1 | INTRODUCTION

Over the past three decades, considerable progress has been made in understanding the genetics of colorectal cancer (CRC). Much of our knowledge has come from the discovery of predisposing genes for hereditary syndromes (Figure 1). The most significant of these include the identification that mutations in the APC gene predispose to familial adenomatous polyposis (FAP) in 1991,1–3 and that mutations in the mismatch repair genes MLH1, MSH2, and PMS2 cause hereditary nonpolyposis colorectal cancer (HNPCC) in 1993-1994.4–9 The discovery of other genes leading to hamartomatous polyposis syndromes also increased the breadth of our knowledge of how these tumors develop, including STK11 for Peutz-Jeghers syndrome,10,11 SMAD4 and BMPRIA for Juvenile Polyposis,12–14 and PTEN for Cowden syndrome.15–18

In parallel with these discoveries, our understanding of how sporadic colorectal cancers develop has also evolved. This was elucidated by Fearon and Vogelstein in 1990, where they laid out the idea that these cancers develop from an early adenoma, and gradually accumulate key genetic alterations that lead to carcinoma.19 They suggested that an early event was loss of APC function, followed by KRAS mutation, then loss of a gene on 18q (DCC or SMAD4), and finally TP53 mutation to complete the adenoma-to-carcinoma sequence (Figure 2). The specific order and timing of these mutations may not be so clearly defined, but this idea was extremely important to our understanding of how many cancers develop: through a step-wise accumulation of genetic alterations that give cells the potential for unchecked cell growth, invasion, and metastasis. This paper will review how understanding genetics has improved targeted therapy in CRC, and important applications
of how DNA can be used for diagnosis, detection of residual or recurrent disease, and determining prognosis in patients.

2 | TARGETED THERAPY

Knowledge of the genetics of CRC has allowed for targeted therapy, which began in 2004 with an antibody (bevacizumab) directed at vascular endothelial growth factor (VEGF) and another (cetuximab) against the epidermal growth factor receptor (EGFR; for KRAS wild-type tumors) in patients with metastatic colorectal cancer. Humanized antibodies cause lower rates of hypersensitivity reactions in patients, and these became available for EGFR (panitumumab) in 2006 and showed efficacy for metastatic CRC (mCRC), and to VEGF receptor 2F (ramucirumab) in 2015. These important pathways and targets are summarized in Figure 3.

2.1 | EGFR

The epidermal growth factor receptor is an ErbB receptor on the plasma membrane, which is a tyrosine kinase. When activated, it stimulates three different pathways, the Ras pathway, PI3-K pathway, and the JAK-STAT pathway, which lead to increased growth, survival, and invasion of these tumors. EGFR is overexpressed in about 25% to 77% of colorectal cancers, which can be targeted using cetuximab and panitumumab if patients are RAS wild-type. One example of their efficacy comes from the TAILOR trial, which randomized patients with mCRC with wild-type RAS (wtRAS) to receive cetuximab with FOLFOX vs FOLFOX alone. Overall survival (OS) was significantly improved with the addition of cetuximab, although only by 3 months (20.7 vs 17.8 mo, hazard ratio 0.76). Checking for RAS mutations is important, as several studies have demonstrated that patients with RAS mutations do not benefit from receiving anti-EGFR therapy in addition to chemotherapy for mCRC. Another important factor predicting efficacy of anti-EGFR therapy in wtRAS mCRC is the location of the primary tumors. A recent meta-analysis including data from four randomized control trials of mCRC revealed significantly improved OS with anti-EGFR plus chemotherapy over chemotherapy alone for left-sided tumors (HR 0.70), but not for right-sided tumors (HR 0.99), even though progression-free survival (PFS) and objective response rates (ORR) were improved in patients with tumors derived from both sides. This suggests that the benefits of anti-EGFR therapy are clearer for patients with metastatic left-sided tumors, but could still be an option for carefully selected patients.
patients with right-sided cancers. Although cetuximab may be useful in patients with mCRC, two large trials adding cetuximab to FOLFOX in the adjuvant setting for stage III patients showed no improvement in disease-free survival (DFS) compared to FOLFOX alone.

Human epidermal growth factor 2, also known as HER2 and ErbB2, differs from other EGFRs in that it does not bind ligand, and amplification leads to receptor activation. In CRC, HER2 amplification only occurs in about 5% of cancers, but can be a cause for unresponsiveness to anti-EGFR treatment. The HERACLES-A trial enrolled 35 patients with metastatic CRC who were KRAS wild-type but HER2 positive to receive treatment with trastuzumab (HER2 antibody) and lapatinib (an EGFR and HER2 tyrosine kinase inhibitor). Complete responses were seen in 3% of patients, partial responses in 25%, but 19% had progression in the central nervous system. Similar responses were seen in another trial of trastuzumab with conjugated deruxtecan in patients with wtRAS and wtBRAF (DESTINY-CRC01), confirming that anti-HER2 therapy may be another option for carefully selected patients with metastatic CRC. Acquired HER2 amplification is one mechanism by which tumors may develop resistance to anti-EGFR therapy, and therefore there may be a role for retesting tumors in these patients.

2.2 | BRAF

About half of patients with wtRAS metastatic CRC do not respond to cetuximab, and these patients may have a mutation in the BRAF gene that leads to resistance. This is most commonly a V600E mutation, which is present in about 5%-10% of colorectal cancers. BRAF inhibitors alone have not shown good activity against metastatic CRC, but response is improved when combined with anti-EGFR therapy, and is enhanced further by addition of a MEK inhibitor. The drug encorafenib is a BRAF inhibitor, and binimetinib is a MEK inhibitor, and these were tested in the BEACON 3 trial. This randomized patients with mCRC with BRAF V600E mutation to three arms after progression on other therapies: (a) cetuximab, encorafenib, and binimetinib plus chemotherapy (irinotecan or FOLFIRI); (b) cetuximab and encorafenib plus chemotherapy; and (c) cetuximab alone plus chemotherapy. This trial showed that the combination of three or two of these drugs led to improvement of median OS over cetuximab alone (median of 9.0, 8.4, and 5.4 mo, respectively). Although these responses were significant, they were modest and only improved OS by 3-4 months, but exemplify the notion of how resistance to one pathway may be overcome by targeting another component of the pathway.

2.3 | VEGF

As mentioned above, another target for therapy are angiogenesis inhibitors. VEGF is made by tumors, binds to the VEGF receptor, and stimulates tumor angiogenesis and proliferation. This binding is inhibited by bevacizumab, and several trials have looked at the utility

**FIGURE 3** Key pathways in colorectal carcinogenesis. Epithelial growth factor (EGF) binds to the epithelial growth factor receptor (EGFR), which activates the Jak/STAT, Ras/Raf/Mek/Erk, and PI3K/Akt/mTOR pathways. Antibodies targeting EGFR include cetuximab and panitumumab. Vascular endothelial growth factor (VEGF) binds to the vascular endothelial growth factor receptor (VEGFR) which activates the PI3K/Akt/mTOR pathway. Bevacizumab is an antibody that targets VEGF itself, and ramucirumab targets the receptor VEGFR-2.
of bevacizumab in patients with mCRC. One was the AVF2107 trial, which randomized patients to FOLFIRI with or without bevacizumab for previously untreated mCRC. It found an improvement in OS from 15.6 to 20.3 months with bevacizumab. In the BRITE trial, patients who developed progression after first-line chemotherapy were either not treated, or given chemotherapy with or without bevacizumab. The no treatment group had a median OS of 3.6 months after first progression, vs 9.5 months for chemotherapy alone without bevacizumab, and 19.2 months for chemotherapy plus bevacizumab. In this study, tumors were not tested for \textit{KRAS} or \textit{BRAF} mutation or for mismatch repair (MMR) deficiency. When given in the adjuvant setting for high-risk stage II or stage III CRC, bevacizumab plus FOLFOX did not improve DFS relative to FOLFOX alone. These studies confirmed that anti-VEGF therapy is another very valuable modality for patients with mCRC (but not stage II or III), especially those with \textit{KRAS} mutation or those with \textit{wtKRAS} and right-sided tumors, which are not likely to respond to cetuximab.

2.4 | Mismatch repair deficiency

Approximately 15% of colorectal cancers are deficient in DNA mismatch repair (dMMR) function due to either germline (HNPCC, Lynch Syndrome) or somatic alterations of \textit{MSH2}, \textit{MLH1}, \textit{PMS2}, and \textit{MSH6}. These tumors display microsatellite instability, which means that short tandem repeat DNA sequences have unfaithful replication, which is indicative of numerous alterations throughout the genome. A small fraction of patients with microsatellite instability who do not have mutations in these genes have been reported to have mutations in the DNA polymerase genes \textit{POLE} or \textit{POLD1}. These patients differ from HNPCC patients in that they generally have multiple adenomas as well, and not all tumors show microsatellite instability. When dMMR leads to changes within coding sequences of genes, this may lead to expression of many neoantigens on the cell surface. Tumors harboring these mutations tend to be more right-sided, mucinous, and less well-differentiated. A meta-analysis of early studies suggested that patients with dMMR tumors have better prognosis, but are less responsive to 5-FU chemotherapy. These tumors have higher infiltration of lymphocytes, and it has been shown that CRC neoantigens can induce cytotoxic T-cell responses. Since patients whose tumors are dMMR have many mutations leading to neoantigens, it is logical that these tumors are more immunogenic. Immune checkpoint inhibitors (ICIs) have shown efficacy in some tumors, most notably melanoma. One current therapy (ipilimumab) is directed at the cytotoxic T lymphocyte antigen 4 (CTLA-4) receptor, which inhibits antigen presenting cells from being able to mediate T-cell activation when delivering neoantigens (Figure 4A). Other therapies (Nivolumab, Pembrolizumab) bind to the
programmed cell death protein 1 (PD-1) receptors on T-cells, through which tumor cells can evade activation of T-cells by expressing the PD-L1 ligand; when these antibodies bind to the PD-1 receptor, T-cells can recognize neoantigens and become activated (Figure 4B).

Early studies with ICIs in CRC patients showed minimal responses, until patients were stratified by MMR status. In a trial where patients with progressive mCRC were treated with the PD-1 antibody Pembrolizumab, the PFS at 20 weeks of patients with dMMR CRC was 78% (seven of nine patients) and 11% for patients with MMR-proficient tumors (two of 18 patients), with a hazard ratio of 0.10 for progression or death between the two groups.44 A follow-up study from the same group looked at 86 patients with dMMR tumors and progressive disease from 12 different sites, and treated them with PD-1 inhibitors. They saw radiographic responses in 53% of patients and complete responses in 21%. The mean time to observing a response was 21 weeks and for complete response was 42 weeks. More in-depth analysis of select patients with responses demonstrated expansion of T-cells recognizing mutation-associated neoantigens, and the authors suggested that patients with tumors refractory to treatment be tested for MMR status, as PD-1 blockade might be an effective strategy irrespective of tumor type.45

Further information regarding the responses of patients with dMMR CRC to ICIs came from the Checkpoint-142 trial. In an open label, phase II trial of patients with dMMR recurrent or mCRC receiving at least one previous chemotherapy regimen, 74 patients were given the PD-1 antibody Nivolumab every 2 weeks. The objective response rate was 32% (3% complete response, 30% partial response, 34% stable disease), and the therapy was well-tolerated.46 Another cohort of 119 patients was given the combination of nivolumab and the CTLA-4 antibody Ipilimumab, and the objective response rate was improved to 55% (3% complete response, 51% partial response, 31% stable disease). Both PFS (71% at 12 months) and OS (85% at a median of 13.4 months of follow-up) were improved with the combination treatment over giving Nivolumab alone (PFS 50% at 12 months, and OS 73%). Responses were durable and were seen in patients with and without KRAS or BRAF mutations.47

KEYNOTE-164 was another open label, phase II clinical trial that enrolled patients with dMMR mCRC into two groups. Cohort A patients (n = 61) had received ≥2 previous therapies with or without EGFR or VEGFR inhibitors, and Cohort B (n = 63) had ≥1 line of previous chemotherapy. Patients received the PD-1 antibody Pembrolizumab for up to 2 years. The objective response rates were 33% in both Cohort A (3% complete, 30% partial, 18% stable disease) and B (8% complete, 25% partial, 24% stable disease), PFS was 2.3 and 4.1 months, and median survival was 31.4 months and not reached, respectively. The therapy was tolerated fairly well, with 13%-16% of patients having grade 3-4 adverse events. Responses were durable, and not dependent on KRAS or BRAF status.48

The more recent KEYNOTE-177 trial randomized patients with dMMR mCRC to Pembrolizumab alone or chemotherapy (5-fluorouracil based, ± cetuximab or bevacizumab). Median PFS was improved in the Pembrolizumab only group over the chemotherapy group (16.5 and 8.2 mo, respectively, HR 0.60), with ORR of 44% and 33%. In those responding to Pembrolizumab, 83% continued past 24 months, suggesting that ICIs might be considered for first-line treatment of patients with dMMR mCRC without chemotherapy.49

In summary, many new options for therapy in patients with advanced, progressive, or mCRC have become available over the past decades. A good algorithm for considering options for these patients is shown in Figure 5. In general, it is important to know the status of the tumor for KRAS (wild-type or mutant), BRAF (wild-type of mutant), and for MMR (deficient or proficient). Patients with mCRC which are dMMR may have good and durable responses to ICIs, and these have recently been approved for first-line therapy in patients with these tumors, as opposed to giving these agents after failure of response to first-line chemotherapy. If patients are MMR-proficient, then patients...
who have left-sided KRAS wild-type tumors may respond to anti-EGFR therapy (cetuximab, panitumumab). If they do not respond, then this may be due to a BRAF mutation, and therefore adding a BRAF inhibitor (vemurafenib, encorafenib) to anti-EGFR therapy may also be helpful; adding a MEK inhibitor (binimetinib) is not currently approved. Patients with HER2-positive tumors who do not respond to EGFR therapy may benefit from HER2 antibody or inhibitor therapy (trastuzumab, lapatinib). In patients who are KRAS mutant and MMR-proficient, the addition of VEGFR antibody (bevacizumab, regorafenib, ramucirumab) to chemotherapy regimens may have some survival benefit.

3 | FECAL DNA TESTING

Knowledge of the mutations leading to CRC has also shown utility for testing in exfoliated cells as a screening tool for CRC.\(^{50,51}\) This has become widely available in the past few years, and one study reported a sensitivity of 85% for CRC and 54% for advanced adenomas, with a specificity of 90%.\(^{52}\) Although potentially useful for patients who cannot undergo colonoscopy, the cost effectiveness for screening the population is a point of debate.\(^{53}\)

4 | GERMLINE DNA-TESTING

The importance of medical genetics consultation and testing for germline mutations in patients with a strong family history of CRC cannot be overemphasized, and has been covered in detail elsewhere.\(^{54}\) Genetic consultation should also be considered in younger patients developing CRC, particularly those in their third to fifth decades who may not quite meet the indications for genetic testing.

5 | CIRCULATING TUMOR DNA (ctDNA)

One of the more exciting recent developments with enormous potential applications for managing patients with CRC has been the ability to sensitively detect tumor DNA in the plasma of patients. This is known as circulating tumor DNA (ctDNA) and has also been referred to as "liquid biopsy."\(^{55,56}\) The utility of these techniques is to determine whether patients have successfully achieved complete resection, for the detection of residual or recurrent disease, and to assess response to therapy.

5.1 | Assays used to test for ctDNA

Tumor cells will lyse and with this, release DNA from their cells. This DNA will end up in the bloodstream, and can be isolated from the plasma. DNA in the bloodstream derives from a variety of sources, which can include both normal and malignant cells. The latter usually have specific mutations, which are most efficiently searched for in DNA extracted from the plasma (Figure 6). These fragments are generally 150-200 bp in length, and ctDNA represents about 0.1%-10% of circulating free DNA.\(^{55}\) The first approach for detecting ctDNA in CRC patients targeted known mutations common to tumors using specific oligonucleotides directed at genes such as KRAS, TP53, BRAF, or methylation of specific genes.\(^{57}\) The advent of next-generation sequencing (NGS) approaches allowed for unbiased sequencing of ctDNA for identification of novel mutations, but is enhanced by a two-step process of adding unique bar codes to each fragment, then a second amplification step to ensure that all daughter sequences are essentially the same, in order to reduce transcription errors or incorrect base calling.\(^{58}\) Another NGS approach sequences normal and tumor tissues from each patient to identify the most common mutations, then develops a personalized, 16-gene multiplex PCR assay for the patient.\(^{59}\)

Another method for studying ctDNA is by methylation profiling, which has the potential to survey more sites in the genome. Previous bisulfite conversion methods led to degradation of limited amounts of ctDNA, so an improved method using DNA immunoprecipitation and high-throughput sequencing (cfMeDIP-seq) has been developed, which shows promise for identifying alterations within ctDNA.\(^{60}\) Instead of examining the genome at increasingly finer detail, some have instead used ultra-low pass whole-genome sequencing where the coverage is 0.1x instead of the usual 30x used for reliable mutation detection. This method reduces the cost of sequencing, allows for more samples to be tested in each sequencing run, and uses imputation to fill in missing information. This method has been used to determine tumor fraction that correlates with disease burden, and is therefore another promising method for following cancer patients.\(^{61,62}\)

In patients with resectable tumors, ctDNA can be used for diagnosis and determination of the mutational profile to be followed. After surgery, it can be used to determine the adequacy of resection and help select patients for adjuvant therapy. In patients receiving adjuvant
therapy or therapy for metastatic disease, ctDNA can be used to monitor the response to treatment as well as for the emergence of resistance to therapy (Figure 7). In some situations, specific chemotherapeutics or targeted therapies can potentially be employed based upon the mutations that become present over time or with rising levels of ctDNA. Measuring ctDNA is emerging as a more sensitive method for detecting recurrence or progression than CEA or conventional imaging.

As with all tests, the sensitivities and specificities of various assays are important since one is trying to detect changes in a very small amount of ctDNA representing just a fraction of cell-free DNA. False-negative results are more likely when there is a lower burden of disease, and are also affected by signal-to-noise ratio and filtering algorithms. False-positives results can result from tumor heterogeneity, or may also come from DNA within normal cells. Clonal hematopoiesis, where there is selective expansion of cells acquiring somatic mutations, and rare germline variants may be sources of these false positives. Positive predictive value also decreases when the allelic fraction of mutations is less than 1%, and there may be considerable variability across platforms. Different applications using ctDNA will be discussed below, as well as ongoing trials incorporating this technology for making treatment decisions.

5.2 | ctDNA to detect residual disease after resection

About 30%-50% of patients with stage I-III CRC will develop recurrence, and one of the more useful applications of ctDNA is to determine the adequacy of surgical resection, and therefore who might benefit from adjuvant therapy. Several studies have shown correlation of detectable ctDNA with positive margins and metastases.

In stage II colon cancer, adjuvant therapy is sometimes recommended for patients with high-risk characteristics. These include T4 tumors, perforation, obstruction, inadequate nodal harvest, lymphovascular invasion, and poor-differentiation. Since recurrence is rare and difficult to foresee, ctDNA could be useful to determine which patients are predicted to have residual disease and will therefore be likely to recur. One of the earlier and best studies to look at this was by Tie et al, where they evaluated 250 patients with resected Stage II colon cancer between 2011 and 2014. They performed genome sequencing of the primary tumor in 231 patients, and found at least one somatic mutation in 230 (99.6%) patients. Most of these were within 15 genes (such as TP53 with nine variants, APC with eight variants, and KRAS with three variants), and they designed personalized assays for the mutations in each person (called Safe-SeqS). They then quantified ctDNA at 4-10 weeks postoperatively, then every 3 months. They followed these patients, 52 of whom received chemotherapy at their clinician’s discretion and 178 who did not. Over a 2-year period, 34 patients (15%) developed recurrence. Of those that did not have chemotherapy, 14/178 had detectable ctDNA. In these 14 patients, 11 (79%) developed recurrence. When contrasted with the 164 patients without detectable ctDNA, only 16 (10%) developed recurrence. This difference was highly significant, with a hazard rate of 18 for those with positive ctDNA. They concluded that detection of ctDNA is an indication of tumor cells remaining after surgery. They suggested that ctDNA should be used to stage patients, like a CT scan, but that it is not perfect, as it was only positive postoperatively in 48% of people who developed recurrence. However, 97% of patients who did not experience recurrence had no ctDNA, and therefore this had even better detection levels than CT scans. Patients with ctDNA were at extremely high risk for recurrence when not treated by chemotherapy,
which was even higher than for Stage III patients routinely treated with adjuvant chemotherapy. Patients who were positive after adjuvant chemotherapy were also at very high risk for recurrence. They concluded that ctDNA analysis is an attractive biomarker for clinical trials.

5.3 | ctDNA to assess treatment response

Besides selecting patients who might benefit from adjuvant therapy, ctDNA may also be a good way to assess a patient’s response to therapy. Our current tools include imaging using the RECIST 1.1 criteria, and CEA levels when these are elevated preoperatively. ctDNA can also be helpful to determine whether the mutational status of a tumor has changed, and therefore that resistance to therapy may be developing. The decision to give chemotherapy or targeted therapy has traditionally been based upon several factors, such as high-risk stage II lesions, most stage III and stage IV tumors, and rising CEA levels. Not all stage II or III patients will benefit from adjuvant therapy, however, and therefore using ctDNA after surgery or in follow-up may be a logical approach to select patients for treatment. Escalation or de-escalation approaches are being suggested in clinical trials for those who are ctDNA-positive vs ctDNA-negative, respectively, to increase efficacy for those who need more therapy and to spare toxicity in those who do not need it.

5.4 | Stage II patients

In the study by Tie et al of patients with stage II disease, 52 patients were treated with chemotherapy, of which six patients were ctDNA positive (12%) and 46 were negative. In those six positive patients, the ctDNA changed from positive to negative in five of six patients with chemotherapy. Of these five, two later became ctDNA positive, and both developed recurrence radiologically. Two of the three others stayed negative, while the other had a radiologic recurrence but ctDNA remained negative. The recurrence-free rate was about 85% in those who remained ctDNA negative after chemotherapy (41 patients), and this was zero in those where ctDNA did not disappear (three patients), for a hazard ratio of 11. At the time of radiologic recurrence, 23/27 patients had positive ctDNA, while only 11/27 had elevated CEA levels. The mean time that ctDNA became positive was 167 days before radiologic recurrence, in contrast to 61 days for CEA. The overall sensitivity of ctDNA was 48% and specificity was 100% in determining recurrence at 36 months postoperatively. This study nicely demonstrated the utility of using ctDNA in follow-up of patients with stage II colorectal cancer.

5.5 | Stage III patients

Tie et al also prospectively studied 100 patients with stage III CRC with R0 resection and no metastatic disease. All patients received 24 weeks of adjuvant chemotherapy, and they collected ctDNA samples 4-10 weeks after surgery and 6 weeks after chemotherapy. They found positive ctDNA in 20/96 patients post-surgically, and in 15/88 completing chemoradiation; 24 patients developed recurrence at a median follow-up of 28.9 months. Patients with positive postoperative ctDNA had a 3.8-fold increased hazard ratio of recurrence (47% were recurrence free [RFS] vs 76% in postoperative negative patients), and if positive after chemotherapy, a 6.8-fold higher hazard ratio (30% 3 year RFS in positive patients vs 77% if negative). Half of the post-surgically positive patients converted to negative after chemotherapy and recurrence-free rates at 3 years were 60% vs 40% if they remained positive (HR 3.7, P = .04). This study showed that ctDNA was diagnostic for recurrence after surgical resection and chemotherapy for stage III colon cancer. These findings have given rise to multiple prospective trials utilizing ctDNA-informed adjuvant therapy.

5.6 | Neoadjuvant therapy in rectal cancer

Further information on using ctDNA to assess treatment response came from patients with locally advanced rectal cancer, as defined by MRI or EUS (T3-4N0 or N1-2). Blood was collected for ctDNA before and after neoadjuvant chemoradiotherapy, and then again after surgery. Of 200 patients enrolled, 162 completed the study with a mean of 24 months of follow-up. Mutations in ctDNA were detected in 77% of patients before chemoradiotherapy, 8% after chemoradiotherapy, and 12% after surgery. There was no difference in RFS by baseline ctDNA status, but after chemoradiotherapy, recurrence was seen in 6/12 (50%) with positive ctDNA vs 15/132 (11%) with negative ctDNA; RFS at 3 years was 50% and 85% (HR of 6.6), respectively. After surgery, 11/19 (58%) with positive and 12/140 (9%) with negative ctDNA had recurrence; RFS at 3 years was 33% and 87%, respectively (HR of 13.0). There was no association between pathologic complete response (pCR) after chemoradiotherapy and ctDNA status, and they concluded that in this short interval after treatment, ctDNA is not predictive of pCR and therefore cannot be used for selecting patients for a watch and wait strategy. Once again, this study demonstrated the value of ctDNA in identifying those patients at high risk for recurrence.

5.7 | Stage IV patients

cDNA may have utility in determining which patients may have resectable disease and those most likely to recur after surgery. Bhangal et al studied methylation status of 48 CRC-associated genes in 34 patients with colorectal liver metastases (CRLM). Samples were collected at baseline then before each cycle of chemotherapy, and patients with partial response or stable disease were selected for resection. Four methylation markers were highly sensitive (SEPT9, DCC, BOLL, and SFRP2) and were seen in all patients at baseline.
These biomarkers correlated better with tumor volume than CEA, were useful for predicting which patients would be unresectable after one cycle of chemotherapy (n = 12), and those that would have pathologic response to therapy (n = 13). Scholer et al looked at sequential ctDNA levels in 26 patients after curative intent CRC resection, and selected a group where roughly half developed recurrence and half no recurrence at 3 years.\textsuperscript{72} ctDNA was detected in 74% of patients preoperatively, which increased with stage of disease; CEA was elevated in only 55% of these patients. They found that ctDNA was detectable in all 14 patients that recurred, but in none of the 12 patients that did not relapse at 3 years. ctDNA also suggested recurrence 9.4 months prior to CT scans. They examined 23 other patients with liver metastases resected for curative intent, and found that all seven patients who were ctDNA positive after resection recurred vs 50% of those that were ctDNA negative post-resection. These studies show that ctDNA levels reflect tumor volume before and after surgery, and are prognostic for resectability and recurrence. This performed better than CEA and was detected earlier relative to CT scans.

Jones et al performed a meta-analysis of studies examining ctDNA in patients with stage IV colorectal cancer, which included 24 reports with 2700 total patients with unresectable disease.\textsuperscript{73} Six of these studies were randomized prospective trials and 17 were prospective cohort studies. The rate of ctDNA positivity before treatment was 81% over 20 studies, and three found a correlation between ctDNA levels and radiologic responses. In 21 studies assessing survival, the hazard rate of ctDNA positivity was 2.2 for OS and 3.15 for PFS. Higher levels of ctDNA before treatment were associated with poorer survival, and those patients who had reduced ctDNA after one cycle of chemotherapy had improved survival. Although further studies are warranted, this paper suggested that ctDNA levels could be used for escalation or de-escalation of therapy, reduced radiologic surveillance, and switching of therapies when there is no response suggested by ctDNA.

### 6 | ONGOING RANDOMIZED TRIALS ASSESSING CTDNA

There are a number of trials that are accruing patients using ctDNA for selecting which patients will get adjuvant therapy. These include patients from resected stage II through stage IV colon cancer, and locally advanced rectal cancer. Several of the ongoing, prospective, randomized trials using ctDNA to inform adjuvant treatment will be discussed below.

For stage II disease, the COBRA trial (NCT04068103; https://clinicaltrials.gov/ct2/show/record/NCT04068103) is a Phase III trial enrolling patients who have resected stage IIA colon cancer, with or without adjuvant therapy. It has two arms, one where patients will undergo active surveillance (and ctDNA tested later), and the other arm, where ctDNA will be tested at baseline. If ctDNA is detectable, patients will receive FOLFOX or capecitabine/oxaliplatin chemotherapy for 6 months, and patients where ctDNA is not detected will have active surveillance. The endpoints will be RFS and OS, and they aim to enroll 1400 patients. Patients with stage IIA CRC are considered to be at low-risk and normally would not receive adjuvant chemotherapy. This trial should help to determine whether patients with detectable ctDNA will benefit from being treated with adjuvant chemotherapy.

The DYNAMIC-II trial (ACTRN1261500381583; https://www.australianclinicaltrials.gov.au/anzctr/trial/ACTRN1261500381583) will study patients with resected stage II CRC (T3-4N0M0; including rectal cancers if not treated preoperatively or with intent to treat with postoperative therapy) to determine the utility of ctDNA to guide the use of adjuvant chemotherapy. In one group of patients, ctDNA results will be used to guide therapy, while in the other, the results will not be disclosed and treatment will be at the discretion of their physicians. Those with detectable ctDNA will be given 5-FU based chemotherapy, and those with negative ctDNA will not receive chemotherapy. Patients will be followed every 3 months for 2 years, then every 6 months for 5 years with the endpoint being RFS.

The BESPOKE trial (NCT04264702; https://clinicaltrials.gov/ct2/show/NCT04264702) is recruiting patients with resected stage II or III CRC in a case-control, prospective manner. The first group will have formalin-fixed paraffin-embedded (FFPE) tissue and whole blood samples tested using the SIGNATERA™ ctDNA test, and there will be a historic matched control group. ctDNA results will be used by clinicians to determine who is treated with adjuvant chemotherapy, and the endpoint will be to determine the number of patients having adjuvant treatment adjusted using ctDNA results, the rates of recurrence, OS, the response of ctDNA to adjuvant therapy, and patient-reported outcomes. They hope to enroll 1000 patients who will be followed for up to 2 years with periodic whole blood collection.

The DYNAMIC-III trial (ACTRN12617001566325; http://www.anzctr.org.au/Trial/Registration/TrialReview.aspx?id=373948&isReview=true) will study 1000 patients with resected stage III CRC (also including rectal cancers if not treated preoperatively or with intent to treat with postoperative therapy) to determine the utility of ctDNA to guide the use of adjuvant chemotherapy. Patients will be randomized to treatment based upon ctDNA results in one group and standard of care without looking at these results in the other. Patients will be stratified into low-risk (T1-3N0) and high-risk groups (T4 ± N2). ctDNA will be determined from FFPE tumors and blood within 5-6 weeks postoperatively, then adjuvant therapy started in ctDNA-positive patients and escalated, and therapy de-escalated in the ctDNA-negative group. The trial will evaluate the impact of this escalation and de-escalation strategy based on ctDNA (non-inferiority), and also look at RFS and OS over 5 years of follow-up.

The CIRCULATE-Japan (https://www.annalsofoncology.org/article/S0923-7534(20)39501-6/fulltext) is a prospective, multicenter, randomized trial examining the use of ctDNA in patients with resectable Stage II-IV CRC to help guide treatment strategies. This
study will use the SIGNATERA™ ctDNA test and collect samples before surgery. 1 month postoperatively, then every 3 months. ctDNA levels will be correlated with DFS and OS, recurrence found on imaging, clinical characteristics, and gene mutations. The study aims to include 2500 patients from approximately 150 cancer centers across Japan.

The DYNAMIC Rectal trial (ACTRN12617001560381; http://www.anzctr.org.au/Trial/Registration/TrialReview.aspx?ACTRN=12617001560381) is evaluating the use of ctDNA in patients with locally advanced rectal cancer who have undergone preoperative chemoradiation followed by surgery. ctDNA samples are collected at 4 and 7 weeks after surgery then patients are randomized to either the ctDNA-informed arm or standard of care arm. Patients with positive ctDNA or high-risk tumor features with negative ctDNA results will have 4 months of chemotherapy, while in the standard of care arm, those with high-risk features will receive chemotherapy. Patients will be followed by imaging and CEA levels out to 5 years and the endpoints will be the number of patients treated by chemotherapy, as well as how ctDNA results correlate with RFS and OS.

7 | SUMMARY

Understanding the spectrum of genetic alterations leading to CRC has allowed for refinement of therapies for patients. Besides traditional chemotherapeutic and radiation therapy options, this has led to a number of pathway-targeted therapies tailored to the genetic characteristics of the tumor. The introduction of these treatments has allowed patients who fail other therapies or have metastatic disease to live longer. To take full advantage of targeted therapies, tumors should at the minimum be tested for KRAS and BRAF mutations, as well as MMR status. With the improved throughput of next-generation sequencing methods, monitoring of ctDNA will soon become the standard for assessing complete resection of tumors, detecting recurrence, and response to therapy. It may also be useful for identifying the rare subset of patients with actionable mutations which may respond toagnostically approved drugs. With the maturation of ongoing clinical trials, ctDNA may soon be used to determine which patients should receive adjuvant therapy and those who can be spared, or those who will benefit from changing the therapeutic regimen. It will likely also impact on surveillance for CRC patients, potentially reducing the frequency of radiologic and blood tests.

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ORCID

James R. Howe

https://orcid.org/0000-0001-5312-5972

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