Tissue-Free Liquid Biopsies Combining Genomic and Methylation Signals for Minimal Residual Disease Detection in Patients with Early Colorectal Cancer from the UK TRACC Part B Study

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ABSTRACT

Purpose: The absence of postoperative circulating tumor DNA (ctDNA) identifies patients with resected colorectal cancer (CRC) with low recurrence risk for adjuvant chemotherapy (ACT) de-escalation. Our study presents the largest resected CRC cohort to date with tissue-free minimal residual disease (MRD) detection.

Experimental Design: TRACC (tracking mutations in cell-free tumor DNA to predict relapse in early colorectal cancer) included patients with stage I to III resectable CRC. Prospective longitudinal plasma collection for ctDNA occurred pre- and postsurgery, post-ACT, every 3 months for year 1 and every 6 months in years 2 and 3 with imaging annually. The Guardant Reveal assay evaluated genomic and methylation signals. The primary endpoint was 2-year recurrence-free survival (RFS) by postoperative ctDNA detection (NCT04050345).

Results: Between December 2016 and August 2022, 1,203 were patients enrolled. Plasma samples ($n = 997$) from 214 patients were analyzed. One hundred forty-three patients were evaluable for the primary endpoint; 92 (64.3%) colon, 51 (35.7%) rectal; two (1.4%) stage I, 64 (44.8%) stage II, and 77 (53.8%) stage III. Median follow-up was 30.3 months (95% CI, 29.5–31.3). Two-year RFS was 91.1% in patients with ctDNA not detected postoperatively and 50.4% in those with ctDNA detected [HR, 6.5 (2.96–14.5); $P < 0.0001$]. Landmark negative predictive value (NPV) was 91.2% (95% CI, 83.9–95.9). Longitudinal sensitivity and specificity were 62.1% (95% CI, 42.2–79.3) and 85.9% (95% CI, 78.9–91.3), respectively. The median lead time from ctDNA detection to radiological recurrence was 7.3 months (IQR, 3.3–12.5; $n = 9$).

Conclusions: Tissue-free MRD detection with longitudinal sampling predicts recurrence in patients with stage I to III CRC without the need for tissue sequencing. The UK TRACC Part C study is currently investigating the potential for ACT de-escalation in patients with undetectable postoperative ctDNA, given the high NPV indicating a low likelihood of residual disease.

Introduction

Colorectal cancer (CRC) is the third most diagnosed cancer and the second leading cause of cancer-related deaths worldwide (1). The management of CRC has significant social and economic impact. Approximately 60% of cases are stage I to III (2). Patients undergo curative surgery, adding multi-modality neoadjuvant therapy in those with locally advanced rectal cancer. Adjuvant chemotherapy (ACT) is recommended for those with high-risk stage II and stage III disease to help reduce recurrence risk (3). Depending on clinico-histopathologic features, ACT is offered in a "one-size-fits-all" approach, frequently involving doublet oxaliplatin and fluoropyrimidine chemotherapy. However, in stage III disease, more than 50% of patients may be cured by surgery alone, up to 30% may recur despite ACT, and approximately 15% to 20% benefit from ACT (4, 5). The absolute benefit of ACT is even lower among patients with stage II disease. Prognostic biomarkers of recurrence and predictive biomarkers of ACT benefit are urgently required to

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The investigators of the TRACC Part B trial are listed in Appendix 1.

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Translational Relevance

Tissue-free approaches to ctDNA detection for MRD are more practical but have been considered less sensitive than tumor-informed approaches. We conducted an extensive analysis of the tissue-free approach in the largest cohort of patients with resected colorectal cancer to date. Our data support a high level of performance comparable with tissue-informed approaches, with a lower false-negative rate than some tissue-informed studies. We observed high specificity, which may be affected by duration of follow-up, immune cell clearance of MRD, clonal hematopoiesis of indeterminate potential (CHIP), or technical artifacts. We showed postoperative ctDNA detection using a tissue-free assay is the single most significant prognostic variable. Thus, our data have informed development of one of the few tissue-free de-escalation MRD interventional studies—TRACC Part C—to guide chemotherapy decisions with the potential to significantly reduce unnecessary chemotherapy, protecting patients from toxicity and transforming patient care.

individualize treatment and spare patients from unnecessary chemotherapy with associated short- and long-term toxicity. ctDNA is emerging as a biomarker to detect minimal residual disease (MRD) to stratify recurrence risk and guide ACT decisions. To date, observational studies have shown patients without detectable MRD (ctDNA not detected postoperatively) have a significantly improved recurrence-free survival (RFS; ref. 6, 7). ctDNA clearance rates seem to range from 31% to 87% (8–10) following ACT, compared with 12.2% to 20% (11) without ACT, with superior disease-free survival in patients who achieve clearance (12). Large-scale translational observational studies are necessary to demonstrate ctDNA assays perform well, are reproducible, and correlate with clinical outcomes to define the next generation of trials for interventions related to MRD and molecular recurrence (13).

Most global observational studies in early-stage CRC have used tumor-informed ctDNA assays to detect MRD. The primary tumor is sequenced to varying degrees (from limited gene panels to whole exome sequencing) to detect tumor-specific mutations, generating bespoke, predefined panels to inform detection in plasma. This approach is multi-step and time-consuming. In addition, it relies on obtaining and transferring tumor tissue and is challenging in the adjuvant setting given the short time frame to commence ACT (14). Tissue-free assays offer a practical opportunity to analyze plasma without a priori knowledge of mutations in tissue, circumventing the need for tumor analysis, resulting in faster turnaround times, and facilitating rapid treatment decisions after surgery. This non-invasive approach has greater potential for implementation in real-world practice. Detection of cancer signals with a tissue-free approach can be through genomic, methylation or fragmentomic analysis, or a combination of these methods to improve sensitivity (15–17). Methylation patterns in tumor samples also correlate with survival outcomes (18). Combining genomic and epigenomic signals optimizes MRD detection. Technology in the field is constantly evolving, facilitating more sensitive and practical means of MRD detection.

Herein, we report results from the UK TRACC Part B study of longitudinal plasma samples for MRD in patients with resected stage I to III CRC using a tissue-free assay combining genomic and methylation signals (Guardant Reveal).

Materials and Methods

Patient cohort and samples

TRACC Part B is an ongoing multicenter, prospective, observational, translational research study, investigating the use of MRD detection to predict recurrence in patients with curatively resected early-stage CRC. Adults aged ≥18 years with a new histologic diagnosis of stage I to III CRC and no radiological metastatic disease or other malignancy within 5 years of study recruitment were eligible to enroll. Patients with locally advanced rectal cancer receiving neoadjuvant radiotherapy ± concurrent chemotherapy were also eligible. The study was approved by the London Westminster Research and Ethics Committee (REC No. 15/LO/1576) and is being delivered following Good Clinical Practice and the Declaration of Helsinki. Between December 2016 and August 2022, 1,203 patients were enrolled across 36 UK sites and provided written informed consent before study procedures took place. All patients received standard-of-care management according to their local hospital policy without patient or clinician knowledge of ctDNA results. Baseline and postoperative clinical and histopathologic characteristics and data on anticancer therapy and surgery were entered into the database.

Processing for up to 1,000 time points was available for this preliminary analysis with additional analyses planned for the remaining time points. Consequently, 214 patients with a postoperative sample and additional blood samples available for testing were selected from the first 850 patients recruited. Other samples had been used in other cohorts including analyses for institutional assay validation, not reported here. Within this framework, we included patients with stage II and III disease followed by stage I, until sample allocation was exhausted. This equated to 997 blood samples processed using the tissue-free Guardant Reveal ctDNA assay by Guardant Health designed for MRD detection.

Sample collection

Blood samples were prospectively collected using standard collection and storage procedures according to the laboratory manual pre- and postoperatively, every 3 months for year 1, every 6 months for years 2 and 3, and annually for years 4 and 5 until discharge or recurrence. Time points were designed to align with standard-of-care assessments (**Fig. 1A**). Baseline blood samples were taken within 4 weeks before neoadjuvant radiotherapy or chemoradiotherapy (CRT) if applicable, or before surgery. Patients with postoperative blood samples taken 3 to 12 weeks after surgery but before ACT were included in the primary analysis population. Blood samples were taken within 10 weeks after clinically or radiologically confirmed recurrence. Blood samples for carcinoembryonic antigen (CEA) were also taken at these time points according to standard operating procedures across all centers. Patients underwent annual computed tomography (CT) surveillance imaging for 3 years after surgery. Month 3, 9, and 30 blood samples for stage I patients and postoperative imaging were optional. At each time point, 30 to 50 mL of whole blood was collected for circulating free DNA (cfDNA) in three to five Cell-Free DNA BCT tubes (Streck Corporate). Streck tubes were inverted eight to 10 times to mix the blood and preservative and transferred for plasma and DNA extraction at room temperature.

Plasma and cfDNA extraction and quantification

Participating centers transferred blood samples to a central laboratory hub at the Royal Marsden Hospital for processing and storage. Whole blood samples underwent double centrifugation,

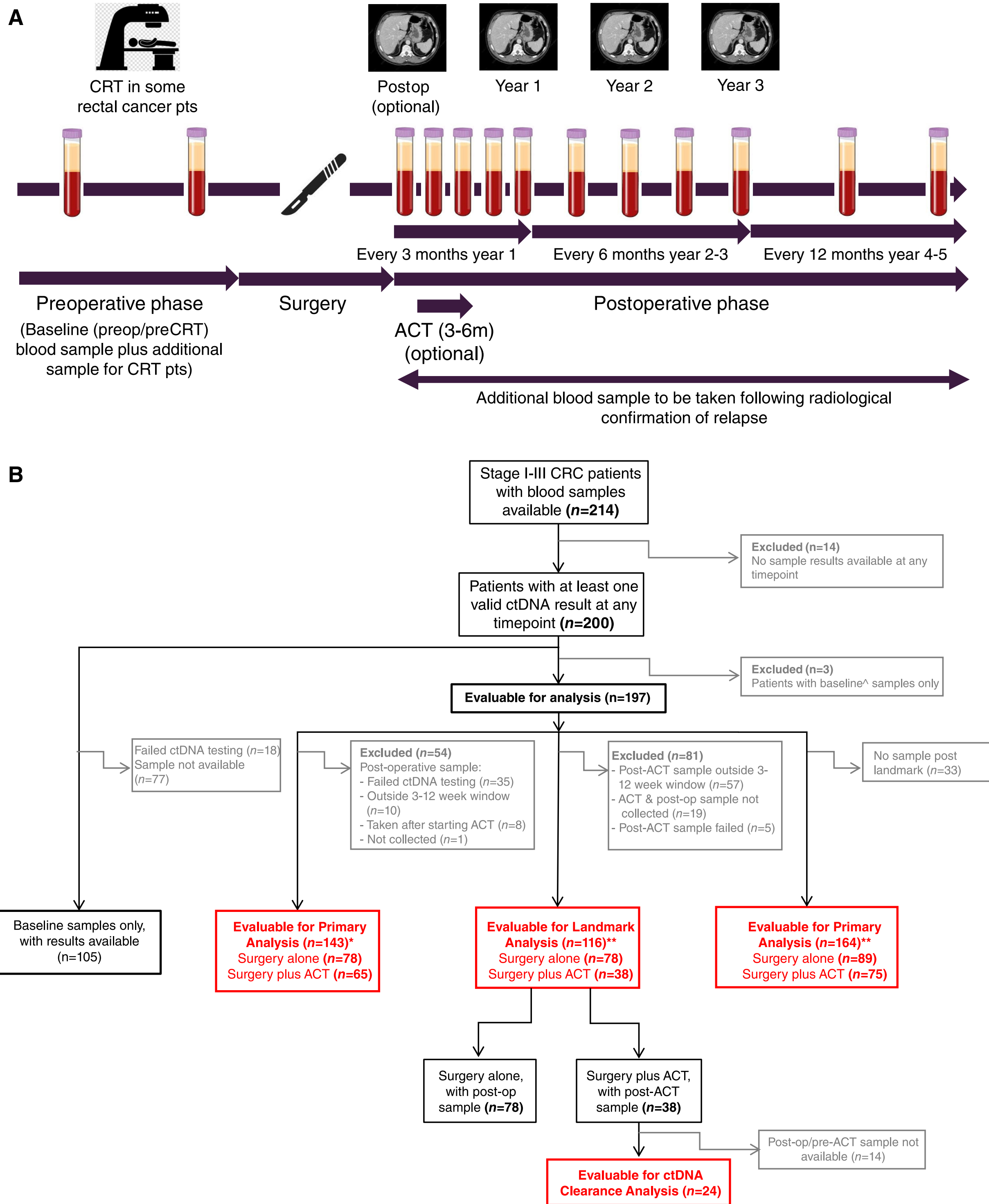

Figure 1.
A, Time points for blood sample collection and CT imaging. **B,** CONSORT diagram and analysis populations.

both for 10 minutes at 1,600 × *g*, and plasma was immediately collected into 2-mL cryovials and stored at −80°C. Plasma samples were transferred to Guardant Health for sequencing (n = 933). Circulating free DNA was extracted from 1.5- to 10-mL plasma, similar to previously described methods (19). For 64 samples, cfDNA had already been extracted from 4 mL of plasma using the

QIAsymphony SP Circulating DNA kit on the fully automated QIAsymphony SP Instrument, following the manufacturer's protocol. Samples were quantified using the Qubit dsDNA HS Assay Kit. A minimum input of 4-mL plasma or 10-ng cfDNA was recommended for submission.

Tissue-free MRD analysis

Extracted cfDNA (median yield, 31 ng; range, 0.2–109 ng; maximum input, 100 ng) was analyzed using the tissue-free, multiomic next-generation sequencing (NGS) platform, Guardant Reveal (version 1.2; Guardant Health, Redwood City, CA), which assesses cfDNA for the presence of tumor-derived genomic alterations and epigenomic (methylation) signatures (20). Circulating free DNA was partitioned by methylation status, and each partition was uniquely barcoded. Barcoded partitions were pooled and processed for the remainder of library preparation and enrichment. Library-prepared samples were enriched using a ~45-kb somatic panel covering common truncal mutations in CRC and a ~450-kb epigenomic panel covering regions that are differentially methylated in CRC and amplified before being sequenced (NovaSeq 6000). NGS data were evaluated using a bioinformatic caller designed for binary classification of each sample as "ctDNA detected" or "ctDNA not detected" based on a predefined threshold that the presence of a genomic and/or epigenomic signal is likely tumor derived.

Tissue analysis

Tissue samples were available and successfully processed retrospectively for 153 of 214 patients. We performed NGS on tissue samples using a validated commercially available assay (TissueNext, Guardant Health), which was used to molecularly characterize the cohort. Results were not used to inform or confirm ctDNA detection in blood. Archival formalin-fixed and paraffin-embedded unstained slides from the resection specimen (or biopsy specimen in patients receiving CRT) were transferred for testing. Formalin-fixed and paraffin-embedded slides met the minimum requirements for analysis (volume ≥ 1 mm^3; area: ≥ 25 mm^2; eight to 10 slides, or less with a total area of 400 mm^2, 5-μm thick with tumor fraction ≥10%). Tumor DNA was extracted, and 84 genes were analyzed for single nucleotide variants, 20 for amplifications, and 12 for fusions; results included assessment of microsatellite instability-high and tumor mutational burden (mutations per megabase). The most clinically informative molecular variants (i.e., *RAS*, *RAF,* and microsatellite instability status) detected are reported.

Statistical analysis

Patients with blood sample results available 3 to 12 weeks postoperatively were included in the primary analysis population and evaluable for the primary endpoint. The landmark analysis population included patients with blood sample results available 3 to 12 weeks after completing definitive treatment: postoperatively in patients undergoing surgery alone and post-ACT in those receiving ACT. The longitudinal analysis population included patients with at least one blood sample result available after completing definitive treatment up to 10 weeks following radiologically confirmed recurrence, regardless of sample result availability at postoperative and landmark time points.

The primary endpoint was 2-year RFS, measured from the date of surgery to clinical or radiological recurrence or death due to CRC and censored at the last follow-up or non-CRC death, according to postoperative ctDNA status in the primary analysis population. The main secondary endpoints were 2-year RFS according to landmark ctDNA status in the landmark analysis population, sensitivity, specificity, and NPV in the primary, landmark, and longitudinal populations, and lead time from ctDNA detection to radiological recurrence in the primary analysis population. Survival estimates by ctDNA status were calculated using Kaplan–Meier and hazard ratios (HR) using Cox regression. A univariate analysis was performed in the primary analysis population to evaluate the association between clinico-histopathologic prognostic factors and RFS; factors found to be significantly associated with RFS in the univariable Cox regression model (P value < 0.005) were then evaluated in a multivariable Cox regression model. All analyses were carried out using STATA V18 (StataCorp. 2023. Stata Statistical Software: Release 18. College Station, TX: StataCorp LLC). Materials, data, and protocols described are available upon reasonable request.

Data availability

Raw sequencing data for this study were generated at Guardant Health and are not available due to patient confidentiality reasons. Patient-level outcomes and derived data (overall test results) supporting the findings of this study can be made available upon reasonable request to the corresponding author.

Results

Analysis populations and patient characteristics

We analyzed 997 serial blood samples for MRD in 214 patients with resected stage I to III CRC (**Fig. 1B**). Patients with no successful sample result available at any time point ($n = 14$) or with only baseline results ($n = 3$) were excluded. It was not possible to generate a ctDNA result for 170/997 (17%) samples, with 46/170 (27%) occurring at the postoperative time point. Most sample failures were associated with low cfDNA yield (≤15 ng) occurring in 103/170 (60.6%) quality control failing samples. Other reasons for failure included failed enrichment ($n = 43$), failed methylation partitioning ($n = 11$), low coverage ($n = 3$), contamination ($n = 7$), and GC bias ($n = 3$). Failed samples were excluded from the analysis because it was not possible to determine ctDNA detection. Baseline and postoperative characteristics for the primary and overall analysis populations are shown in **Table 1**. Data cut-off was August 10, 2022. Except for fewer stage I (4.2% vs. ~23%) and a higher proportion of low-risk stage III patients (40.7% vs. ~23%), the primary tumor site and stage characteristics of this cohort were representative of all patients recruited to TRACC Part B. A comparison of our cohort with prevalence in the general population is given in Supplementary Table S1.

Postoperative ctDNA analysis

In the primary analysis population, the overall 3- to 12-week postoperative ctDNA detection rate was 21/143 (14.7%). The ctDNA detection rate was 9.4% in stage II (8.6% in low risk, 10.3% in high risk) and 19.5% in stage III (19.1% and 20.0% in low and high risk, respectively). Two stage I patients were included in the postoperative analysis; neither had ctDNA detected. In the primary analysis population ($n = 143$), the median time from surgery to the postoperative blood sample was 6 weeks [interquartile range (IQR), 4–7]. Median follow-up was 30.3 months from surgery [95% confidence interval (CI), 29.5–31.3; range, 3.0–56.4 months]. Two-year RFS (time from surgery to recurrence or death from CRC) was 50.4% (95% CI, 27.2–69.7) in ctDNA-detected patients and 91.1% (95% CI, 84.1–95.1) in those with ctDNA not detected [HR, 6.5, (95% CI, 3.0–14.5); $P < 0.0001$; **Fig. 2A**]. Overall, 26 (18.2%) patients experienced an RFS event, of whom 11

Table 1. Baseline and postoperative characteristics in the primary and overall analysis populations.

Characteristics	Primary analysis population (*N* = 143)	Overall analysis population (*N* = 214)
Age at diagnosis		
Median (range)	67 (30–88)	66 (28–88)
Sex—*n* (%)		
Female	59 (41.3)	86 (40.2)
Male	84 (58.7)	128 (59.8)
Cohort—*n* (%)		
STS	115 (80.4)	175 (81.8)
CRT	28 (19.6)	39 (18.2)
Primary—*n* (%)		
Colon	92 (64.3)	137 (64.0)
Rectal	51 (35.7)	77 (36.0)
Side—*n* (%)		
Left	94 (65.7)	143 (66.8)
Right	49 (34.3)	71 (33.2)
Stage—*n* (%)[a]		
I	2 (1.4)	9 (4.2)
II	64 (44.8)	86 (40.2)
Low risk	35 (24.5)	47 (22.0)
High risk	29 (20.3)	39 (18.2)
III	77 (53.8)	119 (55.6)
Low risk	47 (32.9)	76 (35.5)
High risk	30 (21.0)	43 (20.1)
Histology—*n* (%)[b]		
Adenocarcinoma	136 (97.8)	206 (98.6)
Mucinous	21 (15.1)	32 (15.3)
Signet ring cell	3 (2.2)	3 (1.4)
High-grade dysplasia	2 (1.5)	5 (2.5)
Other (neuroendocrine differentiation)	1 (0.7)	1 (0.5)
Adjuvant treatment—*n* (%)		
No	78 (54.5)	108 (50.5)
Yes	65 (45.5)	106 (49.5)
BRAF status—*n* (%)		
Wild-type	84 (58.7)	124 (57.9)
Mutant	19 (13.3)	28 (13.1)
Unknown	40 (28.0)	62[c] (29.0)
RAS status—*n* (%)		
Wild-type	62 (43.4)	90 (42.1)
Mutant	41 (28.0)	62 (29.0)
Unknown	40 (28.0)	62[c] (29.0)
T stage—*n* (%)[e]		
T0[d]	5 (3.5)	6 (2.8)
T1	2 (1.4)	4 (1.9)
T2	12 (8.4)	25 (11.7)
T3	103 (72.0)	150 (70.1)
T4	21 (14.7)	29 (13.6)
N stage—*n* (%)[e]		
N0	77 (53.8)	113 (52.8)
N1	49 (34.3)	77 (36.0)
N2	17 (11.9)	24 (11.2)
EMVI—*n* (%)[e]		
Positive	49 (34.3)	74 (34.6)
Negative	85 (59.4)	126 (58.9)
Unknown/missing	9 (6.3)	14 (6.5)
LVI—*n* (%)[e]		
Yes	34 (23.8)	50 (23.4)
No	91 (63.6)	137 (64.0)
Unknown/missing	18 (12.6)	27 (12.6)
PNI—*n* (%)[e]		
Yes	24 (16.8)	29 (13.6)
No	97 (67.8)	148 (69.2)
Unknown/missing	22 (15.4)	37 (17.3)
MSI/MMR—*n* (%)		
MSS/MMRp	110 (76.9)	166 (77.6)
MSI-H/MMRd	21 (14.7)	29 (13.6)
Unknown	12 (8.4)	19 (8.9)
Resection margin—*n* (%)		
R0	130 (90.9)	193 (90.2)
R1	6 (4.2)	10 (4.7)
RX	3 (2.1)	6 (2.8)
Unknown/missing	4 (2.8)	5 (2.3)

(Continued on the following column)

Abbreviations: CRT, chemoradiotherapy; EMVI, extramural venous invasion; LVI, lymphovascular invasion; MMR, mismatch repair; MMRd, mismatch repair deficient; MMRp, mismatch repair proficient; MMS, microsatellite stable; MSI, microsatellite instability; MSI-H, microsatellite instability-high; PNI, perineural invasion; STS, straight to surgery.
[a]For STS patients, postsurgery pathologic stage was used; for CRT patients, baseline radiological stage was used unless upstaged by postsurgery pathologic stage.
[b]More than one option can be selected.
[c]No sample available/sample failure.
[d]Patients undergoing neoadjuvant CRT.
[e]Pathologic.

(42.3%) had ctDNA detected postoperatively. Sensitivity and specificity of the postoperative ctDNA time point for clinically/radiologically confirmed recurrence was 41.7% (95% CI, 22.1–63.4) and 90.8% (95% CI, 84.1–95.3), respectively, with an NPV of 88.5% (95% CI, 81.5–93.6), noting that 45.5% of patients subsequently received ACT.

In a univariate analysis, postoperative ctDNA status was the single most significant prognostic indicator for RFS [HR, 6.55 (2.95–14.51); $P < 0.0001$]. This held true in a multivariate analysis once possible collinearity among factors (e.g., N stage) was accounted for [HR, 4.80 (1.64–14.04); $P = 0.004$; **Table 2**].

Fifteen of 122 (12.3%) patients with ctDNA not detected postoperatively in the primary analysis population experienced recurrence or died due to CRC. Fourteen patients had at least one additional sample available (including during ACT), of whom five had ctDNA detected before recurrence (35.7%) and three had ctDNA detected after recurrence (21.4%; **Fig. 2B**). In seven patients without ctDNA detected, median time from the last negative sample to recurrence was 10.9 months (IQR, 3.0–21.7). Ten of 21 (47.6%) patients with ctDNA detected postoperatively have not experienced recurrence or died. Six became negative during the follow-up. In those who did not, the median time from the last positive result to the previous contact was 12.7 months (range, 6.0–28.5). Overall, the median lead time from ctDNA detection to recurrence in the primary analysis population was 7.3 months (IQR, 3.3–12.5; $n = 9$).

Baseline ctDNA analysis

Of 105 sample results available at baseline, the detection rate was 4/5 (80%) in patients with stage I disease, 29/46 (63.0%) in those with stage II, and 35/54 (64.8%) in patients with stage III

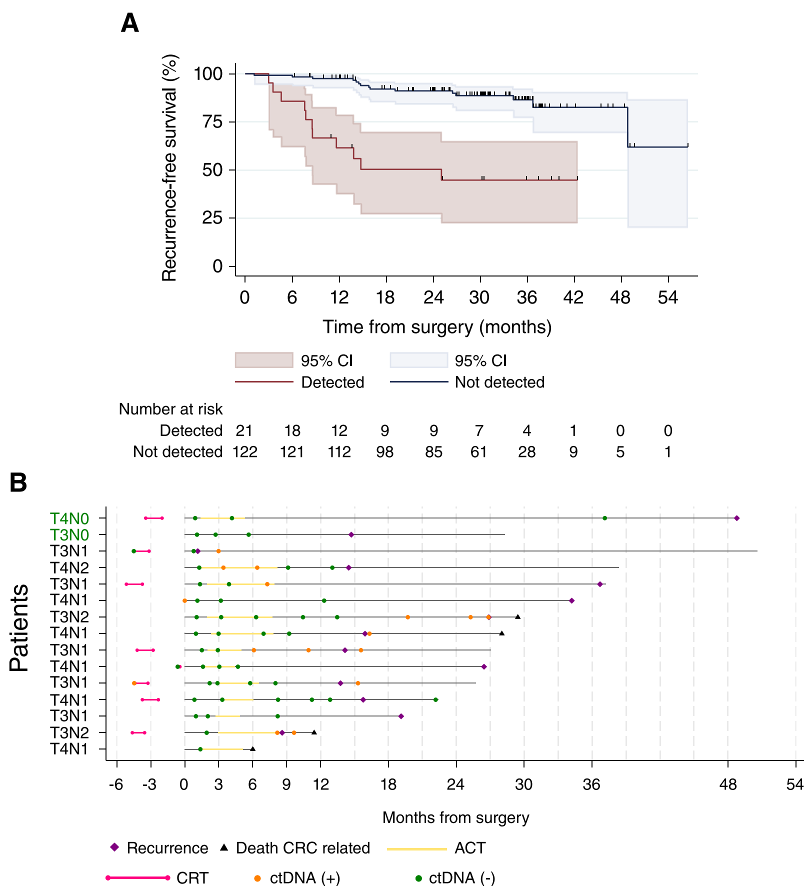

Figure 2.
A, RFS by postoperative ctDNA status ($n = 143$). **B,** Longitudinal ctDNA results for patients with ctDNA not detected postoperatively who experienced recurrence or died due to CRC in the primary analysis population ($n = 15$).

disease (Supplementary Fig. S1). In the primary analysis population, 12 patients with recurrence had a baseline sample result, of whom nine (75.0%) had ctDNA detected (one of three baseline ctDNA-negative patients with recurrence had detectable ctDNA postoperatively). In 68 ctDNA positive samples, 44 (64.7%) were detected by genomic and methylation calls, seven (10.3%) were

Table 2. Univariate and multivariate analysis in the primary analysis population.

	Analysis				
	Univariate			Multivariate (N = 121)	
Variable	***N***	**HR (95% CI)**	***P* value**	**HR (95% CI)**	***P* value**
Age at diagnosis	143	0.98 (0.95–1.01)	0.225	—	
Sex, male vs. female	143	1.17 (0.53–2.61)	0.691	—	
Primary site, rectal vs. colon	143	2.06 (0.95–4.46)	0.067	—	
T4 vs. T0–T3[a]	143	4.17 (1.88–9.25)	<0.0001	—	
N stage, N2 vs. N1–N0[a]	143	4.96 (2.12–11.59)	<0.0001	2.71 (0.87–8.56)	0.090
LVI, positive vs. negative[a]	125	3.43 (1.39–8.47)	0.007	—	
EMVI, positive vs. negative[a]	134	3.78 (1.58–9.05)	0.003	—	
PNI, positive vs. negative[a]	121	3.97 (1.52–10.29)	0.005	3.43 (1.26–9.30)	0.015
RAS status, WT vs. MT	103	1.27 (0.50–3.06)	0.706	—	
BRAF status, WT vs. MT	103	1.94 (0.45–8.40)	0.374	—	
MMR/MSI status (MMRd/MSI-H vs. MMRp/MSS)	131	0.22 (0.03–1.65)	0.142	—	
ACT, yes vs. no	143	2.88 (1.25–6.65)	0.013	—	
Postoperative ctDNA status, positive vs. negative	143	6.55 (2.95–14.51)	<0.0001	4.80 (1.64–14.04)	0.004

Abbreviations: ACT, adjuvant chemotherapy; EMVI, extramural venous invasion; HR, hazard ratio; LVI, lymphovascular invasion; MMR, mismatch repair; MMRd, mismatch repair deficient; MMRp, mismatch repair proficient; MSI, microsatellite instability; MSI-H, microsatellite instability-high; MSS, microsatellite stable; MT, mutant; PNI, perineural invasion; WT, wild-type.
[a]Pathologic.

detected by methylation only, and 17 (25%) were detected by genomic-only calls.

Landmark ctDNA analysis

In the landmark analysis population, 116 patients were evaluable; 78 underwent surgery alone and 38 received ACT. Two-year RFS (time from the end of definitive treatment to recurrence or death from CRC) was 50.0% (95% CI, 22.9–72.2) in patients with ctDNA detected at the landmark time point and 92.4% (95% CI, 84.7–96.3) in patients with ctDNA not detected [HR, 7.39 (2.74–19.9); $P < 0.0001$; **Fig. 3A**]. Sixteen (13.8%) patients experienced an RFS event, of whom seven (43.8%) had landmark ctDNA detected. Landmark ctDNA sensitivity and specificity was 43.8% (95% CI, 19.8–70.1) and 93.0% (95% CI, 86.1–97.1), respectively, with an NPV of 91.2% (95% CI, 83.9–95.9).

Nine of 102 (8.8%) patients with ctDNA not detected at the landmark time point experienced recurrence or died from CRC; five had follow-up samples available, of whom four remained negative throughout. Seven of 14 (50%) patients with ctDNA detected have not experienced recurrence or died, of whom four subsequently became negative.

ctDNA clearance

Twenty-four patients had paired ctDNA results pre- and post-ACT. Two patients had ctDNA detected after surgery and after ACT, one of whom experienced recurrence. Of the remaining 22 patients in whom ctDNA was not detected postoperatively, 21 remained negative following ACT; of these, five experienced recurrence. One patient who had stage III rectal adenocarcinoma responding poorly to neoadjuvant CRT, converted from negative to positive, and experienced recurrence ~12 months postsurgery.

Longitudinal ctDNA analysis

In the longitudinal analysis population ($n = 164$), 37 (22.6%) patients had ctDNA detected in at least one follow-up time point. Twenty-nine patients experienced recurrence; 18 (62.1%) had ctDNA detected (**Fig. 3B**). Of 525 samples from 135 patients who have not experienced recurrence or died, 40 samples from 19 patients (14.1%) had ctDNA detected, of whom 18 patients remained in follow-up at data cut-off (**Fig. 3C**). Patients with ctDNA detected who have not recurred were less likely to have risk factors for recurrence, including being negative for pathologic extramural venous invasion (EMVI; 55.6% vs. 27.8%), lymphovascular invasion (LVI; 72.2% vs. 33.3%) and PNI (72.2% vs. 38.9%) compared with patients with ctDNA detected who experienced recurrence (Supplementary Tables S2 and S3).

We plotted dynamic changes in genomic and methylation signals over time in 18 patients remaining in follow-up who had ctDNA detected but have not experienced recurrence. In 12 of 18 patients, ctDNA was persistently detected or detected in the most recent samples available. One patient with persistently positive samples was later diagnosed with lymphoma. Given the overlap in genomic and methylation signatures between CRC and other tumor types, these results are likely true positives correctly identifying malignancy, just not recurrent CRC. Three of the persistently positive cases had putative CHIP alterations (TP53 mutation detected in the absence of methylation detection). Eight patients may have had insufficient follow-up to observe recurrence. At least three of these had likely true positive results based on increasing genomic and epigenomic signals over time (Supplementary Fig. S2). In six of 18 cases, ctDNA detection was transient (i.e., followed by a ctDNA negative result without intervening treatment), sometimes involving samples collected shortly after surgery or ACT. Such cases are putative false positives; however, we cannot entirely rule out the possibility that some may be true positives with the associated phenomenon of immune surveillance clearing MRD, which could appear as "self-clearance" without intervening therapy and has been described in other CRC cohorts (12, 11). We also observed an association between high cfDNA input (>90 ng) and putative false-positive calls. High cfDNA yield was more common in samples collected during or within the first 3 weeks following ACT, within the first 3 weeks after surgery, and at the time of recurrence.

Eleven of 127 (8.7%) patients who did not have ctDNA detected in the longitudinal analysis population experienced recurrence. In this group, clinico-histopathologic features included a higher rate of positive pathologic EMVI [5/11 patients (45.5%)] compared with those who did not have ctDNA detected during follow-up and did not experience recurrence [28/108 patients (25.9%)]. There was also a higher proportion of LVI (45.5% vs. 18.5%) and PNI (27.3% vs. 9.3%; Supplementary Tables S2 and S3). Relapse sites and ctDNA status in the longitudinal analysis population are shown in **Fig. 3D**.

The sensitivity and specificity of follow-up ctDNA in the longitudinal analysis population were 62.1% (95% CI, 42.2–79.3) and 85.9% (95% CI, 78.9–91.3), respectively (Supplementary Fig. S3). Median lead time from ctDNA detection to recurrence in this population was 4.2 months (IQR, 2.7–7.2; $n = 10$). A summary of results for each population is given in Supplementary Table S4.

Genotypic and epigenetic considerations

Across samples in which ctDNA was detected from all available time points, 78/166 (47.0%) were detected by both genomic and epigenomic signals. A total of 39 (23.5%) and 49 (29.5%) samples had ctDNA detected by genomic-only and methylation-only calls, respectively. The breakdown of detection by calling method in patients with ctDNA detected according to the analysis population is given in Supplementary Fig. S4. The association of *BRAF* mutated and microsatellite instability-high (MSI-H)/mismatch repair deficient (MMRd) disease with ctDNA detection is described in Supplementary Material S1.

Discussion

This preliminary analysis of 997 blood samples from 214 patients recruited to TRACC Part B represents the largest cohort of patients with early-stage resected CRC analyzed by a tissue-free ctDNA assay combining genomic and epigenomic signals to our knowledge. Our data demonstrate ctDNA detection postoperatively by a tissue-free assay detects patients at high recurrence risk. After adjustment for confounders, postoperative ctDNA was the single most statistically significant predictor of recurrence in multivariate analysis. Two-year RFS was significantly better in patients in whom postoperative ctDNA was not detected compared with those who had ctDNA detected, and NPV was high at 88.5% postoperatively and 91.2% following definitive treatment. Both factors support prospectively investigating the use of postoperative ctDNA status to guide adjuvant treatment decisions with a tissue-free assay. The next generation of adjuvant clinical trials are investigating treatment de-escalation and escalation strategies based on postoperative ctDNA status; personalizing treatment decisions using the strongest prognostic biomarker in this disease.

Sensitivity and specificity rates, now validated in a larger cohort with long median duration follow-up of more than 30 months, were comparable to those seen in a previous study by Parikh and colleagues (20) using the same assay. Outcomes, including the use of postoperative ctDNA as a significant prognostic factor for recurrence, were similar to studies incorporating tumor-informed approaches, including Galaxy (CIRCULATE-Japan; ref. 12). The addition of longitudinal sampling improved sensitivity by 20.4% compared with a

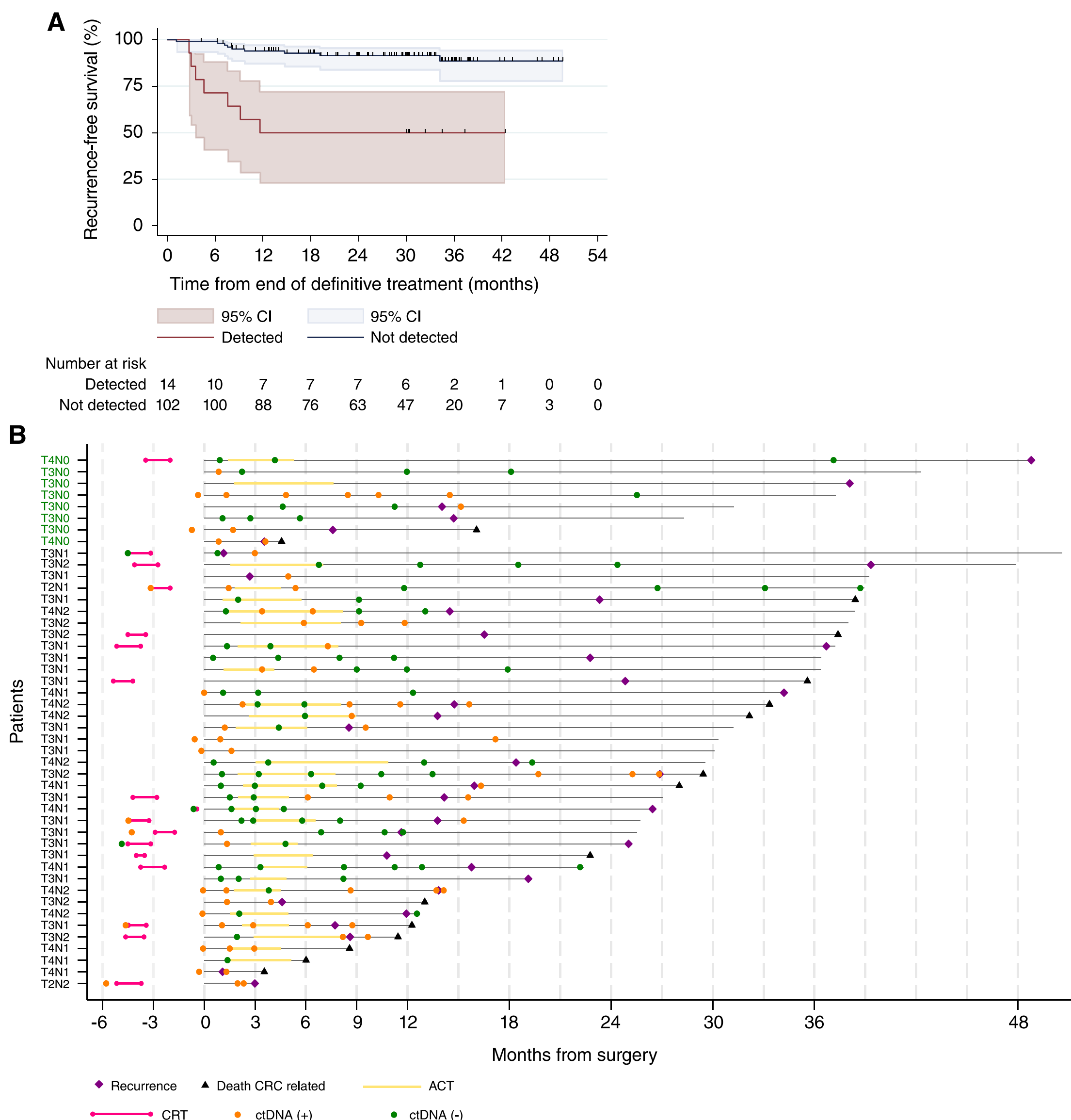

Figure 3.

A, RFS by landmark ctDNA status ($n = 116$). **B,** Longitudinal ctDNA results for patients with ctDNA detected at landmark time point, plus patients who experienced recurrence or died due to CRC regardless of landmark ctDNA status ($n = 46$). (*Continued on the following page.*)

single postoperative time point. While most ctDNA-positive samples were detected by combined signals, unique methylation aspects of the tissue-free assay allowed some to be detected by methylation-only calls, improving detection rates. Techniques to refine the bioinformatics algorithms for tumor-derived genomic and methylation detection are being developed, as are broader methylation panels (e.g., Guardant Infinity) to enhance sensitivity, specificity, and ctDNA quantitation further. Such techniques are important for early detection and MRD in which tumor volume is low and detection is challenging due to low ctDNA concentrations (21). Additionally, dynamic methylation changes can be detected over time, which offer evolutionary insights and potential implications for future management of metastatic disease (22). Longitudinal specificity, reported at the patient level, was lower compared with other analysis populations as it is impacted by one positive time point in a patient who did not experience recurrence, even though multiple negative results may follow. Follow-up for longitudinal samples is also shorter. The number of patients with *BRAF* mutant and MSI-H/MMRd status was too small in this cohort to conclude their role in prognostication when combined with postoperative ctDNA.

In the longitudinal analysis population, patients in whom ctDNA was persistently not detected were less likely to experience recurrence. In 11 (8.7%) patients who did, however, rates of negative postoperative EMVI and LVI were proportionately higher than those in whom ctDNA was detected. The most common recurrence sites included nodal, peritoneum, and local sites. Detection of ctDNA from some metastatic sites is challenging, with peritoneal and intrathoracic metastases shedding lower levels of ctDNA compared with the liver (23, 24), possibly

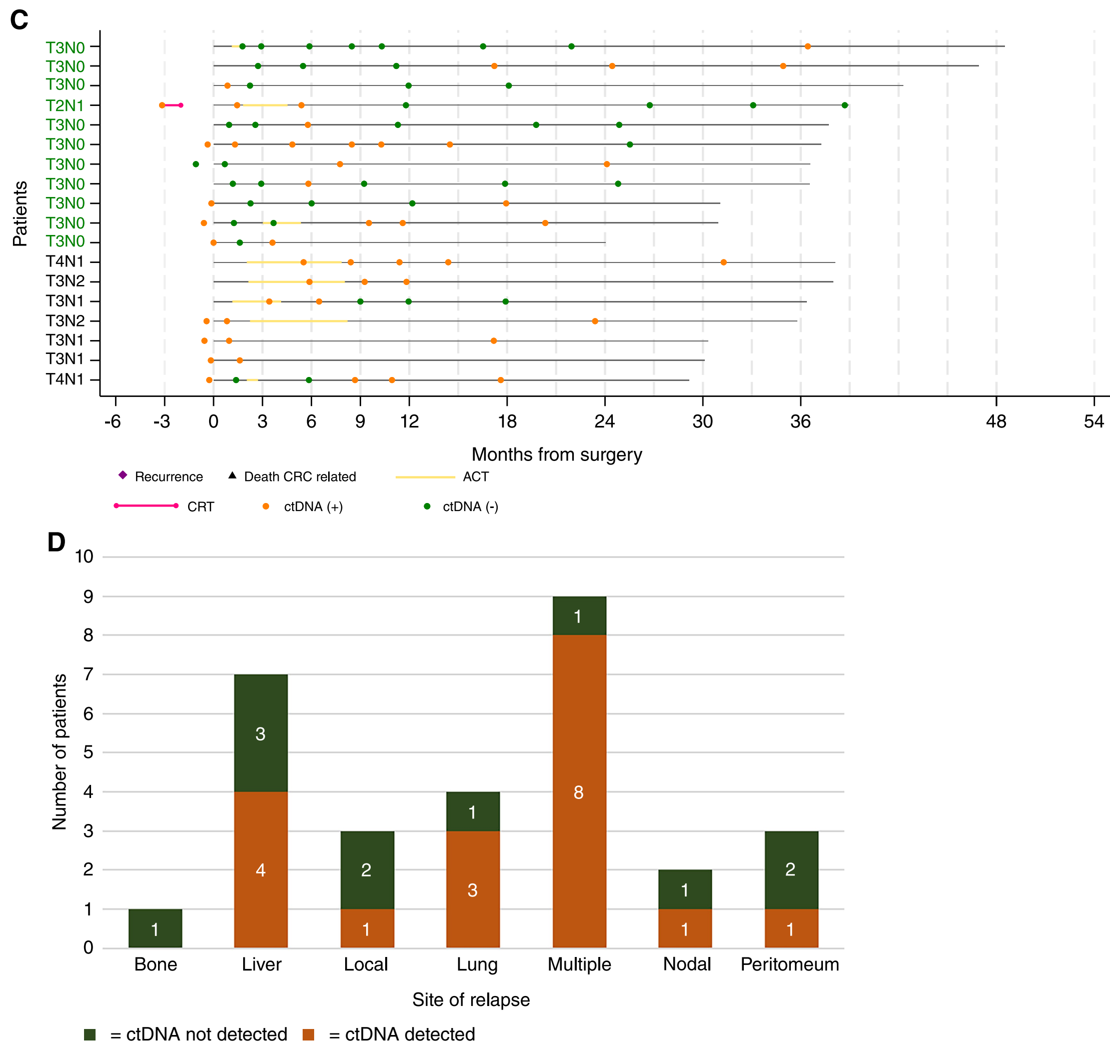

Figure 3.
(*Continued.*) **C,** Longitudinal ctDNA results for patients with ctDNA detected in longitudinal time points who did not experience recurrence or die from CRC and remained in follow-up in the longitudinal analysis population ($n = 18$). **D,** Relapse sites and ctDNA status in patients with recurrence in the longitudinal analysis population ($n = 29$).

explaining why some patients seemed to have false-negative results. In a recent meta-analysis, patients with oligometastatic disease undergoing locoregional treatment who had ctDNA detected before treatment had a shorter RFS compared with those who did not have ctDNA detected (25). Therefore, a "false-negative" result in patients with recurrence (which mimics the oligometastatic setting in many cases) may confer better long-term outcomes. Further research is required to confirm this holds true in patients experiencing recurrence. Preanalytic factors may also explain persistent negative results, for example, infrequent sampling causing missed opportunities for detection.

Disease recurrence occurs more commonly within the first 2 years after surgery (26). A total of 18/126 (14.3%) patients with ctDNA detected during follow-up in the longitudinal analysis population did not experience recurrence. As shown, these may be explained by true positives in patients with insufficient follow-up, timing of blood draw in relation to treatment milestones, second primary or metachronous tumors, and bioinformatic factors leading to incorrect classification of putative CHIP alterations as tumor derived. Following this study, an update to the assay's bioinformatics algorithm was developed using additional samples not evaluated as part of TRACC Part B. This algorithm recalibrated the calling threshold for high cfDNA yield samples and set more stringent criteria for calling samples with genomic-only signals as positive among other refinements. The updated algorithm, when applied retrospectively to this cohort, improved specificity without a notable impact on sensitivity. Hence, there are multiple potential mechanisms for discordance between ctDNA results and clinical outcomes; analysis of larger observational cohorts will help to further determine the influence of biological versus assay-driven factors.

Our UK-wide study was prospectively designed with robust laboratory specifications for sample collection at set time points. The selection of patients in this cohort was not random due to sample availability, calling into question the extent to which it fully represents patients recruited to TRACC Part B and the overall CRC population. Two-year RFS for

landmark ctDNA detected patients in this cohort, compared with that of Parikh and colleagues (20) using the same assay, was 50% versus 0% respectively, which may be explained by the lower-risk population in this cohort. Positive predictive value in any assay with <100% specificity varies based on the a priori recurrence risk. Nakamura and colleagues (27) reported a 2-year landmark (post-ACT) RFS of 14.3% for patients with detected ctDNA, compared to 91.3% for patients without detected ctDNA, using an updated version of the Reveal assay that leverages a larger 15-Mb epigenomic panel with specificity >97%. In line with other observational studies, limitations included missing and failed samples, most commonly due to patients not attending for blood sampling during the COVID-19 pandemic and low cfDNA yield (<15 ng), respectively, causing sample attrition. This emphasizes the importance of optimization of confounders, for example, maximizing plasma volume by completely filling Streck tubes to ensure accurate results. Analysis of larger cohorts will further improve the understanding of assay performance and clinical utility. TRACC Part B has now recruited more than 1,600 patients at the time of publication providing additional samples for this purpose.

In conclusion, our analysis of MRD using a tissue-free approach to detect ctDNA in a large UK prospective cohort of patients with resected early-stage CRC demonstrated improved 2-year RFS in patients with ctDNA not detected at postoperative and landmark time points. Postoperative ctDNA status was the strongest statistically significant predictor of recurrence. We were able to uniquely investigate the impact of combining methylation with genomic signals to improve ctDNA detection. These factors support the use of ctDNA for personalization of treatment in the adjuvant setting. Postoperative NPV was high supporting the use of ctDNA in de-escalation of ACT strategies in patients in whom ctDNA is not detected, currently being investigated in the UK TRACC Part C randomized study (28). The pragmatic tissue-free approach offers a fast turnaround of results, essential for adjuvant studies, and facilitates potential adoption into future healthcare. Questions remain about the characteristics of the small proportion of patients who experience recurrence despite undetectable ctDNA throughout follow-up, as well as those in whom ctDNA is detected but who remain disease-free. Reasons for this include clinicohistopathologic, biological (including immune surveillance and evolutionary considerations), pre-analytic, and bio-informatic factors. Detailed analysis of these domains is ongoing in the remaining several thousand TRACC Part B samples. The UK TRACC Part C randomized de-escalation study has now adopted the tissue-free Guardant Reveal assay on the larger Guardant Infinity platform to reflect the advancing technology with higher specificity thresholding and broader methylation and genomic configuration that maintains high sensitivity.

Authors' Disclosures

F.M. Marti reports personal fees from Servier and nonfinancial support from Takeda outside the submitted work. L. Bucheit is an employee of Guardant Health. T. Rich is an employee and stockholder of Guardant Health. I. Chau reports personal fees from Eli-Lilly, Bristol Myers Squibb, MSD, Roche, Merck Serono, AstraZeneca, Oncxerna, Boehringer Ingelheim, Incyte, Astella, GSK, Sotio, Eisai, Daiichi-Sankyo, Taiho, Servier, Seagen, Turning Point Therapeutics, Novartis, Takeda, and Elevation Oncology and grants from Janssen-Cilag and Eli Lilly outside the submitted work. D. Cunningham reports grants from Clovis, Eli Lilly, 4SC, Bayer, Celgene, and Roche outside the submitted work and is on the scientific advisory board for OVIBIO. N. Starling reports personal fees from Merck, Novartis, MSD Oncology, Eli Lilly, Pierre Fabre, Amgen, Eli Lilly Bangladesh, GSK, Seagen, BMS, AstraZeneca, and Servier, as well as other support from Merck, AstraZeneca, BMS, Pfizer, Guardant Health, Pfizer, Servier, AstraZeneca, MSD Oncology, Novartis, Guardant Health, Gilead, Seagen, and Janssen outside the submitted work. No disclosures were reported by the other authors.

Authors' Contributions

S. Slater: Data curation, formal analysis, investigation, visualization, methodology, writing–original draft, writing–review and editing, study design. **A. Bryant:** Data curation, funding acquisition, project administration. **M. Aresu:** Formal analysis, statistical analysis plan, editing the draft. **R. Begum:** Data curation, project administration, biospecimen sample coordination. **H.-C. Chen:** Data curation, project administration. **C. Peckitt:** Formal analysis, statistical analysis plan. **R. Lazaro-Alcausi:** Data curation, patient recruitment. **P. Carter:** Funding acquisition. **G. Anandappa:** Data curation, study design, patient recruitment. **S. Khakoo:** Conceptualization, data curation, study design, patient recruitment. **L. Melcher:** Data curation, patient recruitment. **V. Potter:** Data curation, Patient recruitment. **F.M. Marti:** Data curation, patient recruitment. **J. Huang:** Data curation, patient recruitment. **G. Branagan:** Data curation, patient recruitment. **N. George:** Data curation, patient recruitment. **M. Abulafi:** Data curation, patient recruitment. **S. Duff:** Data curation, patient recruitment. **A. Raja:** Data curation, patient recruitment. **A. Gupta:** Data curation, patient recruitment. **N. West:** Data curation, patient recruitment. **L. Bucheit:** Resources, formal analysis, investigation, writing–review and editing. **T. Rich:** Resources, formal analysis, investigation, writing–review and editing. **I. Chau:** Conceptualization, resources, supervision, funding acquisition, visualization, writing–review and editing, study design. **D. Cunningham:** Conceptualization, resources, supervision, funding acquisition, visualization, writing–review and editing, study design. **N. Starling:** Conceptualization, resources, supervision, funding acquisition, visualization, writing–review and editing, study design.

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Note

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