

# Circulating tumor DNA in early-stage colon cancer: ready for prime time or needing refinement?

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**Abstract:** Liquid biopsies are the detection of molecular information in fluids from patients with cancer. In colorectal cancer (CRC), the most promising liquid biopsy strategy is the use of circulating tumor DNA (ctDNA) from plasma. In early-stage CRC, the potential for ctDNA to impact care stems from the detection of minimal residual disease (MRD) to guide adjuvant therapy after curative intent treatment and in identifying recurrences during surveillance. As for any new diagnostic test, ctDNA assays must overcome pre-analytical and analytical challenges before clinical implementation. We will discuss important logistical and assay considerations that clinicians and patients should understand when assessing ctDNA assays. We will also delve into important concepts to aid in interpreting ctDNA results and potential incidental findings that may arise. Sequencing errors, germline variants, and clonal hematopoiesis of indeterminate potential (CHIP) must be addressed to properly interpret results. CHIP is also an important consideration that impacts patient prognosis through association with cardiovascular and hematologic diseases. With this background in place, we next review the best available evidence for the use of ctDNA in early-stage colon cancer. Observational cohorts have established MRD after surgery as a significant prognostic factor for recurrence in stage II and III colon cancer. It also has the ability to anticipate clinical recurrence before standard investigations when used in surveillance. The first and only interventional randomized trial to date evaluating ctDNA is DYNAMIC. The study demonstrated the noninferiority of a MRD detection-guided approach in selecting patients with stage II colon cancer for adjuvant treatment. Notwithstanding the important results, there are still important questions to be answered before ctDNA enters prime time in the clinic. However, future appears bright and ongoing trials will help clarify how to best use this technology in early-stage colon cancer.

**Keywords:** ctDNA, plasma, sequencing, colorectal cancer, anti-EGFR, resistance

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## Introduction

Genetic material from neoplastic lesions can be found in various fluids from around the body in patients with cancer. The technology allowing isolation of this genetic material, particularly in the form of circulating tumor DNA (ctDNA) in the blood and other body fluids, has attracted significant attention recently. Several practical applications have been postulated and there are assays available for clinical use, though the optimal setting and intervention with the results of these

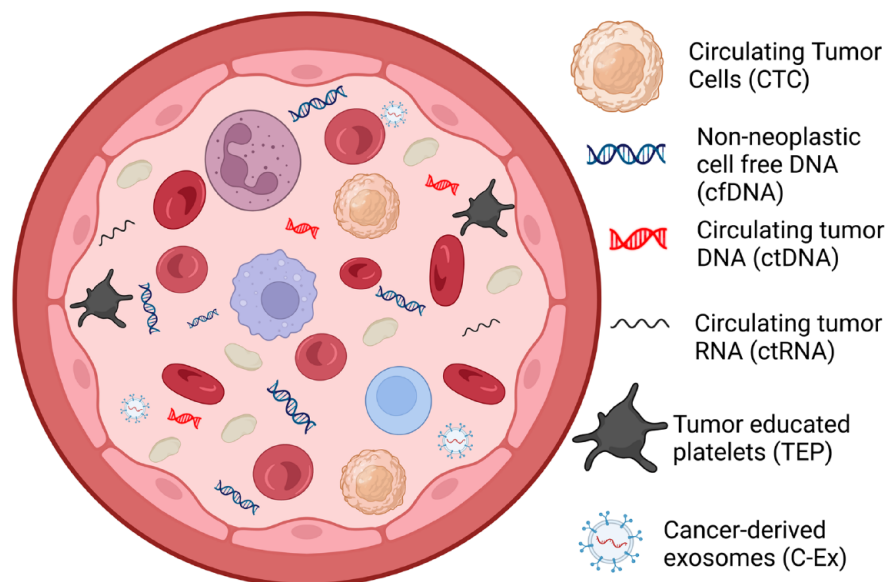
assays is still being validated. In this article, we will review current data regarding the progress to date and challenges that remain before ctDNA can be incorporated into the care of patients with early-stage colon cancer.

## Historical perspectives

The first description of cells from solid tumors in the blood dates back to 1869 during an autopsy of a patient with metastatic breast cancer.<sup>1</sup> In 1948,

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**Figure 1.** Blood analytes potentially surveyable by liquid biopsy.

the presence of circulating cell-free DNA (cfDNA) in the blood of healthy individuals was reported by Mandel and Metais.<sup>2</sup> Later, in 1977, higher levels of cfDNA were found in the blood of patients with metastatic cancer in comparison with non-metastatic cancers and healthy controls and a correlation between the levels of cfDNA and treatment response was described.<sup>3</sup> Nevertheless, it was not until 1994 when the first oncogenic alterations were characterized in cfDNA. *RAS* mutations were detected in blood samples from patients with pancreatic cancer, myelodysplastic syndrome, and acute myeloid leukemia.<sup>4,5</sup> Since then, technologic advances have allowed the development of high-sensitivity assays that enable the identification and measurement of small amounts of cancer-related mutations and broader characterization of cancers with multi-gene panels in fluids from patients with cancer.<sup>6</sup>

### Basic concepts

Liquid biopsy is the process of collecting and analyzing neoplastic material from body fluids such as blood, urine, pleural effusion, ascites, saliva cerebrospinal liquid, and stool. A number of different analytes can be surveyed by different technologies including circulating tumor cells, tumor-educated platelets, exosomes, circulating nucleic acids, proteins, and metabolites (Figure 1).<sup>7</sup>

Cell-free nucleic acids are extracellular fragments of DNA (cfDNA) or RNA (cfRNA) that can be found in body fluids. In patients with cancer, a proportion of the total cell-free nucleic acids is derived from neoplastic cells secondary to apoptosis, necrosis, or tumor secretion. When found in serum, plasma, or lymphatic fluid, tumor-derived nucleic acids are named circulating tumor DNA (ctDNA) and circulating tumor RNA (ctRNA).<sup>8</sup>

ctDNA is the fraction of cfDNA originating from tumor cells.<sup>6</sup> The relative abundance ranges from <0.1% to >10% with a half-life of 114 min.<sup>9,10</sup> Several factors influence the dynamics of ctDNA such as type of cancer, tumor burden, stage, cellular turnover, sites of metastases, treatments, inflammatory processes, infection, and trauma.<sup>9</sup> ctDNA is usually found as small fragments measuring 143–145 base pairs.<sup>11</sup>

There are several possible applications for ctDNA in the management of colorectal cancer (CRC). ctDNA could be implemented to screen for CRC, facilitate genotyping to guide therapy, or provide prognostic information to guide management after surgery.<sup>12</sup> After curative surgery, ctDNA is highly prognostic for recurrence across stages.<sup>13–23</sup> The ability to detect minimal residual disease (MRD) after surgery may allow tailoring of adjuvant chemotherapy regimen and duration to minimize overtreatment and maximize efficacy or may play a role in guiding multi-modality therapy during treatment

of locally advanced rectal cancer.<sup>24–26</sup> Finally, surveillance for recurrent disease after treatment could also be impacted by the incorporation of ctDNA in the follow-up protocol.<sup>17,18,20</sup> In the metastatic setting, ctDNA assays have shown utility in providing comprehensive genomic profiling, monitoring therapeutic response, assessing resistance and clonal evolution, and guiding re-treatment with anti-epidermal growth factor receptor therapy.<sup>27–29</sup> Despite the myriad of potential applications of ctDNA, several challenges exist before this technology can reach prime time. In the next segments, we will discuss those challenges specifically in the setting of early-stage colon cancer.

### Pre-analytical challenges

The pre-analytical phase of a test encompasses the processes that take place before the analysis itself. For ctDNA, the potential scarcity of ctDNA in the blood and its short half-life create several challenges. The two main threats are degradation of ctDNA and contamination from normal non-neoplastic DNA. Standardized procedures for specimen collection, handling, transport, processing, and storage are paramount to decrease those risks.<sup>6,12</sup> Blood is usually drawn from a peripheral vein using needles with a large gauge diameter (<21G) to minimize blood cell lysis, which can be a challenge if blood is collected using a butterfly needle. The necessary volume varies based on particular assay, but typically ranges from 10 to 20 mL, though some tests may use up to 60 mL to increase the stoichiometric probability of finding low allele frequency mutations. Plasma is the preferred blood component for ctDNA analysis due to the fact that it contains much lower DNA derived from leukocyte lysis than serum.<sup>6,12,30,31</sup>

The choice of tubes for collection will depend on the time expected until sample processing. Ethylenediaminetetraacetic acid (EDTA) tubes result in short ctDNA stability and plasma isolation should be performed within 4–6 h when they are used. Their main advantage is a lower cost than other tubes that include stabilizing compounds to prevent DNA degradation. When a more prolonged time until sample processing is necessary, leukocyte stabilization cfDNA collection tubes allow blood to be stored for up to 14 days in temperatures of 6–37°C.<sup>32</sup> These stabilizing tubes ensure less concern about differences in handling across samples but have risen considerably in price as of late, now being 20 times the price of EDTA tube. Whichever tube is utilized,

agitation should be minimized and mixing should be done by inverting the tubes and not shaking. The presence of clotting in collection tubes during phlebotomy typically results in an inadequate sample for analysis.<sup>6,12,30,31</sup>

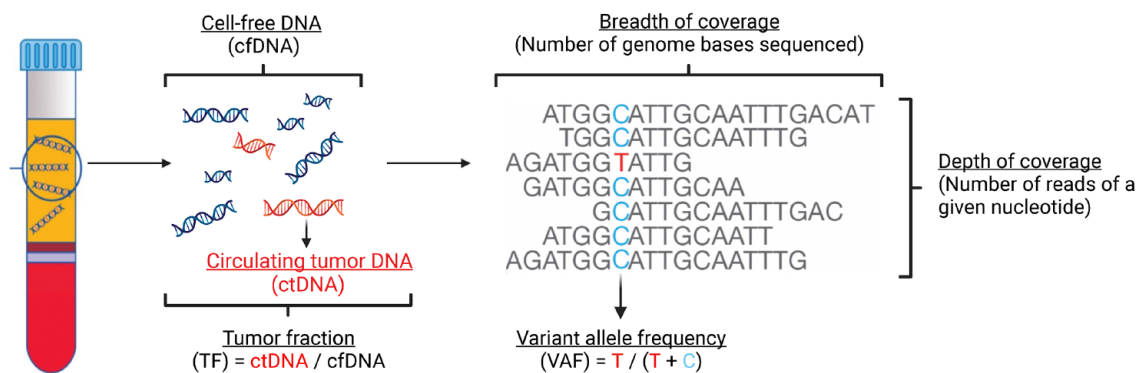
After blood collection, plasma isolation is usually achieved *via* two-step sequential centrifugation at different speeds to concentrate blood cells in the pellet or buffy coat and to eliminate cell organelles and debris, therefore obtaining plasma with higher cfDNA homogeneity and purity. Subsequently, isolated plasma should be frozen preferentially in single use aliquots, usually from 300 µL to 2 mL. This avoids future repeated freeze–thaw cycles of samples, which increase the risk of nucleic acid degradation.<sup>6,12,30,31</sup> The methods of cfDNA extraction and purification are well described and several commercial kits are available with performance thought to be comparable. The choice between methods will vary depending on the objective of the ctDNA assay and is beyond the scope of this review.

### Analytical challenges

Analytical validity refers to the ability of a diagnostic test to reliably predict the presence or absence of a given marker in a reproducible fashion. Like other diagnostic tests, ctDNA assays must be validated, results must be reproducible and high levels of sensitivity (tumor DNA detection) and specificity (low rate of false-positive results) must be achieved. Assay methodology [polymerase chain reaction (PCR) or next-generation sequencing (NGS) based], level of genomic coverage (breadth and depth), and bioinformatics pipeline can all impact assay performance. The desired level of coverage and depth of sequencing depends on the intended clinical use. In the MRD setting, assays are required to reliably detect ctDNA at a variant allele frequency (VAF) of 0.01%, which is the theoretical minimum ctDNA concentration in a standard 10 mL blood sample (one tumor molecule in 10–12,000 DNA molecules). However, in the metastatic setting, low allele frequency mutations may be less important if ctDNA is abundant and a broader panel with more shallow sequencing may be appropriate.

### Sensitivity

Strategies to detect ctDNA can be broadly divided into PCR or NGS-based methodology. Traditionally, PCR provided increased depth of



**Figure 2.** Defining depth and breadth of coverage for ctDNA assays. ctDNA, circulating tumor DNA.

coverage (the number of times a given nucleotide is read during sequencing) and NGS techniques provided increased breadth of coverage (the number of regions that are sequenced) (Figure 2).

PCR techniques, such as Digital Droplet PCR or BEAMing (beads, emulsion, amplification, and magnetics), allow for the detection of one or a few known individual point mutations at VAFs of  $\leq 0.01\%$ .<sup>33</sup> The limited number of alterations targeted with this methodology requires prior tissue sequencing (tumor-informed assays) to enhance sensitivity and specificity when used in the MRD setting. It is inferred that mutations in tumor tissue will be detectable in the bloodstream, and tissue sequencing is used to design a personalized assay. This comes at the cost of increased turnaround time, which could potentially impact patient outcomes if initiation of adjuvant therapy is delayed.<sup>34</sup> Nevertheless, in the DYNAMIC trial, commencement of chemotherapy at a median of 83 days after surgery in the ctDNA-guided arm did not appear to compromise efficacy.<sup>24,25</sup> It remains to be determined if this will hold true in subsequent trials.

NGS technologies allow for broader breadth of coverage, meaning a single ‘off-the-shelf’ panel incorporating a larger number of usual cancer mutations can be used for many patients in a de-personalized fashion. However, sensitivity and specificity are instead limited by the error rate of DNA polymerase. With increasing depth of coverage, as is required for detection of mutations at very low VAFs, sequencing errors can be difficult to distinguish from true mutations. This is addressed through the use of unique molecular identifiers (UMI) and error-suppression

algorithms. UMI is a method by which all DNA molecules on a NGS library are made unique. This can be done by different processes, one of which is molecular barcoding, the addition of a random short sequence of nucleotides that are individual to each DNA molecule. The presence of these specific barcodes on the original DNA fragment allows bioinformatic filtering of duplicate reads and PCR errors, optimizing distinction between true variant alleles present in the original sample, from errors introduced during the process of NGS. Sequencing with the use of UMI can increase specificity and sensitivity of variant call and detection. This is particularly important in the setting of very low input samples and detection of very low VAFs such as MRD testing.<sup>35,36</sup> NGS panels may also be paired with tissue-based sequencing as a priori knowledge of a mutation in a cancer reduces the possibility that a low allele frequency mutation occurred by chance.

Other approaches to improve NGS performance include combining hotspot panels with DNA methylation and the assessment of DNA fragmentation patterns to characterize the fragmentome.<sup>37–39</sup> Methylation, one of the most studied epigenetic phenomenon in humans, is the covalent addition of a methyl group to position 5 carbon of the DNA cytosine ring. This is done by methyltransferase enzymes and usually causes gene silencing. Aberrant DNA methylation is thought to occur at very early stages of cancer. Usually, a global hypomethylation pattern, resulting in genomic instability and activation of silenced oncogenes, associated with focal promoter hypermethylation of tumor suppressor genes is observed in cancer, resulting in a distinct methylome when compared with normal

**Table 1.** Differences between ctDNA analysis methods.

Assay type	Tumor informed	Tumor agnostic
Methodology	A tumor tissue sample undergoes profiling (targeted panel <i>versus</i> exome <i>versus</i> genome) and a subsequent personalized assay is created to interrogate plasma	NGS panel for frequent mutations and/or a methylation signature
Turnaround time	About 4–5 weeks	About 1 week
Cost	Potentially higher for first assay, with subsequent assays being lower in cost	Potentially lower initial assay but no reduction in costs for subsequent assays
Sensitivity	Theoretically highest	High
Need for tissue sample	Yes	No

ctDNA, circulating tumor DNA; NGS, next generation sequencing.

cells.<sup>37,39,40</sup> This unique methylation signature can be surveyed in ctDNA from patients with CRC and has been shown to increase sensitivity by 25–36%, when combined with a plasma only NGS panel.<sup>19,23</sup> Similarly, fragmentation pattern of plasma DNA is variable and is related to several factors such as nucleosome organization, chromatin structure, gene expression, and nuclease content of the tissue of origin. This results in particular patterns of characteristics such as fragment size, nucleotide motifs at the fragment end, and presence of jagged (single stranded) ends that can help distinguish ctDNA from cfDNA. Therefore, DNA fragmentation signatures are also under investigation as a mean to increase the accuracy of NGS panels.<sup>39</sup>

Nowadays, both PCR and NGS can reliably detect ctDNA at very low VAFs and the number of assays currently available or in development have exponentially increased, with wide variability in the level of genomic coverage. Confirming analytical validity for each assay remains an unmet need in view of the lack of an established standard. Prospectively comparing assays is unrealistic and cross-trial comparisons are fraught considering the heterogeneity that can be introduced from patient selection, sample collection, and analysis. Efforts may be better placed on prospectively establishing the desired assay characteristics according to each clinical setting. Once this is established, analytical validation could be confirmed *in vitro* with established reference DNA samples for quality control, as is done with other diagnostic tests. Table 1 summarizes the

major differences between ctDNA analysis methods.

With recent advances in sequencing, biology plays an increasing role in establishing the limits of ctDNA use. Among each phlebotomy sample, there may not be cancer-derived DNA due to the random distribution of ctDNA in a patient's total blood volume. This is a particular problem when trying to identify microscopic disease, such as after curative surgeries. Increasing sample volume by drawing a larger volume of blood or through serial monitoring may improve sensitivity.<sup>13–25</sup> Parikh *et al.* showed that incorporating serial longitudinal samples improved sensitivity from 55.6% to 69%.<sup>19</sup> The appropriate time of blood collection is also still unclear. Several factors interfere with the kinetics of ctDNA and therefore influence test sensitivity and validity. For example, trauma-induced DNA release from normal tissue can dilute ctDNA and persists for up to 4 weeks after surgery and be associated with false-negative results.<sup>41</sup> The Colon and Rectal–Anal Task Forces of the United States National Cancer Institute (NCI) for ctDNA applications and integration in CRC recommended minimum standard timepoints for sample collection. Overall, the collection should be done at least 4–8 weeks after surgical resection with a curative intent for detection of MRD, whereas an interval of 2–8 weeks was postulated for the first sample after completion of all potentially curative therapies. Preoperative samples, although recommended in the NCI whitepaper, have never been shown to improve the utility in the MRD setting and should

## Clonal hematopoiesis (CH)

The acquisition of somatic mutations leading to the clonal expansion of hematopoietic stem cells

	ARCH	CHIP	CCUS	MDS (Low risk)	MDS (High risk)	sAML
VAF	< 2%	> 2%	> 2%	> 2%	>> 2%	>>> 2%
No of lesions	+	+	++	+++	++++	++++
Cytopenias	-	-	+	+	++	++
Dysplasia	-	-	-	+	+	+
BM blasts	< 5%	< 5%	< 5%	< 5%	5-19%	> 20%
BM failure						
Prognosis						

ARCH: Age-related clonal hematopoiesis  
 CHIP: Clonal hematopoiesis of indeterminate potential  
 CCUS: Clonal cytopenia of undetermined significance  
 MDS: Myelodysplastic syndrome  
 sAML: Secondary acute myeloid leukemia  
 VAF: Variant allele frequency  
 BM: Bone marrow



**Figure 3.** Spectrum of clonal hematopoiesis identifiable in ctDNA. ctDNA, circulating tumor DNA.

not be mandatory given the time and expenses associated with it.

Finally, factors impacting the release and clearance of tumor DNA in the bloodstream remain poorly understood. Tumor vascularity, metabolic activity, and subclonal evolution are likely to affect DNA shedding by tumor cells.<sup>42</sup> Isolated peritoneal metastases, lung metastases, and brain metastases are associated with lower detectable VAFs in the blood and mutations may not be detectable if these are the only sites of metastasis.<sup>43</sup> In addition, relying on ctDNA testing for MRD detection and adjuvant therapy decision-making after surgery is questionable for the small but clinically relevant proportion of patients with undetectable ctDNA prior to curative-intent surgery, and it has been suggested to exclude these patients from clinical trials currently underway to test ctDNA-based adjuvant strategies.<sup>12</sup>

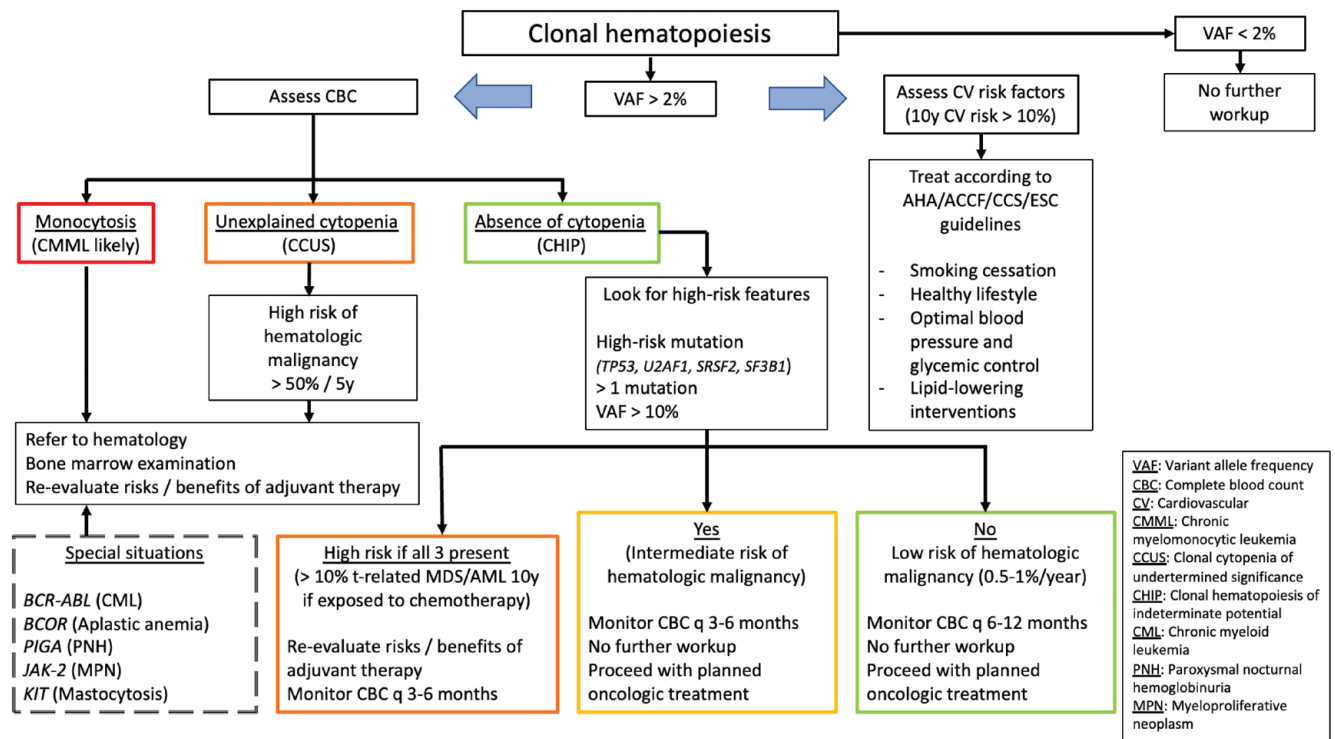
### Incidental findings

Assays are tracking an increasing number of variants as a mean to increase sensitivity. This increased breadth of coverage leads to an increased rate of incidental findings to be interpreted and addressed. Detected variants can be cancer derived, germline, or due to clonal hemat-

opoiesis, with the latter two possibilities potentially confounding patient management.

Clonal hematopoiesis is the acquisition of somatic mutations that lead to clonal expansion of hematopoietic stem cells. It encompasses a spectrum of clinical entities (Figure 3) with the potential to evolve to bone marrow failure or acute myeloid leukemia in a minority of patients. It is also a pervasive biological phenomenon that can confuse interpretation of ctDNA results. Clonal hematopoiesis at a VAF > 2% without unexplained cytopenia is called clonal hematopoiesis of indeterminate potential (CHIP).

CHIP is detectable in 20–30% of patients with solid tumors.<sup>44–46</sup> Its prevalence varies by primary tumor type and increases with age and cancer treatment. CHIP variants occur more frequently in myeloid driver genes and epigenetic regulators, with mutations in *DNMT3A*, *TET2*, and *ASXL1* being the most common. More importantly, mutations in DNA repair genes such as *TP53*, *ATM*, and *CHEK2* are overrepresented in patients with prior oncologic treatments and can be misinterpreted as tumor-derived variants.<sup>47,48</sup> Some of these genes are potential therapeutic targets, emphasizing the importance of accurate variant interpretation. Determining whether a variant is



**Figure 4.** Proposed algorithm for dealing with clonal hematopoiesis detected in ctDNA. ctDNA, circulating tumor DNA.

due to CHIP can be done bioinformatically in some settings or with matched solid tumor or whole blood sequencing.

In the general population, CHIP is associated with increased risk of major cardiovascular events.<sup>49–51</sup> The magnitude of this risk is similar or higher than the risk conferred by traditional cardiac risk factors such as hypertension, diabetes, or smoking. The absolute 10-year risk is over 10%, this is considered high risk according to international cardiovascular societies guidelines.<sup>52,53</sup> Although there is currently no prospective data to guide management of cardiovascular risk factors in this setting, most experts recommend risk reduction interventions per usual guidelines, including optimal blood pressure, lipids, and glycemic control.<sup>52–56</sup>

CHIP is also associated with an increased risk of myeloid hematologic neoplasm.<sup>49,57</sup> The relative risk is significant [hazard ratio (HR) > 10], but this translates into an absolute risk of 0.5–1%/year. Patients with multiple mutations, increased clone size (VAF > 10%), or mutations in *TP53* and/or spliceosome genes (*U2AF1*, *SF3B1*, and *SRSF2*) are at highest risk and need to be

monitored more closely.<sup>46,58–61</sup> The detection of clonal hematopoiesis with unexplained cytopenia is termed clonal cytopenia of undetermined significance. Some of these patients have a previously undiagnosed concurrent hematologic neoplasm, and in those who do not, the probability of developing an overt myeloid malignancy within 5 years is >50%.<sup>59,61</sup> These patients should be referred for bone marrow examination and hematology assessment. Similarly, the presence of unexplained peripheral monocytosis with detection of a variant in myeloid genes confers a prognosis equivalent to chronic myelomonocytic leukemia and should be managed as such.<sup>62</sup> Given the increasing complexity of prognostication and risk mitigations strategies, many centers are now implementing specialized clinics to provide comprehensive multidisciplinary recommendations.<sup>56</sup> Our approach for medical oncologists is summarized in Figure 4.

Other important incidental findings are germline variants and sequencing errors. There are three criteria that help distinguish a variant as somatic: VAF substantially less than 50%; commonly recurring somatic variant with clinical significance in cancer; and not commonly observed in

population databases.<sup>63</sup> Although helpful, these criteria are not perfect and sequencing germline DNA can help to verify if a variant is germline. On the other hand, sequencing errors can be minimized by the technique of molecular barcoding of the initial DNA prior to sequencing as previously explained.

After extraction and sequencing, DNA is analyzed and compared with a reference genome through a chosen bioinformatic pipeline, which can include computational filtering algorithms. This step can also help differentiate mutations from sequencing errors, CHIP variants, and germline mutations. A well-validated and robust pipeline helps mitigate the risks associated with incidental findings, however are often proprietary for each commercial assay and may be a 'black-box' for test users that is difficult to cross compare and externally validate.

### Clinical challenges

In a seminal paper, Diehl *et al.* showed that ctDNA detection using a BEAMing PCR test for four genes (*APC*, *KRAS*, *PIK3CA*, and *TP53*) was highly prognostic for disease-free survival (DFS) and ctDNA levels reflected tumor burden and response to therapies in CRC.<sup>10</sup> Since then, there has been a multiplication of observational studies highlighting the prognostic utility of ctDNA post-curative intent surgery and for detection of submicroscopic disease that cannot be detected radiographically, also known as MRD. In the next section, we will review the relevant data divided by stage.<sup>13–25</sup>

#### Stage II colon cancer

Tie *et al.*'s 2016 biomarker study using the Safe-Sequencing System (SafeSeqS) assay in 230 patients was the first to evaluate ctDNA in patients with stage II colon cancer.<sup>13</sup> In the group of patients not treated with adjuvant chemotherapy, 14/178 (7.9%) had ctDNA detected postoperatively and 11 (79%) of those relapsed with a median follow-up of 27 months. On the other hand, only 16/164 (9.8%) patients without detectable ctDNA recurred during the same period [HR, 18; 95% confidence interval (CI), 7.9–40;  $p < 0.001$ ]. As for the group treated with adjuvant chemotherapy, the presence of ctDNA was observed in 6/52 (11%) patients postoperatively. Five of those had ctDNA clearance during chemotherapy, but in two, ctDNA became

detectable again after completion of adjuvant chemotherapy and both relapsed radiographically. From the three remaining patients, two continued recurrence free at 16 and 34 months and one recurred despite ctDNA remaining undetectable. ctDNA positivity after adjuvant chemotherapy was associated with very poor recurrence-free survival (RFS) (HR, 11; 95% CI, 1.8–68;  $p = 0.001$ ). Finally, postoperative ctDNA status had a greater impact on RFS than any individual or combination of clinicopathological risk factors and remained an independent predictor of RFS after multivariable analysis.<sup>13</sup>

More recently, Tie *et al.* presented results from the first randomized controlled trial using ctDNA to guide adjuvant treatment in patients with stage II colon cancer.<sup>24</sup> DYNAMIC was a phase II, multicenter, randomized controlled trial investigating the noninferiority of a ctDNA-guided approach, in comparison with the current standard approach, in selecting patients for adjuvant treatment. A total of 455 patients were randomized 2:1 to ctDNA guided or standard management. Tumor and plasma were analyzed using SafeSeqS tumor-informed ctDNA assay. Plasma was obtained for testing at 4 and 7 weeks after surgery with results made available to treating clinicians between 8 and 10 weeks after the procedure. Patients with at least one positive test received adjuvant treatment with regimen chosen by treating clinicians.<sup>24</sup>

After a median follow-up of 37 months, noninferiority of ctDNA guided management to standard management was confirmed with an absolute difference in 2-year RFS of 1.1% (95% CI, –4.1 to 6.2). The 2-year RFS was 93.5% with ctDNA-guided therapy versus 92.4% with standard treatment selection (HR, 0.96; 95% CI, 0.51–1.82). In addition, significantly less patients received adjuvant chemotherapy in the ctDNA-guided arm, 15% versus 28% (RR, 1.82; 95% CI, 1.25–2.65), but with more oxaliplatin-based chemotherapy use (62% versus 10%). Interestingly, the median time to start treatment after surgery was longer in the ctDNA group, 83 days versus 53 days, highlighting the logistical challenge of the tumor-informed assays.<sup>24</sup>

An important post hoc exploratory analysis comparing baseline clinicopathologic risk features and outcomes was performed in the subgroup of patients ctDNA negative after surgery. The 3-year RFS observed was higher among patients with



clinical low-risk than those with high-risk disease, 96.7% *versus* 85.1% (HR, 3.04; 95% CI, 1.26–7.34). A similar result was observed for patients with pathological T3 and T4 stage tumors, 94.2% *versus* 81.3% (HR, 2.6; 95% CI, 1.01–6.71).<sup>24</sup> Taken together, these findings indicate that ctDNA testing may not be able to solely outperform the traditional prognostic markers for the entire population of stage II CRC and there may still be some utility in clinical features.

Additional exploratory analysis from the ctDNA-guided treatment cohort shows further relevant information. First, the test was more sensitive for predicting distant than locoregional recurrence. Postoperative ctDNA was negative in all eight patients with exclusive locoregional recurrence, while 8 of 15 patients with distant recurrence had a positive ctDNA after surgery ( $p = 0.02$ ). Second, an impressive 87% of patients achieved ctDNA clearance with adjuvant chemotherapy and that was associated with favorable outcomes. The 2-year RFS was 97% for patients who cleared ctDNA, whereas the 1-year RFS for persistently detectable ctDNA was 20% (HR, 55.7; 95% CI, 5.8–532.2;  $p < 0.001$ ). Finally, carcinoembryonic antigen (CEA) measurements did not add prognostic value for patients who are ctDNA negative.<sup>25</sup>

### Stage III colon cancer

To date, there are no prospective randomized trials evaluating ctDNA in stage III colon cancer that have reported results; however, there are many observational studies. Tie *et al.* prospectively evaluated 100 consecutive Australian patients with resected stage III colon cancer planned for 6 months of adjuvant chemotherapy.<sup>14</sup> Serial plasma samples were collected after surgery and after completion of chemotherapy with the tumor-informed Safe-SeqS assay, similar to their stage II study above. At least one somatic mutation was identified in the tumor tissue of all 96 patients with sufficient tissue to sequence. ctDNA was detected in 20/96 (21%) patients postoperatively and no association was found between detectable ctDNA and baseline clinical or pathologic factors. However, ctDNA was associated with an increased risk of recurrence (HR, 3.8; 95% CI, 2.4–21.0;  $p < 0.001$ ), even after multivariate analysis including routine clinicopathologic features (HR, 7.5; 95% CI, 3.5–16.1;  $p < 0.001$ ). After adjuvant chemotherapy, 13/78 (17%) patients with available samples had detectable ctDNA, which was also

associated with recurrence (HR, 6.8; 95% CI, 11.0–157.0;  $p < 0.001$ ). Lack of ctDNA clearance with adjuvant chemotherapy was associated with worse 3-year RFS (HR, 3.7; 95% CI, 1.1–17.0;  $p = 0.04$ ).<sup>14</sup>

Henriksen *et al.* published a 168 patient study in stage III CRC using a tissue-informed multiplex-PCR sequencing assay that follows 16 patient-specific somatic mutations (Signatera multiplex-PCR assay, Natera Inc. Austin, Texas, USA).<sup>15</sup> Plasma samples were collected postoperatively a median of 2 weeks after surgery (interquartile range, 2–4 weeks). Among patients with successful sequencing, the recurrence rate was 80% (16/20) for patients with detectable ctDNA, whereas it was 18% (22/120) for patients with undetectable ctDNA. RFS was significantly shorter when ctDNA was detected after surgery (HR = 7.0; 95% CI, 3.7–13.5;  $p < 0.001$ ). On a multivariable analysis, ctDNA was the strongest predictor of recurrence when controlling for clinicopathologic features in the postoperative setting (HR = 30.97; 95% CI, 10.63–90.20;  $p < 0.001$ ). Interestingly, the levels of cfDNA were higher in the 22 ctDNA-negative patients who recurred than in the 20 ctDNA-positive patients ( $p = 0.015$ ). The authors hypothesized that trauma from recent surgery resulting in massive DNA shedding could have diluted the ctDNA below the detection level. This was confirmed later, when samples for 15 of those patients collected more than 2 months after surgery were analyzed. cfDNA was found to have decreased while the ctDNA detection rate increased from 0% to 80% (12/15 patients). Among patients with detectable ctDNA postoperatively, adjuvant chemotherapy was able to permanently clear only 23% (3/13 patients) with 36 months of follow-up. Meanwhile, 100% (10/10) of patients who had a transient clearance or who did not clear ctDNA with adjuvant therapy relapsed. Persistence of ctDNA after completing adjuvant treatment was highly prognostic for RFS (HR = 94.25; 95% CI, 15.74–564.30;  $p < 0.001$ ) and its longitudinal measurement was the only significant predictor of RFS in multivariable analysis involving baseline clinicopathologic features and CEA serial surveillance (HR = 40.7; 95% CI, 11.6–143;  $p < .001$ ). Finally, the presence of ctDNA in the post-adjuvant setting identified recurrence with a lead time of 9.8 months compared with conventional surveillance.<sup>15</sup>

Taieb *et al.* have also presented retrospective analysis of 1017 participants of the IDEA-France

trial.<sup>16</sup> They used a tumor-agnostic digital droplet PCR test to detect methylation of the *WIF1* and *NPY* genes. Overall, 140/1017 (13.8%) were ctDNA-positive postoperatively. With a median follow-up of 6.6 years, the 3-year DFS rate was 66.4% for ctDNA-positive patients and 76.7% for ctDNA-negative patients (HR=1.46, 95% CI, 1.08–1.97,  $p=0.015$ ). Moreover, the 5-year overall survival was, respectively, 81% and 87% (HR=1.56, 95% CI, 1.08–2.26;  $p=0.018$ ). The ctDNA status was confirmed on multivariate analysis as an independent prognostic marker for DFS (HR=1.55, 95% CI, 1.13–2.12,  $p=0.006$ ) and OS (HR=1.65, 95% CI, 1.12–2.43,  $p=0.011$ ). Interestingly, ctDNA was prognostic in patients treated with 3 months of chemotherapy and with high-risk stage III cancer (T4 and/or N2), but not in those treated for 6 months and with low-risk stage III cancer (T1-3/N1).<sup>16</sup> This might indirectly suggest that 6 months of adjuvant systemic treatment, in this case with FOLFOX (90% received either 3 or 6 months of FOLFOX), may be able to clear a significantly higher proportion of patients with detectable ctDNA after surgery, the incremental benefit being most significant among patients with lower baseline disease risk.

#### Combined stage studies

Several observational trials have evaluated the use of ctDNA in a combined stage population of patients with CRC and the most relevant data are summarized in Table 2.<sup>17–23</sup> The heterogeneity between study populations, as well as the difference in assays, treatments and follow-up protocol makes it challenging to analyze them combined. Nevertheless, the findings are compatible with what have been previously presented in stage specific studies. Postoperative ctDNA positivity ranges from 10% to 25% and ctDNA clearance from about 16% to 30% – with noticeable exception of higher clearance from Kotaka *et al.* (68%) and Anandappa *et al.* (57%). Once again, ctDNA detection after completing curative intent treatment was significantly correlated with worse outcomes in all cohorts, usually preceding clinical recurrence by about 3–9 months.

#### Surveillance after curative intent treatment

The use of ctDNA as a surveillance strategy after curative intent treatment of colon cancer was evaluated in several of the previously discussed studies. Longitudinal measurements were made

at different time points and performance was compared with conventional tests and clinical recurrence. Overall, the results appear to indicate that ctDNA is more sensitive for recurrence detection than standard investigations such as CEA measurements and computed tomography (CT) scans.<sup>14,15,17–21</sup>

Nevertheless, a recent retrospective cohort challenged this assumption. A total of 48 patients with stages II–IV CRC treated with curative intention were followed for 2 years. They underwent periodic ctDNA testing in addition to CT scans and CEA per standard of care in the United States, where ctDNA is reimbursed in stage II/III settings with certain assays. In all, 15 patients relapsed on follow-up. Numerically, ctDNA did not perform better than imaging in detecting recurrence and there were no statistically significant differences in sensitivity between ctDNA and conventional tests. More importantly, the study failed to show any potential increase in curative intent treatment after disease relapses with use of ctDNA.<sup>64</sup> The contrasting data highlight the paramount necessity of well-designed randomized trials to further explore the role of ctDNA in monitoring for recurrence after curative intent treatment of CRC.

#### Is ctDNA ready for prime time in the management of early-stage colon cancer?

The final major challenge for any diagnostic test before incorporation into clinical practice is establishing clinical utility. Studies to date demonstrate strong prognostic utility of ctDNA detection in early-stage CRC. However, sensitivity is not perfect, and it can be estimated that approximately 10% of patients with stage II and 20–25% of patients with stage III or high-risk stage II colon cancer will have a disease recurrence despite a negative ctDNA result in the studies presented in the prior sections of this review. On the other hand, detectable ctDNA is not just a risk factor for recurrence, but essentially identifies persistent subclinical disease, heralding recurrence with a lead time of up to 9 months.<sup>15,17–25</sup>

Nevertheless, a test is only useful if it can be used to change management. Therefore, clinical utility beyond prognostication is still lacking for most indications and can only be proven by well-designed randomized controlled trials. In resected CRC, the most important themes are escalation and de-escalation of adjuvant treatment in

**Table 2.** Selected observational studies in mixed stage CRC.

Study (ctDNA assay)	Number of patients	Stage distribution	Timing of blood collection	Preoperative ctDNA sensitivity	Postoperative ctDNA positivity	ctDNA clearance after chemotherapy	Recurrence outcomes	Additional findings
Reinert <i>et al.</i> <sup>17</sup> , 2019 (H/Seq 2500 system, Illumina Inc.)	130	Stage I = 5 (4%) Stage II = 39 (31.2%) Stage III = 81 (64.8%) *5 patients excluded	- Up to 14 days before surgery; - 30 days after surgery; - q3 months up to 36 months	Stage I = 40% Stage II = 92% Stage III = 90%	10/94 (10.6%)	3/10 (30%)	Surveillance test: - ctDNA +: 14/15 relapsed (93.3%) - ctDNA -: 2/60 relapsed (3.3%) - ctDNA lead time for recurrence detection: 8.7 months	- Post-op ctDNA + recurrence risk: HR, 7.2; 95% CI, 2.7–19.0; <i>p</i> < 0.001 - Post-adjuvant ctDNA + recurrence risk: HR 17.5; 95% CI, 5.4–56.5; <i>p</i> < 0.001 - Follow-up ctDNA + recurrence risk: HR, 43.5; 95% CI, 9.8–193.5; <i>p</i> < 0.001 - ctDNA was the only significant prognostic factor for relapse on multivariate analyses
Wang <i>et al.</i> <sup>18</sup> , 2019 (Safe-SeqS assay)	58	Stage I = 9 (15.5%) Stage II = 21 (36.2%) Stage III = 28 (48.3%)	- 30 days after surgery; - q3–6 months on follow-up	-	-	-	Surveillance test: - ctDNA +: 10/13 relapsed (76.9%) - ctDNA -: 0/45 relapsed (0%) - ctDNA lead time for recurrence detection: 3 months	- 3 patients who were ctDNA + post-op spontaneously cleared ctDNA and did not relapse - 2/18 patients treated with ACT were ctDNA + after completing systemic treatment. Both relapsed (100% relapse rate when ctDNA positive after ACT) - ctDNA has higher sensitivity for recurrence detection than CEA (100% versus 60%)
Parikh <i>et al.</i> <sup>19</sup> , 2021 (Guardant Reveal)	84	Stage I = 8 (9.5%) Stage II = 20 (23.8%) Stage III = 40 (47.6%) Stage IV = 16 (19%)	- Preoperatively - 4 weeks after surgery - 4 weeks after adjuvant treatment	-	17/70 (24.2%)* *Post all curative intent treatment	1/6 (16.6%)	Surveillance test: - ctDNA sensitivity for recurrence: 69% HR, 12.26 ( <i>p</i> < 0.0001) - ctDNA specificity for recurrence: 100%	- Plasma only assay with integrated epigenomic signature - Landmark ctDNA negative predict value: 75.5% - Landmark ctDNA positive predict value: 100% - ctDNA + Stage I–III RFS 13.8, 95% CI 2.85–67.42, <i>p</i> < 0.001
Henriksen <i>et al.</i> <sup>20</sup> , 2021 (Signatera, bespoke mPCR NGS)	260	Stage I = 4 (1.5%) Stage II = 90 (34.6%) Stage III = 166 (63.8%)	- q3 months for 3 years	-	20/218 (9.2%)	5/20 (25.0%)	Postoperative test: - ctDNA +: 15/20 relapsed (75.0%) - ctDNA -: 27/198 relapsed (13.6%) - ctDNA lead time for recurrence detection: 8 months	ctDNA outperformed CEA in predicting recurrence after surgery: HR 7.1; 95% CI, 3.4–15; <i>p</i> < 0.001 - Longitudinal ctDNA + post treatment recurrence risk: HR 36; 95% CI: 16–81; <i>p</i> < 0.001
Anandappa <i>et al.</i> <sup>21</sup> , 2021 (Signatera, bespoke mPCR NGS)	122	Stage II = 58 (47.5%) Stage III = 64 (52.5%)	- Preoperatively or before neoadjuvant chemoradiation; - after surgery	All stages = 93.4%	14/107 (13.1%)	8/14 (57.1%)	Postoperative test - ctDNA +: 6/14 relapsed (42.9%) - ctDNA -: 8/93 relapsed (8.6%)	RFS ctDNA + HR: 28.8, 95% CI: 3.5–234.1 <i>p</i> < 0.001

(Continued)

Table 2. (Continued)

Study (ctDNA assay)	Number of patients	Stage distribution	Timing of blood collection	Preoperative ctDNA sensitivity	Postoperative ctDNA positivity	ctDNA clearance after chemotherapy	Recurrence outcomes	Additional findings
Kotaka et al. <sup>22</sup> , 2022 (Signatera, bespoke mPCR NGS)	1365	Stage I = 116 (8.5%) Stage II = 478 (35.0%) Stage III = 503 (36.9%) Stage IV = 268 (19.6%)	- Preoperatively: 4, 12, 24, 36, 48, 72, and 96 weeks after surgery	-	188/1040 (18.1%)	68%	- 6 months DFS rates according to ctDNA Dynamic from week 4 post-op to week 12 post-op: - Negative to negative: 98% - Positive to negative: 100% - Negative to positive: 59% - Positive to positive: 45%	- 6 months DFS superior for patients who cleared ctDNA after completion of adjuvant therapy when compared with those who remained ctDNA + after it: HR of 52.3, 95% CI, 7.2–380.5, $p < 0.001$ - 6 months ctDNA clearance rate with or without adjuvant chemotherapy (stages II and III): 33% versus 0% $p < 0.001$ - No benefit of adjuvant chemotherapy for patients who were post op ctDNA--HR, 1.3; 95% CI, 0.5–3.6, $p = 0.63$
Tsukada et al. <sup>23</sup> , 2022 (Guardant Reveal)	100	Stage II = 45 (45%) Stage III = 48 (48%) *7 patients excluded	Preoperatively - 4 weeks after surgery - q3 months year 1 - q6 months years 2–5	Stage II = 86% Stage III = 83% All stages = 84%	Stage II = 19% Stage III = 29% All stages = 25%	-	ctDNA sensitivity for recurrence: - Single timepoint: 50% (6/12) - Longitudinal: 91% (10/11) ctDNA lead time for recurrence detection: 6.6 months	- Plasma only assay with integrated epigenomic signature - 1 year DFS ctDNA + HR: 3.49, 95% CI, 0.93–13.10, $p = 0.049$ - MRD had the strongest association with relapse on multivariate analysis

ACT, adjuvant chemotherapy; CI, confidence interval; CRC, colorectal cancer; ctDNA, circulating tumor; DFS, disease-free survival; HR, hazard ratio; RFS, recurrence-free survival.

patients based on ctDNA results after surgery, as well as feasibility and efficacy of surveillance strategy based on ctDNA.

The DYNAMIC randomized trial provided the first glimpse of prospective validation of ctDNA's clinical utility beyond prognostication.<sup>24,25</sup> The decision whether or not to offer adjuvant chemotherapy for a patient with stage II colon cancer is challenging and ctDNA provides a tool to help inform that decision. Although the rates of cure are >80%, there is a group of patients who will recur.<sup>65</sup> DYNAMIC showed that a ctDNA-informed approach could be used to reduce chemotherapy in most patients, while allowing those patients at highest risk of recurrence to receive intensified therapy. This resulted in non-inferior RFS for the overall population, and although not analyzed yet, likely provided a cost-effective tool given the ability to reduce the use of chemotherapy and clinical resources during the course of adjuvant therapy. A closer look at the data highlights that there are still unanswered questions before implementation. DYNAMIC showed that patients with clinical high-risk features, even without detectable postoperative ctDNA, had inferior outcomes, indicating a potential benefit of a combined approach with traditional clinicopathologic criteria.<sup>24</sup> The subgroup of low-risk stage II colon cancer appears to be the one where the use of ctDNA is closer to prime time, given the excellent outcomes reported and the fact that about 10% of them will have ctDNA detected postoperatively, allowing most to avoid adjuvant therapy. Other unanswered questions brought up by the trial are the optimal chemotherapy for ctDNA-positive cancers, duration of therapy, and what is the proportion of patients with detectable ctDNA that will be cured instead of just having delayed radiological recurrence by early treatment. At the end of the day, DYNAMIC provides important information, but more prospective data are needed.

Several trials are currently investigating the unanswered questions as well as different strategies for incorporation of ctDNA in the management of curative intent treatment of colon cancer. In stage II, COBRA (NCT04068103/NRG-GI005), MEDOCC-CrEATE (NL6281/NTR6455), CIRCULATE AIO-KRK-0217 (NCT04089631), PRODIGE 70 – CIRCULATE (NCT04120701), and IMPROVE-IT (NCT03748680), the latter also accruing stage I patients, are evaluating escalation of treatment with adjuvant chemotherapy in

patients with detectable ctDNA after surgery. Other trials such as DYNAMIC III (ACTRN12617001566325), CIRCULATE-US (NCT04068103), CIRCULATE JAPAN/VEGA (UMIN000039205), TRACC (NCT04050345), and PEGASUS (NCT04259944) are studying tailored adjuvant chemotherapy with escalation or de-escalation according to ctDNA results in high-risk stage II or stage III cancers, while the SU2C ACT3 trial evaluates the use of biomarker led therapy in cancers that fail to clear ctDNA during adjuvant therapy (NCT03803553). Finally, IMPROVE IT-2 (NCT04084249) is comparing ctDNA-guided postoperative surveillance to conventional radiological assessments and ALTAIR (NCT04457297) is investigating systemic treatment for patients with molecular relapse (ctDNA detection) during follow-up.

### Conclusions

Overall, the advancements in liquid biopsy and ctDNA have been remarkable. Although we are now able to detect MRD after curative treatment in many patients with CRC, there remain unanswered questions and important steps to optimize the use of ctDNA. Hopefully, as more data from prospective randomized trials emerge and the technology further develops, we will be able to overcome the remaining challenges to fully incorporate ctDNA in the management of patients. We are close, but not quite ready for ctDNA to move into prime time. Nevertheless, future certainly looks bright.

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*Ethics approval and consent to participate*  
Not Applicable.

*Consent for publication*  
Not Applicable.

### Author contribution(s)

**Joao Paulo Solar Vasconcelos:** Conceptualization; Data curation; Methodology; Project administration; Writing – original draft; Writing – review & editing.

**Melina Boutin:** Conceptualization; Methodology; Writing – original draft; Writing – review & editing.

**Jonathan M. Loree:** Conceptualization; Funding acquisition; Methodology; Supervision;

Writing – original draft; Writing – review & editing.

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