Prognostic Significance of Post-Surgery Circulating Tumor DNA in Non-Metastatic Colorectal Cancer: Individual Patient Pooled Analysis of Three Cohort Studies

Short Title: Post-surgery ctDNA in non-metastatic colorectal cancer

Authors: *Jeanne Tie[1,2,3,4], *Joshua D. Cohen[5], Serigne N. Lo[6,7,8], Yuxuan Wang[5], Lu Li[9], Michael Christie[1,4,10], Margaret Lee[1,2,11,12], Rachel Wong[1,11,12], Suzanne Kosmider[2], Iain Skinner[2], Hui Li Wong[1,3], Belinda Lee[1,3], Matthew E. Burge[13], Desmond Yip[14], Christos S. Karapetis[15], Timothy J. Price[16], Niall C. Tebbutt[17], Andrew M. Haydon[18], Janine Ptak[5], Mary J. Schaeffer[5], Natalie Silliman[5], Lisa Dobbyn[5], Maria Popoli[5], Cristian Tomasetti[7,19], Nickolas Papadopoulos[5], Kenneth W. Kinzler[5], *Bert Vogelstein[5], *Peter Gibbs[1,2,4]

*JT, JC, BV and PG contributed equally to this work

Affiliations:

1. Division of Personalised Oncology, The Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia
2. Department of Medical Oncology, Western Health, Melbourne, Australia
3. Department of Medical Oncology, Peter MacCallum Cancer Centre, Melbourne, Australia
4. Faculty of Medicine, Dentistry and Health Sciences, University of Melbourne, Australia
5. Ludwig Center and Howard Hughes Medical Institute, Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins Medicine, Baltimore, USA
6. Melanoma Institute Australia, The University of Sydney, North Sydney, Australia
7. Faculty of Medicine and Health, The University of Sydney, Sydney, Australia
8. Institute for Research and Medical Consultations (IRMC), Imam Abdulrahman Bin Faisal University, Dammam, Kingdom of Saudi Arabia
9. Division of Biostatistics & Bioinformatics, Department of Oncology, Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins Medicine, Baltimore, USA
10. Department of Pathology, Royal Melbourne Hospital, Melbourne, Australia
11. Department of Medical Oncology, Eastern Health, Melbourne, Australia
12. Eastern Health Clinical School, Faculty of Medicine, Nursing and Health Sciences, Monash University, Melbourne, Australia
13. Department of Medical Oncology, Royal Brisbane and Women's Hospital, Brisbane, Australia
14. Department of Medical Oncology, Australian National University, Canberra, Australia
15. Department of Medical Oncology, Flinders Medical Centre, Flinders University, Adelaide, Australia

16. Department of Medical Oncology, Queen Elizabeth Hospital, University of Adelaide, Adelaide, Australia
17. Department of Medical Oncology, Olivia Newton-John Cancer and Wellness Centre, Victoria, Australia
18. Department of Medical Oncology, The Alfred Hospital, Melbourne, Australia
19. Department of Biostatistics, Johns Hopkins Bloomberg School of Public Health, Baltimore, USA

Corresponding author: Jeanne Tie, The Walter and Eliza Hall Institute of Medical Research, Australia; e-mail: tie.j@wehi.edu.au; @JeanneTie

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Abbreviations:
cfDNA - cell-free DNA
CI - confidence interval
CRC - colorectal cancer
CRCSS - colorectal cancer-specific survival
ctDNA - circulating tumor DNA
HR - hazard ratio
iAUC - integrated area under the curve
LARC - locally advanced rectal cancer
MAF - mutant allele frequency
MRD - minimal residual disease
N stage - nodal stage
OR - odds ratio
OS - overall survival
RFS - recurrence-free survival
T stage - tumor stage

ABSTRACT

Studies in multiple solid tumor types have demonstrated the prognostic significance of ctDNA analysis after curative intent surgery. A combined analysis of data across completed studies could further our understanding of circulating tumor DNA (ctDNA) as a prognostic marker and inform future trial design. We combined individual patient data from three independent cohort studies of non-metastatic colorectal cancer (CRC). Plasma samples were collected 4-10 weeks after surgery. Mutations in ctDNA were assayed using massively-parallel-sequencing with a technique called SafeSeqS. We analyzed 485 CRC patients (230 stage II colon, 96 stage III colon, 159 locally advanced rectal). ctDNA was detected after surgery in 59 (12%) patients overall (11.0%, 12.5% and 13.8% for samples taken at 4-6, 6-8 and 8-10 weeks; P=0.740). ctDNA detection was associated with poorer 5-year recurrence-free (38.6% vs 85.5%; P<0.001) and overall survival (64.6% vs 89.4%; P<0.001). The predictive accuracy of post-surgery ctDNA for recurrence was higher than that of individual clinico-pathological risk features. Recurrence risk increased exponentially with increasing ctDNA mutant allele fraction (MAF) (hazard ratio, 1.2, 2.5 and 5.8 for MAF of 0.1, 0.5 and 1%). Post-surgery ctDNA was detected in 3 of 20 (15%) patients with loco-regional and 27 of 60 (45%) with distant recurrence (P=0.018). This analysis demonstrates a consistent long-term impact of ctDNA as a prognostic marker across non-metastatic CRC, where ctDNA outperforms other clinico-pathological risk factors and MAF further stratifies recurrence risk. ctDNA is a better predictor of distant versus loco-regional recurrence.

INTRODUCTION

The gold standard for determining prognosis following curative intent surgery for solid tumors remains a careful examination of the resected primary specimen. Unfortunately, this pathology-based staging system remains somewhat imprecise. Many “high risk” patients are not destined to recur because minimal residual disease (MRD) is not present, whereas some “low risk” patients will recur due to unexpected MRD leading to clinical progression. Direct examination for evidence of residual disease post-surgery, rather than estimating the likelihood of such based on the pathology assessment, promises to be a more precise way to inform adjuvant therapy decision-making.

Tumor-specific DNA mutations were initially detected in the cell-free component of the peripheral blood (circulating tumor DNA - ctDNA) of patients with metastatic disease, with potential clinical applications including non-invasive real-time molecular characterization of tumors [1-3] and real-time assessment of tumor bulk.[4-6] The possibility that ctDNA could be a useful prognostic marker of MRD was suggested in an initial series of 18 patients undergoing resection of colorectal cancer (CRC) liver metastases.[7] This promise has been further demonstrated in non-metastatic CRC [8-11] and many other solid tumor types.[12-16] Prospective trials have now been initiated to explore how ctDNA status could optimally inform adjuvant therapy decision making and ultimately improve patient outcomes.[17]

Future prospective studies might be informed by consolidating lessons learnt from completed non-randomised studies. Important questions include those about the optimal

timing of blood collection after surgery, whether quantitative measurement of ctDNA mutation burden can provide further risk stratification when ctDNA is detectable, the predictive performance of ctDNA compared to known clinic-pathologic risk factors, the impact of ctDNA mutation burden on the benefit derived from adjuvant chemotherapy, the correlation between ctDNA detection and sites of recurrence, and the prognostic impact fof ctDNA on long term outcomes, including overall survival. Here we report a combined analysis of three completed cohort studies of patients with non-metastatic CRC.

PATIENTS AND METHODS

Patients and Data Collection

We combined individual patient data from three independent multi-centre cohort studies of stage II colon cancer, stage III colon cancer, and locally advanced rectal cancer. The primary objective of each study was to demonstrate that ctDNA analysis at 4 to 10 weeks after curative intent surgery could be used as an MRD indicator and predict recurrence. The use of adjuvant chemotherapy in all three studies was at clinician discretion, blinded to the ctDNA results. Full study details are provided in the primary publications.[8-10] There was sufficient homogeneity of the populations, ctDNA analysis method and clinical follow-up to enable pooling of the results. The homogeneity test using Higgins' I^2 method by pooling of the clinical outcome results was 0.01% confirming the homogeneity between studies. In brief, patients with histologically confirmed, operable, stage II–III CRC without evidence of metastatic disease were enrolled, unless there was another malignancy diagnosed within

the last three years. Blood samples for ctDNA and CEA analysis were collected four to ten weeks post-surgery, prior to commencing any adjuvant chemotherapy. At each collection time-point, at least 30 mL of blood was drawn into EDTA tubes, centrifuged twice at 1200g and 1800g, and plasma aliquoted into 10 mL tubes for storage at -80^{0}C. Serum CEA was measured by the local diagnostic laboratory at participating site, with CEA concentrations of <5 µg/L considered normal. Recurrence and survival results presented here differ from the initial publications (8-10) due to updated follow-up information.

Circulating Tumor DNA (ctDNA) Analysis

The detection and quantitation of ctDNA were performed using the Safe-Sequencing (Safe-SeqS) assay, an error-reduction technology for the detection of low frequency mutations, which has been described in detail previously.[8, 18] Briefly, formalin-fixed paraffin-embedded tumor tissue from the biopsy or surgical specimen was initially analysed for somatic mutations in 15 genes recurrently mutated in CRC (*SMAD4, TP53, AKT1, APC, BRAF, CTNNB1, ERBB3, FBXW7, HRAS, KRAS, NRAS, PIK3CA, PPP2R1A, RNF43, POLE*). For each patient, the mutation identified in the tumor tissue with the highest mutant allele frequency (MAF) was assessed in cell-free DNA (cfDNA) from the plasma. This analysis revealed whether a detectable amount of ctDNA was present, and if so, to quantify it. Leukocyte DNA was used to exclude variants arising from clonal hematopoiesis as well as constitutional polymorphisms.

Statistical Analysis

All analysis was based on a one-step individual patient data meta-analysis approach by pooling individual patient data from the three studies. Baseline characteristics were summarised using descriptive statistics. Associations between the detection of ctDNA and baseline clinico-pathologic characteristics were tested using univariable logistic regression with random study effect to account for the clustering among patients from the same cohort. We performed pooled analysis separately for three time-to-event endpoints: overall survival (OS), which included death from any cause as an event; CRC–specific survival (CRCSS), which included only death due to recurrent CRC; recurrence-free survival (RFS), which included locoregional and distant recurrence, where deaths without recurrence were censored at the time of death. Loco-regional recurrence included anastomotic, regional nodal, peritoneal and omental relapses. All time-to-event outcomes were summarised graphically over the entire follow-up period using Kaplan–Meier methods along with survival rates at 3- and 5-years. Survival differences by ctDNA status were tested with a log-rank test stratified with cohort as a factor. The impact of ctDNA status in each survival outcome was assessed using both univariable and multivariable Cox proportional hazard models stratified with each cohort factor. Factors that showed a strong or moderate association with outcomes assessed as p-value <0.20 from the univariable analysis were considered potential confounders and included in the multivariable models.

The performance of ctDNA as a marker of RFS outcome was also investigated using Harrell's C-statistics. ctDNA predictive accuracy alone was compared individually to each potential clinical predictor using the integrated area under the receive operating

characteristic curve (iAUC) with 1000× bootstrap resampling. In addition, the incremental value of ctDNA status over all other prognostic factors was assessed by comparing the accuracy of two multivariable models with and without ctDNA status. Difference in performance of risk prediction between models was tested using the likelihood ratio p-value. The pattern of association between ctDNA MAF as a continuous variable and RFS were determined by plotting the log hazard ratio (HR) of ctDNA MAF taking no ctDNA detection as reference. Non-parametric log HR curve and its associated 95% confidence interval (CI) were derived using the smoothHR R package. All the statistical analyses were carried out in SAS 9.4 (SAS Institute Inc., Cary, NC, USA) and R version 3.5.0 (R Foundation for Statistical Computing, Vienna, Austria).

RESULTS

ctDNA Detection, Timing of Blood Collection and Association with Clinical Variables

Table 1 provides a summary of the original studies. A total of 485 patients with stage II–III CRC and known post-surgery ctDNA status were included in our pooled analysis (Table 2). Median patient age was 63 years, 59% were male, 31% had a right-sided primary tumor and 29% had pathologic node-positive disease. ctDNA was detected in plasma samples taken 4 to 10 weeks after surgery in 59/485 (12%) patients. At 4-6, 6-8 and 8-10 weeks after surgery, the ctDNA detection rate was 11.0%, 12.5% and 13.8% respectively (P = 0.740). Post-op ctDNA detection was associated with N stage and lymphovascular invasion but not tumor location, T stage or nodal yield (Table 2).

Survival, Disease Recurrence and Site of Recurrence

Median follow-up time was 47 months (range, 4 to 84 months) in the pooled data. Of 47 deceased patients, 36 (76.6%) had died from CRC relapse and 11 (23.4%) without known disease recurrence. Both OS and CRCSS rates were significantly lower in patients with detectable versus undetectable post-surgery ctDNA (OS: unadjusted HR, 3.77; 95% CI, 1.96-7.25; Figure 1A; CRCSS: HR, 4.84; 95% CI, 2.38-9.85; Figure 1B). The 5-year OS for patients with or without detectable post-surgery ctDNA was 64.9% and 89.4%, respectively. During follow-up, 90 (18.6%) patients experienced CRC recurrence, 93% occurring within three years of surgery. RFS was significantly inferior for patients with positive post-surgery ctDNA (5-year RFS: 38.6% vs 85.5%; HR, 7.56; 95% CI, 4.85-11.79; Figure 1C). ctDNA detection remained a significant prognostic factor with respect to all three endpoints after adjusting for potential confounders (Table 3).

Of the 90 CRC recurrences, 60 (67%) were distant recurrences only, 20 (22%) were locoregional recurrences only and 10 (11%) both. ctDNA was detected post-surgery in 3 of 20 (15%) patients with locoregional recurrence only, 27 of 60 (45%) with distant recurrence only and 5 of 10 (50%) with both (locoregional vs distant recurrence only: P = 0.018; Figure 1D).

Predictive Accuracy and Subgroup Analysis for RFS

The predictive accuracy of post-surgery ctDNA for RFS as assessed by iAUC was higher than that of individual clinico-pathological risk features such as tumor differentiation, T

stage, N stage, lymphovascular invasion and post-surgery CEA (ctDNA iAUC = 0.67; 95% CI, 0.62-0.72; Figure 2A). However, the addition of ctDNA status to all clinical variables significantly improved the accuracy of recurrence prediction (iAUC = 0.76; 95% CI, 0.70-0.83). The univariate HRs for RFS according to the detection of post-surgery ctDNA in various subgroups are shown in Figure 2B. ctDNA detection was associated with poor RFS across all subgroups including N-stage and for patients treated (HR, 4.63; 95% CI, 2.56-8.36) or not treated with adjuvant chemotherapy (HR, 15.2; 95% CI, 7.72-29.7).

ctDNA Mutant Allele Fraction (MAF) and RFS

For the 59 cases with detectable ctDNA, the median MAF was 0.046% (interquartile range, 0.010%-0.191%). Log HRs from the adjusted RFS analyses according to ctDNA MAF as a continuous variable are shown in Figure 3A. The prognostic impact of ctDNA was significant for all ctDNA MAF values. The risk of recurrence increases exponentially as ctDNA MAF becomes higher as compared to patients without detectable ctDNA (hazard ratio, 1.2, 2.5 and 5.8 for MAF of 0.1, 0.5 and 1%). For patients not treated with adjuvant chemotherapy, 3-year RFS was 9% in patients with a MAF > 0.046% compared to 33% for those with a MAF ≤ 0.046% (HR, 6.67; 95% CI, 1.77-25.11; Figure 3B). For chemotherapy treated patients, 3-year RFS was 25% in patients with MAF > 0.046% compared to 70% for those with a MAF ≤ 0.046 (HR, 2.63; 95% CI, 0.98-7.09; Figure 3C). For pathological node-negative patients with undetectable ctDNA, there was no significant difference in RFS between those treated or not treated with adjuvant chemotherapy (HR, 1.07; 95% CI, 0.49-2.33; Figure 3D).

DISCUSSION

Completed studies have repeatedly demonstrated the adverse prognostic significance of detectable ctDNA following curative intent surgery for solid tumors. For CRC this is most striking in patients who do not receive adjuvant chemotherapy, including HRs of 28 (stage II colon) and 22 (rectal cancer).[8, 9] Where adjuvant chemotherapy is administered, as in our stage III cohort, the HRs are more modest given the confounding effect of chemotherapy.[10] When ctDNA remains detectable after completion of adjuvant chemotherapy this suggests failed treatment and associates with a substantially elevated recurrence risk. Here we report a pooled analysis of individual patient data from three non-metastatic CRC cohort studies, focusing on novel endpoints such as the significance of blood sample timing, plasma MAF, and any associations with sites of recurrence.

In the current analysis we found that the percentage of patients with detectable ctDNA increased in proportion to increasing pathology stage, lower for stage II (8.7%) compared to stage III CRC (21%). Notably, subgroup analysis demonstrates that detectable ctDNA in patients with a low risk pathology, such as low risk stage II colon cancer or a rectal patient with a ypN0 disease, is associated with a far greater recurrence risk (57%) than the highest risk pathology with no detectable ctDNA, e.g. ypN+ rectal cancer post chemoradiation or stage III colon cancer with N2 disease (20%). Additionally, the iAUC data comparing the predictive accuracy of ctDNA status and other clinical parameters demonstrates that post-

surgery ctDNA is the single strongest predictor for recurrence. Combining ctDNA status with traditional prognostic markers provides incremental predictive accuracy.

With increasing time from surgery, it is expected that progression of any MRD would lead to a greater proportion of patients having detectable ctDNA, where those with an initial very low "tumor bulk" and minimal release of ctDNA would now have sufficient amount of ctDNA above the detection threshold. Consistent with this, it has been shown that patients with an initially negative ctDNA who later develop distant recurrence, will typically have ctDNA detectable several months before clinically detected disease.[8, 11, 19] In our analysis we found a numerically lower ctDNA detection rate at 4-6 weeks (11%) versus 8-10 weeks (13.8%) but this difference was not statistically significant. We are further exploring the impact of time from surgery and the potential value of analysis at multiple time points (4- and 7-week samples) in our DYNAMIC-II and -RECTAL studies (ACTRN12615000381583; ACTRN12617001560381). Other studies have demonstrated a trauma-induced increase in total cell-free DNA in the first few weeks after surgery[7, 20], diluting any ctDNA and likely making it more difficult to detect.

One reasonable approach would be an initial blood sample at 4 weeks after surgery as a compromise between avoiding the immediate post-operative period and maximizing the time for ctDNA analysis (anticipating a turn-around time of 2 weeks for plasma analysis) and reporting. An early positive test would be actionable and permit the prompt initiation of adjuvant chemotherapy, with data suggesting that the timeliness of adjuvant therapy is

important.[21] Given the potential small increase in detection rate with a blood draw beyond 4 weeks, further sampling in those with an initially negative test who are not planned for adjuvant chemotherapy based on pathology criteria (e.g. low risk stage II CRC) could be considered. Further studies will need to define the impact of chemotherapy commenced beyond the standard 8-week window if patients were to have no detectable ctDNA initially but later return a positive test. This potentially includes patients in routine follow-up months or a year or two after surgery where ctDNA is detectable but no recurrence is evident on imaging. Delayed "adjuvant" chemotherapy may in theory eradicate disease in patients who develop detectable ctDNA during follow-up but without evidence of recurrence on imaging and should be further explored.

While the level of ctDNA can be quantified as MAF, studies to date of non-metastatic CRC have only categorized ctDNA results as either positive or negative. In our analysis we found an exponential increase in recurrence risk with rising ctDNA MAF. The hazard ratio for MAF of 0.1% compared to no detectable ctDNA is 1.2, for 0.5% is 2.5, and for 1.0% is 5.8. In addition to the implications for recurrence risk, the MAF may also correlate with the likelihood of adjuvant chemotherapy eradicating MRD and preventing cancer recurrence. In our series, patients with a ctDNA above the median MAF of 0.046% experienced a 3-year RFS of 9% without chemotherapy and 25% with chemotherapy, whereas for those with MAF < 0.046% the 3-year RFS was 33% without chemotherapy and 70% with chemotherapy. Both groups appear to benefit from adjuvant treatment although the magnitude of benefit is smaller in patients with a higher MAF (absolute risk reduction: 16%

vs 37%). Despite adjuvant therapy, the patients with a MAF above 0.046% have a persistently high risk of recurrence, suggesting they are appropriate for the most aggressive available treatment strategy or enrolment in clinical trials. However, these are preliminary findings from a mixed patient population treated with a variety of chemotherapy regimens. Further analysis by adjuvant therapy delivered and by stage is required before any firm conclusions can be made.

One interesting question raised by these data is why some patients with detectable ctDNA do not recur (in the absence of adjuvant therapy)? ctDNA is not a conventional prognostic marker such as CEA, in which measurable levels are found in normal individuals. The presence of ctDNA, in theory, is a binary metric indicating that a large number of residual metastatic cancer cells exist in the patient's body.[22] There are at least two potential explanations for this unexpected result. First, it is possible that a subset of ctDNA measurements are false positive. Though the technique used (SafeSeqS) minimizes errors in the analytic process, each positive SafeSeqS score is associated with some probability of it being a false positive. The lower the MAF, the greater this probability. Second, it is possible that there are indeed residual cancer cells, but that these small metastatic deposits are eliminated by the immune system. It is now widely accepted that immune checkpoint inhibitors based on enhancement of the natural immune response to tumors can limit cancer growth. Notably, both hypotheses are consistent with the key fact demonstrated herein that higher MAFs are associated with a worse prognosis. Future studies with improved error-reduction techniques may allow distinction between the two possibilities as

well as provide better prognostic metrics for patients with very low levels of residual disease.

We also observed that post surgery ctDNA detection is more commonly associated with disease recurrence at distant sites than locoregional relapses (e.g. peritoneal relapse). This suggests that the amount of tumor DNA released into the circulation may vary by anatomical site. Consistent with this is the reported low ctDNA detection rate in patients with peritoneal metastases in several previous studies of metastatic CRC.[23-25] However, ctDNA analysis remains superior to standard pathology staging as a marker of locoregional disease recurrence.

Mutational heterogeneity within the primary tumor and among metastatic lesions has been extensively documented at the sequence level. This could impact the evaluation of ctDNA, as we only sampled one region of each cancer evaluated. We mitigated this potential problem by focusing on driver gene mutations that are present in clonal fashion in the primary tumor, i.e. truncal mutations that appear to be present in virtually every neoplastic cell within the sampled region. It has been rigorously shown that such truncal driver gene mutations, unlike passenger gene mutations, are nearly always present in all metastatic lesions as well as throughout the primary tumor.[26-29] Additionally, microsatellite instability (MSI) or mismatch repair (MMR) status does not impact this approach to ctDNA analysis - evaluation of clonal driver gene mutations in the plasma of patients with known driver gene mutations in their primary tumors. The number of clonal driver gene mutations is similar in

cancers with and without MSI, though the number of passenger mutations is much larger in MSI cancers than in microsatellite stable cancers. As the major purpose of ctDNA evaluation in our study was to detect the presence of occult metastatic lesions, this strategy was well-suited for our objective. It would not be well-suited for ctDNA evaluation in other clinical scenarios, such as detecting mutations leading to resistance after chemotherapy was initiated.

Whilst adjuvant chemotherapy has consistently been shown to improve survival for unselected patients with stage III colon cancer, the role of adjuvant chemotherapy continues to be debated for patients with stage II colon cancer or following neoadjuvant therapy and surgery for locally advanced rectal cancer. Our preliminary data suggests that being able to define the very high-risk patients (detectable ctDNA) with stage II colon cancer or with locally advanced rectal cancer may define a subset where a significant benefit from adjuvant therapy can be more easily demonstrated. This hypothesis is being tested in randomised studies that have been initiated in Australasia (DYNAMIC and DYNAMIC-RECTAL) and several independent groups in the US and Europe (COBRA: NCT04068103; CIRCULATE: NCT04089631; PRODIGE-70 CIRCULATE: NCT04120701; IMPROVE-IT: NCT03748680). These protocols typically, relative to standard of care, prescribe escalating treatment intensity (more agents or longer duration of therapy) for patients with detectable ctDNA and de-escalating treatment (less agents or shorter duration of therapy) intensity for patients where ctDNA is not detectable. The major justification for reducing the intensity of treatment in patients with undetectable ctDNA is the markedly

reduced recurrence risk in these patients. Our data suggest that patients with node-negative CRC and undetectable ctDNA appear not to benefit from adjuvant chemotherapy (Figure 3D).

Limitations to our study include combining data across studies and disease stages, including locally advanced rectal cancer treated with neoadjuvant chemo-radiation, as well as the lack of representation from stage I disease. For consistency, we have chosen to use pathological stage (yp stage) for the rectal cancer cohort. The relatively low event rates (recurrence and death) limits the power of any subgroup analysis. Only one mutation was analyzed in the peripheral circulation, whereas analysis of multiple variants will likely improve assay sensitivity. Broadly speaking, there are two approaches to ctDNA analysis for detection of MRD after curative resection in early stage cancer, tumor-informed versus tumor-agnostic approaches. In the tumor-informed approach, somatic mutations are first identified in an individual patient's tumor tissue followed by targeted sequencing of plasma DNA using a personalized assay. Several tumor-informed personalized ctDNA assays have been developed (e.g. SafeSeqS, CAPP-Seq, TAm-Seq, TARDIS, Signatera, ArcherDX PCM) with limits of detection below 0.1% VAF.[11, 13, 18, 30-32] For the tumor-agnostic approach, ctDNA analysis is performed without prior knowledge of a patient's tumor mutation profile and often include broad panel-based sequencing or methylation assay (e.g. Guardant Health's LUNAR assay). Though more resource-intense, the tumor-informed approach offers the highest analytical sensitivity and is particularly well-suited for MRD detection. In our studies, we have assessed one clonal driver gene mutation per patient.

When such a mutation is found in plasma following treatment, we have shown that disease nearly always recurs, documenting very high specificity of the approach. However, about half of the patients that recur do not have a detectable mutation in their plasma. There are two potential reasons for "missing" these cases in the tumor-informed ctDNA analysis. One is that the numbers of mutant DNA molecules released into the circulation may be low, and stochastic factors could limit sensitivity. Searching for more than one mutation should, in theory, mitigate this issue. Second, it is possible that essentially no DNA molecules are released into the circulation because the number of cancer cells is so small, and in that case, searching for more mutations would not be of value. With the development of new technologies, these two hypotheses can be tested, and it can be determined whether searching for more mutations raises sensitivity without compromising specificity and thereby provides potential clinical value.

Analysis of ctDNA 4-10 weeks after surgery is a powerful independent prognostic marker. A reasonable starting point for future studies would be a blood draw at 4-5 weeks post-surgery, but earlier and/or repeated samples may add value. It would be reasonable to consider further stratifying ctDNA positive patients by MAF, but currently there is insufficient data to consider allocating more or less aggressive treatment based on the MAF. We would suggest that all patients with detectable ctDNA should receive the most active available adjuvant treatment.

Data Accessibility: The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Conflict of Interest: BV, KWK, & NP are founders of, and hold equity in Thrive Earlier Detection and Personal Genome Diagnostics. KWK & NP are on the Board of Directors of, and consultants to, Thrive Earlier Detection. KWK & BV are consultants to Sysmex, Eisai, Personal Genome Diagnostics and CAGE Pharma and hold equity in CAGE Pharma. KWK, BV, and NP are consultants to and hold equity in NeoPhore. BV is a consultant to and holds equity in Catalio Capital Management. NP is an advisor to and holds equity in CAGE Pharma. The companies named above, as well as other companies, have licensed previously described technologies related to the work described in this paper from Johns Hopkins University. BV, KWK, and NP are inventors on some of these technologies. Licenses to these technologies are or will be associated with equity or royalty payments to the inventors as well as to Johns Hopkins University. CT and the University are entitled to royalty distributions related to technology licensed to Thrive Earlier Detection. CT is a consultant to Thrive. The terms of all these arrangements are being managed by Johns

Hopkins University in accordance with its conflict of interest policies. All other authors declare no competing interests.

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FIGURE LEGENDS

Figure 1. Kaplan–Meier Estimates of Survival and Recurrence, and Pattern of Recurrence According to the Presence or Absence of Post-Surgery ctDNA. (A) Overall survival (B) Colorectal cancer-specific survival (C) Recurrence-free survival (D) Site of recurrence.

Figure 2. Predictive Accuracy of Post-Surgery ctDNA and Risk of Recurrence According to ctDNA Status by Various Clinical Subgroups. (A) Relative predictive accuracy of ctDNA and other clinicopathological features using iAUC with 1000 × bootstrap resampling. (B) Forest plot of RFS and comparison of the presence or absence of post-surgery ctDNA in various subgroups. The grey squares indicate the HRs (ctDNA-positive vs ctDNA-negative) for the subgroup analyses and the horizontal lines indicate the corresponding 95% CIs for the HRs. The vertical line represents an HR of 1.0 (no difference in RFS between ctDNA-positive and -negative patients).

Figure 3. Mutant Allele Fraction (MAF), Recurrence-Free Survival (RFS) and Adjuvant Chemotherapy. (A) Non-parametric estimates of the dependence of RFS according to ctDNA MAF as a continuous variable (log hazard ratio, with 95% confidence limits); reference value = 0 (no detectable ctDNA). (B) Kaplan-Meier estimates of RFS according to median ctDNA MAF cut-off (0.046%) for patients not treated with chemotherapy (C) Kaplan-Meier estimates of RFS according to median ctDNA MAF cut-off (0.046%) for patients

treated with adjuvant chemotherapy (D) Kaplan-Meier estimates of RFS for pathologic node-negative patients with undetectable ctDNA who were treated or not treated with adjuvant chemotherapy.

Table 1: Baseline characteristics of the three independent cohorts included in the pooled analysis

Characteristics	Stage II (N = 230)	Stage III (N = 96)	LARC (N = 159)
Post-Surgery ctDNA Status			
Negative	210 (91.3%)	76 (79.2%)	140 (88.1%)
Positive	20 (8.7%)	20 (20.8%)	19 (11.9%)
Age (years)			
Mean (sd)	64.8 (12.3)	62.2 (11.1)	61.8 (13.3)
Median (range)	65.4 (23.5, 86.8)	64.0 (26.2, 82.3)	62.4 (28.0, 86.4)
Gender			
Female	99 (43.0%)	47 (49.0%)	52 (32.7%)
Male	131 (57.0%)	49 (51.0%)	107 (67.3%)
Tumor Location n (%)			
Left	127 (55.2%)	50 (52.1%)	159 (100%)
Right	103 (44.8%)	46 (47.9%)	0 (0.0%)
Tumor Stage n (%)			
0	0 (0.0%)	0 (0.0%)	36 (22.6%)
T1-3	192 (83.5%)	69 (71.9%)	115 (72.3%)
T4	38 (16.5%)	27 (28.1%)	8 (5.0%)
Nodal Stage n (%)			
N0	230 (100%)	0 (0.0%)	116 (73.0%)
N1	0 (0.0%)	68 (70.8%)	34 (21.4%)
N2	0 (0.0%)	28 (29.2%)	9 (5.7%)
Histologic Differentiation n (%)			
Poor	37 (16.1%)	29 (30.2%)	13 (9.2%)
Moderate	187 (81.3%)	63 (65.6%)	124 (87.9%)
Well	6 (2.6%)	4 (4.2%)	4 (2.8%)
Lymphovascular Invasion n (%)			
No	180 (81.4%)	47 (49.0%)	142 (91.0%)
Yes	41 (18.6%)	49 (51.0%)	14 (9.0%)
Nodes Examined n (%)			
<12	29 (12.6%)	7 (7.3%)	74 (46.5%)
≥12	201 (87.4%)	89 (92.7%)	85 (53.5%)
MMR status n (%)			
Proficient	189 (82.2%)	88 (91.7%)	77 (48.4%)
Deficient	41 (17.8%)	8 (8.3%)	6 (3.8%)
Unknown	0	0	76 (47.8%)
Post-Surgery CEA Elevated n (%)			
No	213 (95.5%)	89 (92.7%)	147 (95.5%)
Yes	10 (4.5%)	7 (7.3%)	7 (4.5%)
Adjuvant Chemotherapy n (%)			
No	178 (77.4%)	1 (1.0%)	57 (35.8%)
Yes	52 (22.6%)	95 (99.0%)	102 (64.2%)

LARC = locally advanced rectal cancer; MMR = mismatch repair

Table 2. Prevalence of Post-Surgery ctDNA According to Clinical Variables.

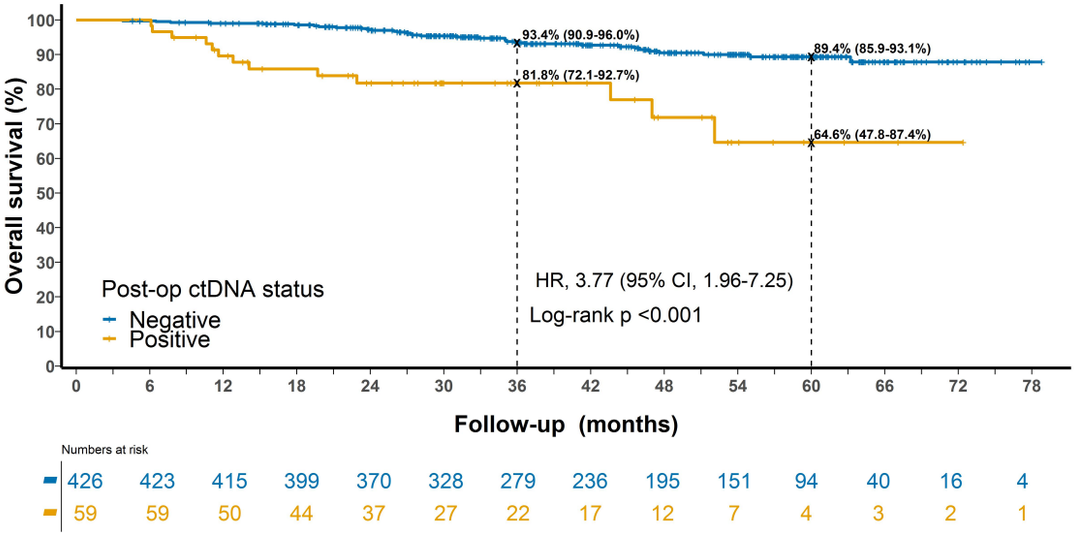
Characteristics	All patients (N = 485)	ctDNA Positive (N = 59)	ctDNA Negative (N = 426)	OR (95% CI) (N = 426)	P-value
Age (years)					
Mean, SD	63.3 (12.5)	62.0 (10.8)	63.5 (12.7)	0.99 (0.97, 1.01)	0.473
Median (range)	64.5(23.5, 86.8)	62.5 (41.2, 86.0)	64.7 (23.5, 86.8)		
Gender					
Female	198 (40.8%)	24 (40.7%)	174 (40.8%)	1	
Male	287 (59.2%)	35 (59.3%)	252 (59.2%)	1.04 (0.59, 1.82)	0.897
Tumor Location, n (%)					
Left	336 (69.3%)	44 (74.6%)	292 (68.5%)	1	
Right	149 (30.7%)	15 (25.4%)	134 (31.5%)	0.68 (0.35, 1.34)	0.267
Tumor Stage, n (%)					
T0	36 (7.4%)	2 (3.4%)	34 (8.0%)	1	
T1-3	376 (77.5%)	45 (76.3%)	331 (77.7%)	2.63 (0.58, 11.86)	0.208
T4	73 (15.1%)	12 (20.3%)	61 (14.3%)	3.58 (0.70, 18.29)	0.126
Nodal Stage, n (%)					
N0	346 (71.3%)	30 (50.8%)	316 (74.2%)	1	
N1	102 (21.0%)	17 (28.8%)	85 (20.0%)	2.11 (1.11, 4.01)	0.023
N2	37 (7.6%)	12 (20.3%)	25 (5.9%)	5.06 (2.30, 11.09)	<.0001
Histologic Differentiation, n (%)					
Poor	79 (16.9%)	9 (15.5%)	70 (17.1%)	1	
Moderate	374 (80.1%)	49 (84.5%)	325 (79.5%)	1.32 (0.60, 2.89)	0.489
Well	14 (3.0%)	0 (0.0%)	14 (3.4%)	NA (NA, NA)	NA
Lymphovascular Invasion, n (%)					
No	369 (78.0%)	34 (57.6%)	335 (80.9%)	1	
Yes	104 (22.0%)	25 (42.4%)	79 (19.1%)	3.00 (1.65, 5.44)	0.0003
Nodes Examined, n (%)					
<12	110 (22.7%)	13 (22.0%)	97 (22.8%)	1	
>=12	375 (77.3%)	46 (78.0%)	329 (77.2%)	1.01 (0.50, 2.04)	0.985
MMR status n (%)					
Proficient	354 (73.0%)	52 (88.1%)	302 (70.9%)	1	
Deficient	55 (11.3%)	3 (5.1%)	52 (12.2%)	0.33 (0.11, 1.01)	0.086
Unknown	76 (15.7%)	4 (6.8%)	72 (16.9%)		
Post-Surgery CEA Elevated, n (%)					
No	449 (94.9%)	46 (78.0%)	403 (97.3%)	1	
Yes	24 (5.1%)	13 (22.0%)	11 (2.7%)	10.13 (4.25, 24.16)	<.0001
Adjuvant Chemotherapy, n (%)					
No	236 (48.7%)	23 (39.0%)	213 (50.0%)	1	
Yes	249 (51.3%)	36 (61.0%)	213 (50.0%)	1.14 (0.59, 2.21)	0.6987

OR = Odds Ratio; MMR = mismatch repair

Table 3. Univariable and multivariable Cox regression for Overall survival, Colorectal Cancer-specific survival and Recurrence-free survival

	Univariable		Multivariable	
Outcome	**HR (95% CI)**	**P-value**	**HR (95% CI)**	**P-value**
Overall survival				
Post-surgery ctDNA (positive vs negative)	3.77 (1.96, 7.25)	<0.001	2.77 (1.2, 6.38)	0.017
Age (>70 vs ≤70 yo)	1.75 (0.98, 3.11)	0.059	1.66 (0.86, 3.21)	0.130
T stage (T0-2 vs T3-4)	11.29 (2.82, 160.9)	0.003	9.5 (1.76, 119.19)	0.013
N stage				
N1 vs N0	3.57 (1.2, 10.6)	0.022	2.06 (0.6, 7.05)	0.247
N2 vs N0	5.92 (3.33, 35.83)	<0.001	5.95 (1.55, 22.84)	0.009
Lymphovascular invasion (Yes vs No)	2.29 (1.19, 4.42)	0.013	1.76 (0.84, 3.69)	0.134
Post-surgery CEA (elevated vs not elevated)	4.7 (2.07, 10.66)	<0.001	4.21 (1.65, 10.74)	0.003
Histologic Differentiation				
Moderate vs Poor	0.97 (0.44, 2.12)	0.930	0.91 (0.4, 2.06)	0.822
Well vs Poor	3.33 (0.99, 11.18)	0.052	6.64 (1.66, 26.61)	0.008
Adjuvant Chemotherapy (Yes vs No)	0.38 (0.17, 0.86)	0.020	0.24 (0.09, 0.63)	0.004
Colorectal cancer-specific survival				
Post-surgery ctDNA (positive vs negative)	4.84 (2.38, 9.85)	<0.001	3.86 (1.5, 9.93)	0.005
Age (>70 vs ≤70 yo)	1.94 (1.01, 3.74)	0.048	1.9 (0.87, 4.13)	0.105
T stage (T0-2 vs T3-4)	8.97 (1.79, 108.7)	0.012	7.04 (1.12, 89.92)	0.039
N stage				
N1 vs N0	1.87 (0.48, 7.24)	0.368	0.82 (0.16, 4.24)	0.813
N2 vs N0	7.9 (2.55, 38.38)	0.001	4.57 (0.86, 24.23)	0.074
Lymphovascular invasion (Yes vs No)	3.51 (1.71, 7.22)	0.001	2.67 (1.15, 6.2)	0.022
Post-surgery CEA (elevated vs not elevated)	6.2 (2.66, 14.43)	<0.001	6.33 (2.34, 17.11)	<0.001
Histologic Differentiation				
Moderate vs Poor	0.83 (0.35, 1.97)	0.679	0.81 (0.32, 2)	0.643
Well vs Poor	4.02 (1.16, 13.92)	0.028	10.07 (2.34, 43.23)	0.002
Adjuvant Chemotherapy (Yes vs No)	0.35 (0.13, 0.94)	0.036	0.2 (0.06, 0.68)	0.009
Recurrence-free survival				
Post-surgery ctDNA (positive vs negative)	7.56 (4.85, 11.79)	<0.001	6.16 (3.67, 10.32)	<0.001
T stage (T0-2 vs T3-4)	4.07 (2.2, 11.67)	<0.001	3.92 (1.43, 10.80))	0.008
N stage				
N1 vs N0	2.08 (1.82, 8.91)	0.001	1.73 (1.06, 7.04)	0.037
N2 vs N0	4.98 (3.26, 19.52)	<0.001	3.84 (1.73, 13.52)	0.003
Lymphovascular invasion (Yes vs No)	2.12 (1.31, 3.45)	0.002	1.26 (0.74, 2.13)	0.395
Post-surgery CEA (elevated vs not elevated)	4.36 (2.4, 7.92)	<0.001	2.38 (1.22, 4.65)	0.011
Histologic Differentiation				
Moderate vs Poor	0.98 (0.56, 1.72)	0.952	0.83 (0.46, 1.47)	0.513
Well vs Poor	1.59 (0.53, 4.78)	0.411	2.48 (0.69, 8.83)	0.162
Adjuvant Chemotherapy (Yes vs No)	0.95 (0.55, 1.64)	0.855	0.56 (0.30, 1.03)	0.062

(A)



Numbers at risk

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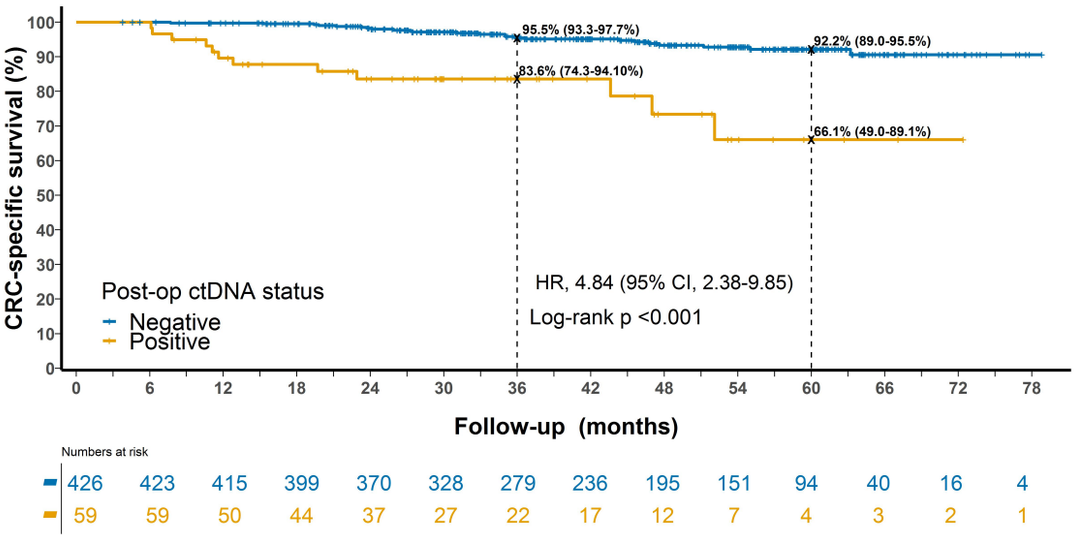
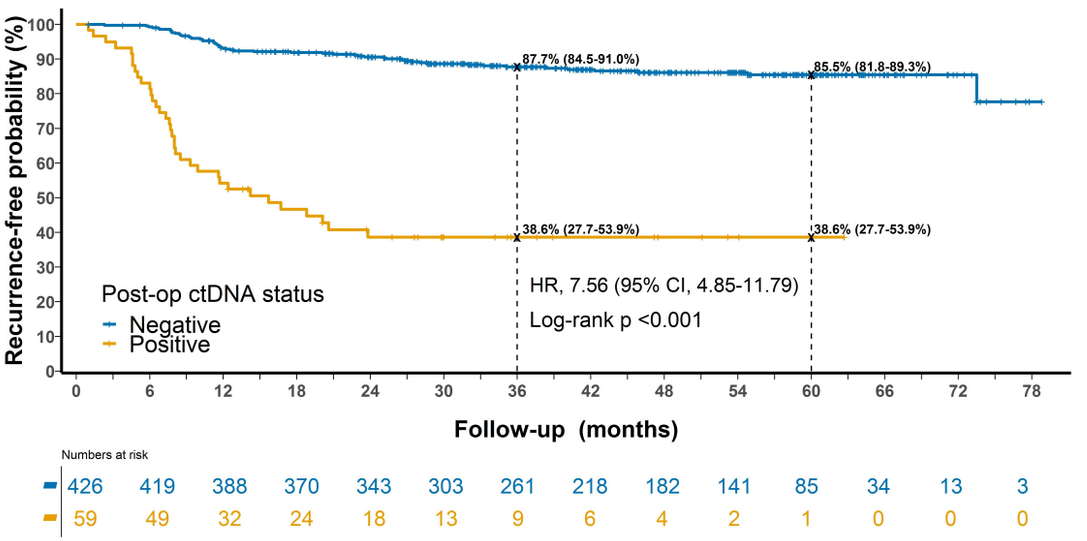
(B)



Numbers at risk

426	423	415	399	370	328	279	236	195	151	94	40	16	4
59	59	50	44	37	27	22	17	12	7	4	3	2	1

(C)



Numbers at risk

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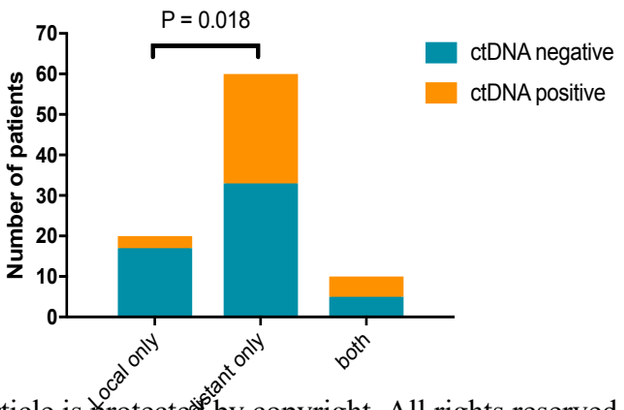
(D)



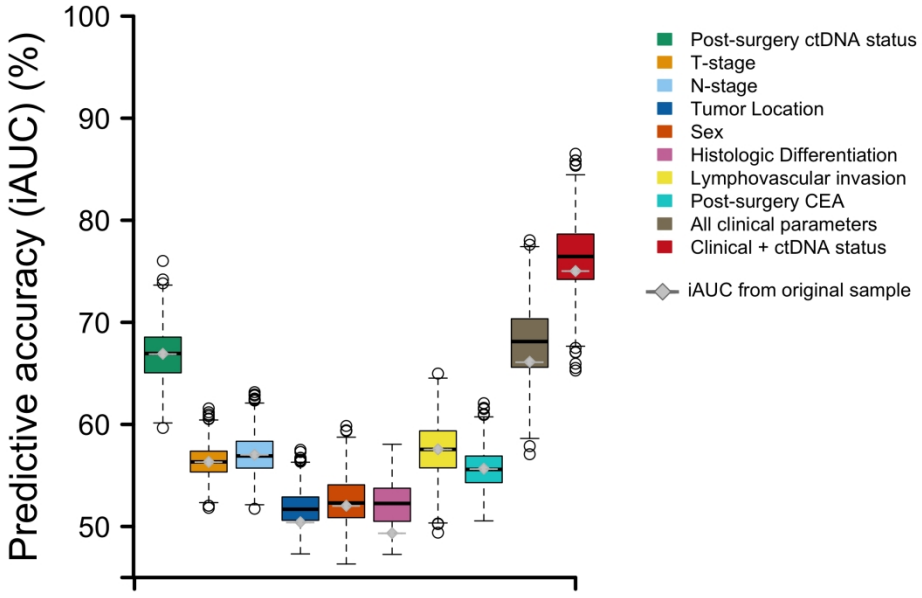


Figure 2

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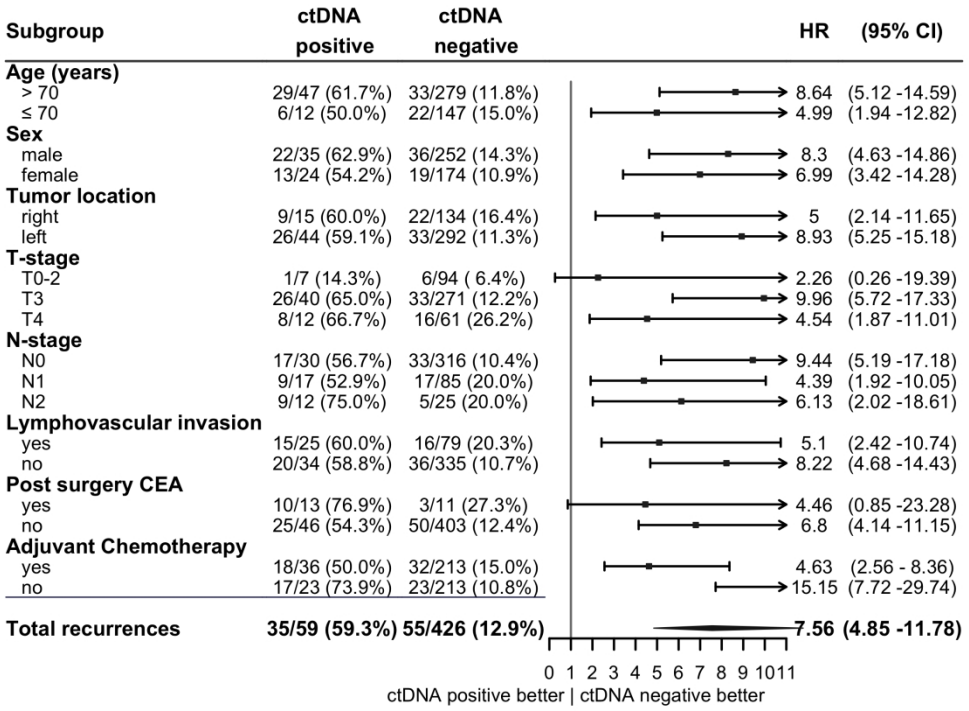

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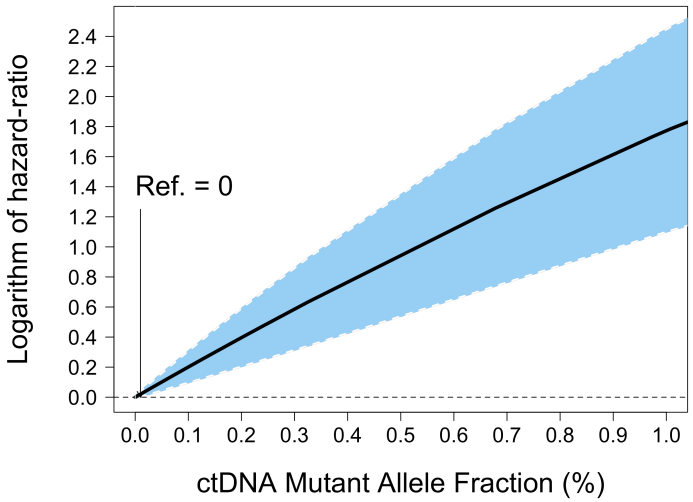

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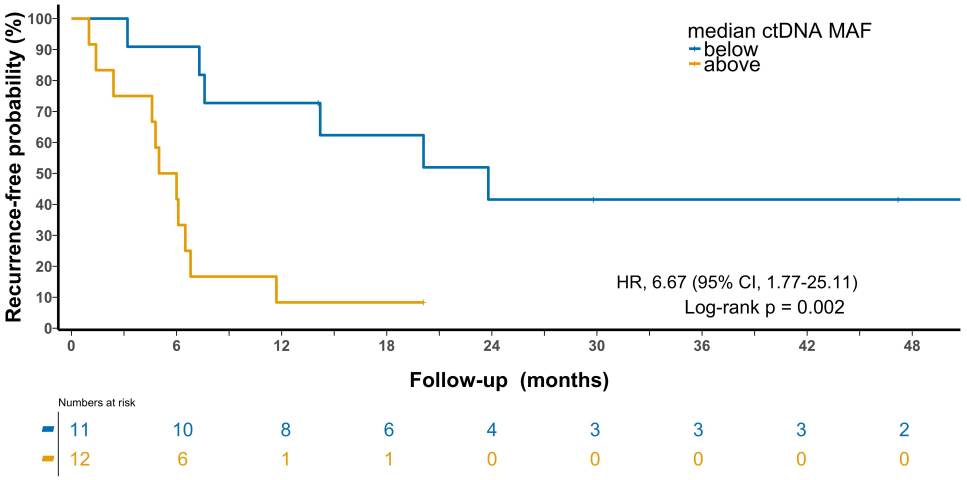
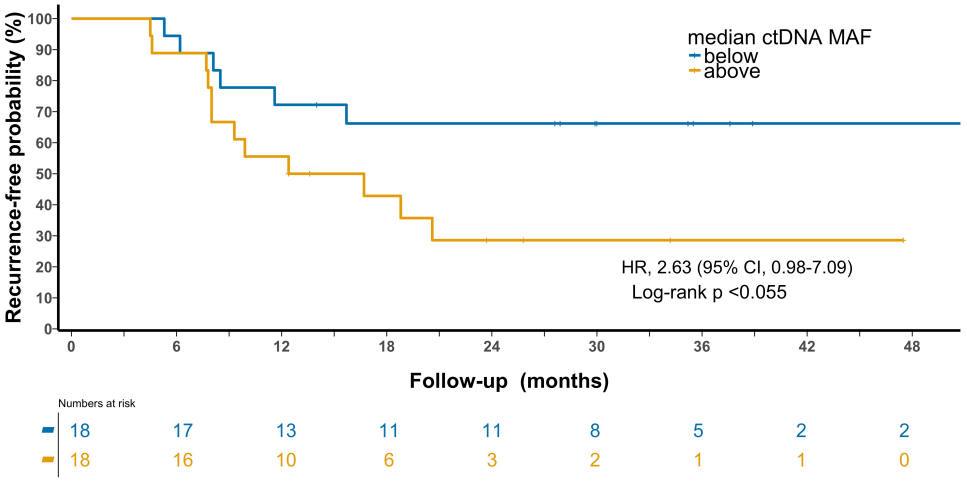


Numbers at risk

below	11	10	8	6	4	3	3	3	2
above	12	6	1	1	0	0	0	0	0

(C)



Numbers at risk

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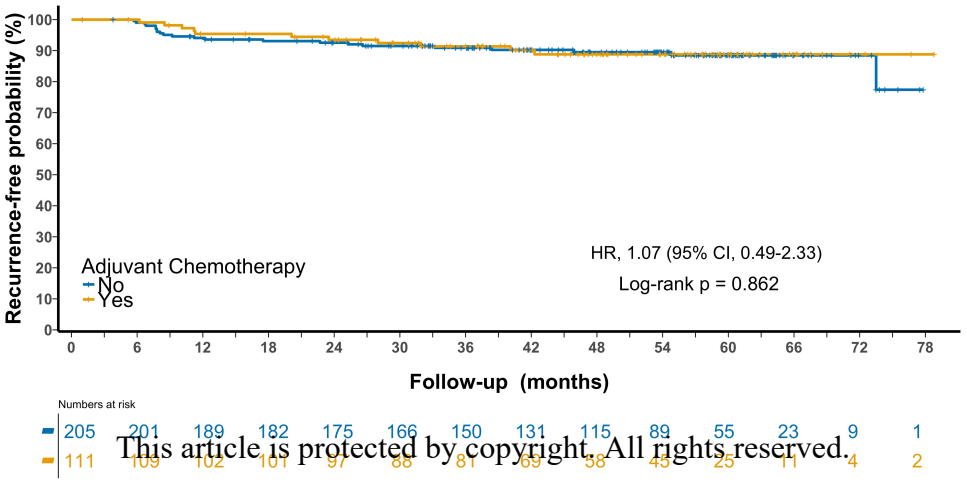
above	18	16	10	6	3	2	1	1	0

(D)



Numbers at risk

No	205	201	189	182	175	166	150	131	115	89	55	23	9	1
Yes	111	109	102	101	97	88	81	69	58	45	25	11	4	2