with potentially resectable CRLM treated with first-line triplet (FOLFIRINOX) or doublet chemotherapy associated with targeted therapy. Assessment through CellSearch at inclusion showed ≥ 1 CTC/7.5 mL in 42% of patients, while 91% of 46 tissue-genotyped *KRAS* MT patients were confirmed by ddPCR. The proportion of patients with $\geq 3/7.5$ mL CTCs decreased during chemotherapy from 19% at baseline to 3% after 4 weeks and 0% before liver surgery; likewise, ctDNA lowered from 91 to 63% and then 19%. Detectable *KRAS* ctDNA after 4 weeks was associated with a lower R0/R1 surgical outcome ($p = 0.01$) and, among patients with R0/R1 resection, detectable ctDNA levels before surgery conferred shorter OS ($p < 0.001$). In multivariate analysis, ≥ 3 CTCs was an independent prognostic factor for OS both at baseline and after 4 weeks of therapy [118]. Onidani and coworkers improved CTC detection using an epithelial cell adhesion molecule-independent approach in a cohort of 20 patients with gastrointestinal cancer, among whom 11 had mCRC (55%), with a median 14.5 CTCs/mL. A combination NGS analysis of genomic profiles obtained from CTCs and ctDNA showed low concordance, suggesting high tumor

Table 2 Comparison of sensitivity for ctDNA and CTCs in mCRC

Study	Detectable ctDNA (%)	Detectable CTCs or CTC-DNA
Betgeowda et al. [30] 5 patients with mCRC	100	60
Germano et al. [117] 20 patients with mCRC	100	37
Bidard et al. [118] 46 patients with mCRC	91	42
Onidani et al. [81] 7 patients with mCRC	100	57

CTCs circulating tumor cells, CTC-DNA DNA extracted from circulating tumor cells, ctDNA circulating tumor DNA, mCRC metastatic colorectal cancer

Table 3 Trials prospectively assessing the role of liquid biopsy in mCRC

Study	Study population	Liquid biopsy analysis	Primary endpoints
Observational studies			
COLOMATE (NCT03765736)	Locally advanced and/or mCRC	ctDNA	To facilitate accrual to molecularly assigned therapies; to facilitate clinically annotated genomic analyses
PERMED01 (NCT02342158)	Locally advanced and metastatic malignancies	ctDNA and CTCs	To facilitate accrual to molecularly assigned therapies
TARGET	Locally advanced and metastatic malignancies	ctDNA	To match patients with a broad range of advanced cancers to early-phase clinical trials based on ctDNA analysis
NCT03594448	MSI mCRC	ctDNA	To test concordance between MSI status in ctDNA and primary tumor tissues; to correlate MSI ctDNA changes with response to therapy
RASINTRO (NCT03259009)	mCRC under treatment with anti-EGFR MoAb rechallenge	ctDNA	To correlate ctDNA RAS MT with PFS of anti-EGFR rechallenge
OPTIMAL-II (NCT03750175)	mCRC under treatment with anti-EGFR MoAb	ctDNA	Feasibility and reliability of ctDNA-based selection of KRAS, NRAS, and BRAF WT Patients with mCRC who will benefit from anti-EGFR MoAb and ctDNA analysis during therapy and at time of progression
NCT03401957	mCRC treated with cetuximab-based infusional 5-fluorouracil regimen as first line	ctDNA	To observe the percentage and the time to onset of RAS MT
PERSEIDA (NCT02792478)	RAS WT mCRC in first-line treatment	ctDNA	To evaluate the appearance of new RAS MT at disease progression and prior to radiological documentation
PREDATOR (ESMO 2020, Loupakis et al)	Oligometastatic CRC treated with curative intent	ctDNA	To evaluate PFS according to post-operative ctDNA status
Interventional studies			
Study and phase of research	Study population	Liquid biopsy analysis	Primary endpoints
NCT03436563	Resected mCRC with detectable ctDNA following resection of all known liver metastases (cohort D)	ctDNA	M7824 (anti-PD-L1/TGF-beta-RII fusion protein) for 6 doses after resection and completion of standard-of-care therapy
Phase Ib/II			
CHRONOS (NCT03227926)	RAS/BRAF WT mCRC previously sensitive to anti-EGFR MoAb and then progressed, after ≥ 1 following line of therapy, with >50% drop in RAS mutational load at serial ctDNA analyses or absent RAS MT/EGFR ectodomain MT ctDNA	ctDNA	Rechallenge of third-line panitumumab monotherapy
Phase II			
NCT03087071	RAS/BRAF WT mCRC with clinical benefit and then progression to anti-EGFR MoAb	ctDNA	Rechallenge of panitumumab monotherapy in patients with EGFR ectodomain MT ctDNA or without neither EGFR ectodomain nor KRAS/NRAS/BRAF MT ctDNA; panitumumab and trametinib in patients with KRAS/NRAS/BRAF MT ctDNA
Phase II			
CAPRI 2-GOIM	RAS/BRAF WT anti-EGFR refractory mCRC	ctDNA	Continuum of treatment with cetuximab if RAS WT ctDNA; second-line FOLFOX and bevacizumab if RAS MT ctDNA and then third-line rechallenge with cetuximab and irinotecan if back to RAS WT ctDNA or standard-of-care therapy if persistent RAS MT ctDNA
			ORR
NCT03844620	Pretreated mCRC	ctDNA	Comparison of either regorafenib or TAS-102 continuation based on early changes in ctDNA analysis or according to standard of care
Phase II			
MoLIMoR (NCT04554836)	RAS MT mCRC	ctDNA	Comparison of FOLFIRI vs FOLFIRI plus intermittent addition of cetuximab when ctDNA analysis demonstrates conversion to RAS WT
Phase II			
VISNU-1 (NCT01640405)	Previously untreated patients with mCRC with ≥ 3 CTCs/7.5 mL	CTCs	Comparison of first-line FOLFOX plus bevacizumab vs FOLFOXIRI plus bevacizumab
Phase III			

Table 3 (continued)

Study	Study population	Liquid biopsy analysis	Primary endpoints
VISNÜ-2 (NCT01640444) Phase II	Previously untreated patients with <i>KRAS</i> WT mCRC with <3 CTCs/7.5 mL	CTCs	Comparison of first-line FOLFIRI plus bevacizumab vs FOLFIRI plus cetuximab based on <i>BRAF</i> and <i>P13K</i> mutational status

CTCs circulating tumor cells, ctDNA circulating tumor DNA, EGFR epidermal growth factor receptor, MoAb monoclonal antibodies, MSI microsatellite instability, MT mutated, ORR overall response rate, PD-L1 programmed death-ligand 1, PFS progression-free survival, TGF transforming growth factor, WT wild-type

heterogeneity. An additional cohort of seven patients with mCRC undergoing anti-EGFR treatment was studied for the appearance of resistance genetic alterations at the time of progression, with 100% ctDNA and 57% CTC-DNA detection [81]. Table 2 summarizes and directly compares the sensitivity for ctDNA and CTCs (or CTC-DNA) reported by these investigations of mCRC, showing higher detection rates for ctDNA.

7 Conclusions

Recent advances in terms of feasibility in the clinic and a faster turnaround time, together with molecular-implemented assessment of both spatial and temporal cancer heterogeneity, made LB a valid noninvasive candidate to challenge traditional tissue biopsy. Circulating tumor cells and circulating DNA are the tumor components most widely studied with this approach, carrying diagnostic, prognostic, and predictive information also in mCRC. Ongoing trials assessing their role in mCRC are reported in Tables 3 and 4. Remarkable future applications of LB could be envisioned all along the continuum of care of patients with mCRC (Fig. 3). In the first-line treatment setting, LB could expedite molecular profiling for the administration of targeted anticancer agents (i.e., anti-EGFR drugs) [86, 119, 120]. Further, prognostically proven ctDNA and CTC levels could become additional decision criteria for de-escalation/intensification of upfront backbone chemotherapy and modulation of post-operative treatment in the oligometastatic setting, even if the ideal cut-off in this regard is missing [29, 55, 60, 63, 75]. In later lines of therapy, ctDNA could inform treatment sequence and drug selection, by identification of the best timing for anti-EGFR retreatment, based on *RAS* and *EGFR* clonal tracking, or identification of mechanisms of resistance for upfront or sequential enhanced targeting [112]. Molecular selection for clinical trials has already been taking advantage of LB owing to its increased feasibility and comprehensiveness for screening criteria [83, 120, 121]. As a future prospective, “molecular” progression will complement radiological evaluation, while computational mathematics already proved capable of predicting time-to-treatment failure in patients with mCRC through clonal tracking forecast [78, 122]. Finally, although not a focus of the present review, LB may have a role as a screening tool in CRC and is currently under intense investigation aiming to guide decisions about adjuvant treatment for patients with stage II–III surgically resected CRC [123, 124]. In conclusion, LB represents a major opportunity for better tailoring the whole patient care in mCRC, with ctDNA especially

Table 4 Ongoing trials retrospectively assessing the role of liquid biopsy in mCRC

Study and phase of research	Study population	Liquid biopsy analysis	Intervention	Primary endpoint	Role of liquid biopsy analysis
CAVE (NCT04561336) Phase II	Pretreated <i>RAS</i> WT mCRC	ctDNA	Combination of avelumab plus cetuximab	OS	To compare OS and PFS in ctDNA <i>RAS/BRAF</i> WT and MT populations
CR-SEQUENCE (NCT03635021) Phase III	Untreated <i>RAS</i> WT left-sided mCRC	ctDNA	FOLFOX plus panitumumab followed by FOLFIRI plus bevacizumab vs FOLFOX plus bevacizumab followed by FOLFIRI plus panitumumab	PFS	To state the clinical impact of clonal dynamics of ctDNA
KISIMA-01 (NCT04046445) Phase Ib	Pretreated mCRC progressed to standard-of-care therapies	ctDNA	ATP128, with or without BI 754091	Safety and tolerability of ATP128 and BI754091	To detect early signal of relapse through ctDNA in patients treated with ATP128 and BI 754091 before and after liver surgery
IMPROVE (NCT04425239) Phase II	Untreated <i>RAS/BRAF</i> WT mCRC	ctDNA	Continuous vs intermittent panitumumab and FOLFIRI in first line	PFS	To evaluate potential biomarkers of primary and secondary resistance analyzing ctDNA
iRE-C (NCT04108481) Phase I–II	Liver predominant mCRC	ctDNA	Yttrium-90 radioembolization (Y90-RE) in combination with durvalumab	Safety and tolerability of Yttrium-90 radioembolization combined with durvalumab and ORR	To determine changes in the expression profile and in ctDNA levels after treatment
NCT04169347 Phase II	Left-sided <i>RAS</i> WT mCRC	ctDNA	FOLFOXIRI plus panitumumab	ORR	To evaluate velocity of response through ctDNA clearance
NCT03829410 Phase I–II	<i>RAS</i> MT mCRC	ctDNA	Onvansertib in combination with FOLFIRI and bevacizumab	Safety and tolerability of onvansertib plus FOLFIRI plus bevacizumab and ORR	To evaluate reduction of <i>RAS</i> MT in ctDNA
AIO-KRK-01160 (NCT04034459) Phase II	<i>BRAF</i> MT mCRC	Not specified	FOLFOXIRI plus bevacizumab vs FOLFOXIRI plus cetuximab in first line	ORR	To investigate molecular biomarkers for the prediction of sensitivity and secondary resistance of cetuximab
NCT04247256 Phase I–II	FOLFIRI-resistant mCRC	ctDNA	Combination of SCO-101 with FOLFIRI rechallenge	Safety and tolerability of SCO-101 and ORR	To evaluate the change in ctDNA from baseline until first computed tomography scan

ctDNA circulating tumor DNA, mCRC metastatic colorectal cancer, MT mutated, ORR overall response rate, OS overall survival, PFS progression-free survival, WT wild-type

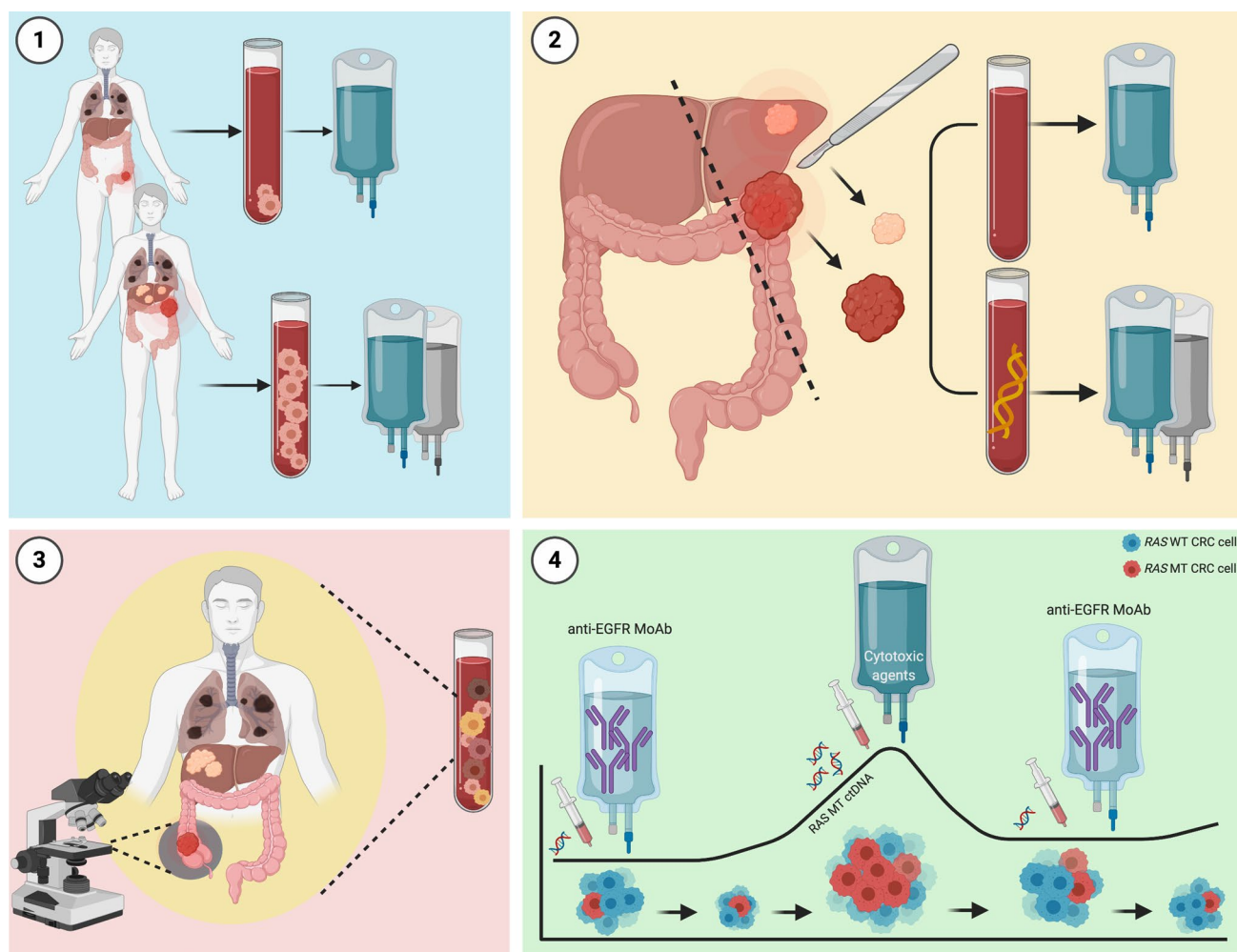


Fig. 3 Overview of potential clinical applicability for circulating tumor cells (CTCs) and circulating tumor DNA (ctDNA) in the continuum of care for patients with metastatic colorectal cancer (mCRC). Circulating tumor cells and ctDNA yield potential clinical applicability in treatment guidance for patients with mCRC: (1) the CTC count may guide treatment selection, with regard to therapy intensification and de-escalation; (2) after surgical resection for oligometastatic colorectal cancer (CRC), post-surgical treatment strategies may be modulated according to minimal residual disease (MRD), as deter-

mined by ctDNA levels in peripheral blood; (3) ctDNA may refine molecular profiling in mCRC by capturing tumor molecular heterogeneity, which is otherwise unachievable by single-tissue biopsy exploring just one site of the disease; and (4) ctDNA allows clonal tracking and real-time monitoring of emerging resistance, guiding treatment selection and rechallenge with anti-epidermal growth factor receptor (EGFR) monoclonal antibodies (MoAb). *MT* mutant. Created with BioRender.com

demonstrating, from initial pioneering studies in this tumor type [11], an ever-increasing acceleration toward multiple clinical applications.

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