Supplementary Appendix

Supplement to: Tie J, Cohen JD, Lahouel K, et al. Circulating tumor DNA analysis guiding adjuvant therapy in stage II colon cancer. N Engl J Med 2022;386:2261-72. DOI: 10.1056/NEJMoa2200075

This appendix has been provided by the authors to give readers additional information about the work.

SUPPLEMENTARY APPENDIX

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Contributors

Institution Name	Principal Investigators
Barwon Health	Madhu Singh
Bendigo Health	Sam Harris
Border Medical Oncology	Craig Underhill
Cabrini Hospital	Jeremy Shapiro
Calvary Mater Newcastle Hospital	Fiona Day
Canberra Hospital	Desmond Yip
Chris O'Brien Lifehouse	Lisa Horvath
Eastern Health	Rachel Wong, Margaret Lee
Epworth Freemasons (Richmond)	Sumitra Ananda
Fiona Stanley Hospital	Adnan Khattak
Flinders Medical Centre	Christos Karapetis
Melbourne Private Hospital	Peter Gibbs
Monash Health	Marion Harris
Nepean Cancer Centre	Jenny Shannon
Newcastle Private Hospital	James Lynam
Northern Hospital	Belinda Lee
Peter MacCallum Cancer Centre	Jeanne Tie
Royal Brisbane and Women's Hospital	Matthew Burge
Royal Hobart Hospital	Louise Nott
South West Health Care	Theresa Hayes
St Vincent's Hospital Melbourne	Sue-Anne McLachlan
Western Health	Peter Gibbs, Susanne Kosmider
Western Private Hospital	Peter Gibbs

Circulating Tumor DNA (ctDNA) Analysis Method

We used a tumor-informed personalized approach for ctDNA analysis, where somatic mutations were first identified by targeted sequencing of each patient's tumor tissue, and the presence of the same mutation(s) was then assessed in the plasma samples. All tumor tissue mutation and ctDNA analyses were performed by the study scientists (JDC, KL, YW, JP, NS, LD, MP, and BV) blinded to the clinical outcome.

Plasma samples were collected for ctDNA analysis from all patients at week-4 and week-7 after surgery. The second week-7 testing was performed to see if this increased test sensitivity, due to an anticipated increase in ctDNA detection rate with time after surgery and with more volume sampled. The ctDNA results for the two timepoints were as follows: 31 patients with week-4 time point positive and week-7 time point positive, 8 patients with week-4 time point positive and week-7 time point negative, and 6 patients with week-4 time point negative and week-7 time point positive.

(A) Tumor tissue mutation analysis

Formalin-fixed paraffin-embedded tumor tissue from the primary tumor was analysed for somatic mutations in 15 genes recurrently mutated in colorectal cancer (*SMAD4, TP53, AKT1, APC, BRAF, CTNNB1, ERBB3, FBXW7, HRAS, KRAS, NRAS, PIK3CA, PPP2R1A, RNF43, POLE*). Tumor sections were macro-dissected under a dissecting microscope to enrich neoplastic cell content. DNA was purified with a Qiagen FFPE Kit (Qiagen cat #56494). Primers were designed and sequencing results analysed as previously described.[1]

(B) Plasma sample collection and mutation analysis

30 to 60ml of blood samples were collected in K2-EDTA tubes and processed within 3 hours by double centrifugation; buffy coat was collected after the first centrifugation. All samples were stored at -80°C prior to extraction and analysis. At least 10 ml of plasma was purified from each patient using the QIAamp Circulating Nucleic Acid kit (Qiagen cat# 55114).

For each patient, at least one mutation identified from targeted sequencing of the tumor tissue was assessed in cell-free DNA (cfDNA) from the plasma. The detection and quantitation of ctDNA were performed using the Safe-Sequencing System error-reduction technology for the detection of low frequency mutations[1-3] with plasma DNA divided into 12, 24, or 95 wells per sample. Leukocyte DNA was used to exclude constitutional polymorphisms.

For plasma DNA samples partitioned into 12 or 24 wells, ctDNA was classified as detectable (ctDNA-positive) or undetectable (ctDNA-negative). This classification was based on exact permutation tests as described previously[1-3] that compared the difference between the average mutant allele frequency (MAF) across the wells containing the sample of interest with that of the wells containing the control sample for each mutation. One-sided p-values were calculated using the permTS function of the R perm package (version 3.5.1). A sample was classified as ctDNA positive if the p-value was <0.1. For plasma samples divided into 95 wells, the mutant allele frequencies of all observed mutations were used to model the amplicon-specific distribution of assay noise. The p-values corresponding to the mutations of interest (i.e., those detected in the primary tumor tissue) were combined using Fisher's method to calculate a final p-value for the patient. A patient was classified as ctDNA positive using the same p-value threshold (<0.1) described above. Further technical details of these assays will be reported elsewhere.

Statistical Method

All statistical analyses adhered to a statistical analysis plan written and made publicly available (medRxiv 2021.09.02.21262816; doi: https://doi.org/10.1101/2021.09.02.21262816) on September 6th, 2021, before the database lock which occurred on October 15th, 2021.

The final approach for the analysis of the primary endpoint and the non-inferiority margin were as defined at the beginning of the trial.

Figure S1. Patient Registration, Randomization and Follow-Up

All eligible patients who provided written informed consent were registered and underwent week-4 and week-7 post-surgery blood draws. Patients were randomized following confirmation of adequate tumor tissue by central pathology review and of a successful week-4 blood draw. Patients who did not have both post-surgery blood draws were excluded from the intention-to-treat (ITT) population. The per-protocol population included patients who had undergone 24-month surveillance imaging (unless recurrence or death occurred prior) and for ctDNA-guided management, ctDNA-positive patients who received at least 12 weeks of chemotherapy or ctDNA-negative patients who received no more than 4 weeks of chemotherapy.

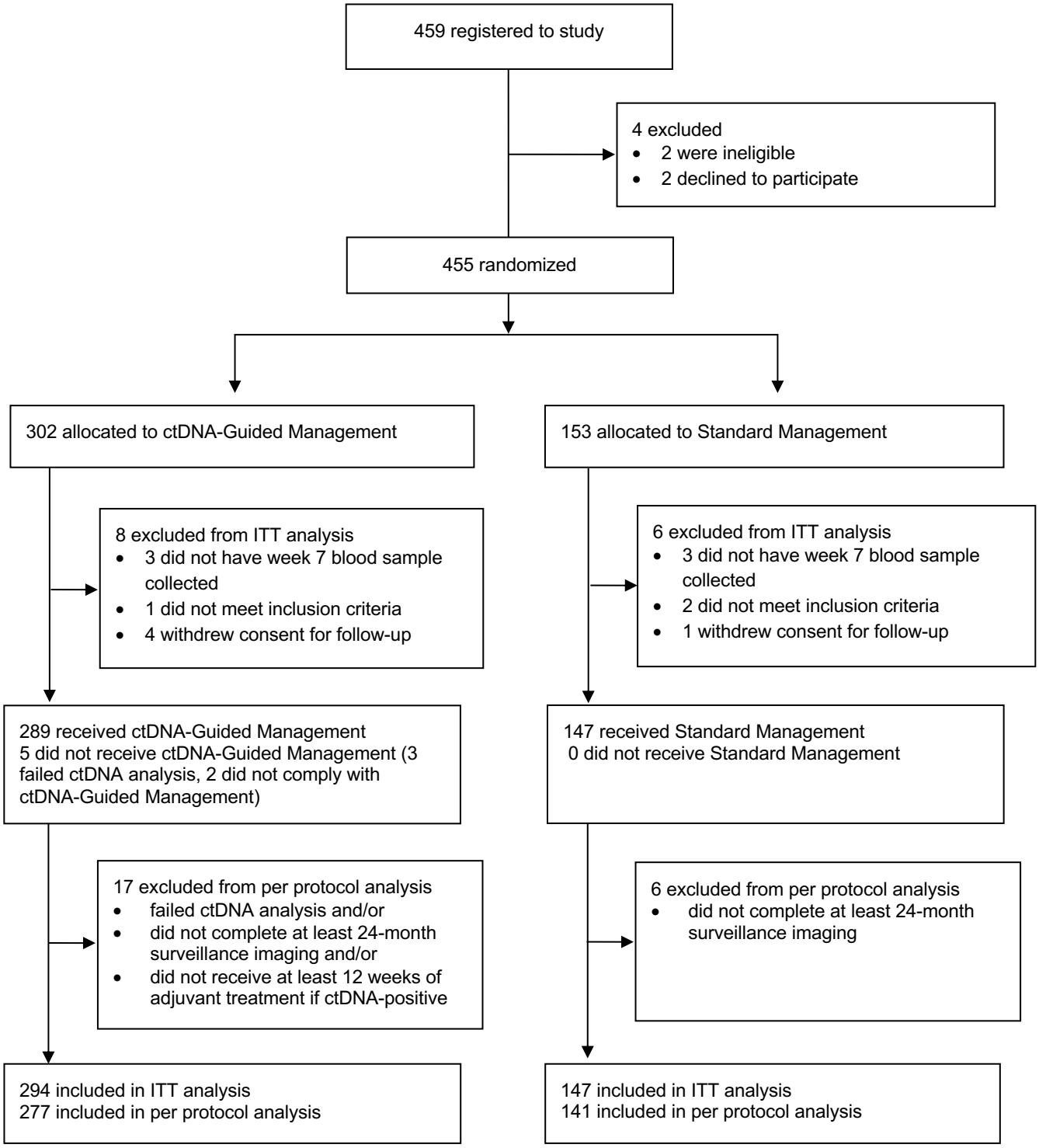

Figure S2. Non-inferiority Margins for Recurrence-Free Survival and Time-to-Recurrence at the 2- and 3-Year Landmarks

The point-estimate differences, 95% confidence intervals and the non-inferiority margins for recurrence-free survival (panel a) and time-to-recurrence (panel b) are shown for the 2- and 3-year landmarks. Non-inferiority is confirmed for 2- and 3-year recurrence-free survival as well as 2-year time-to-recurrence. Non-inferiority is confirmed for each outcome at both point estimates given the 95% CI overlaps with the non-inferiority margin.

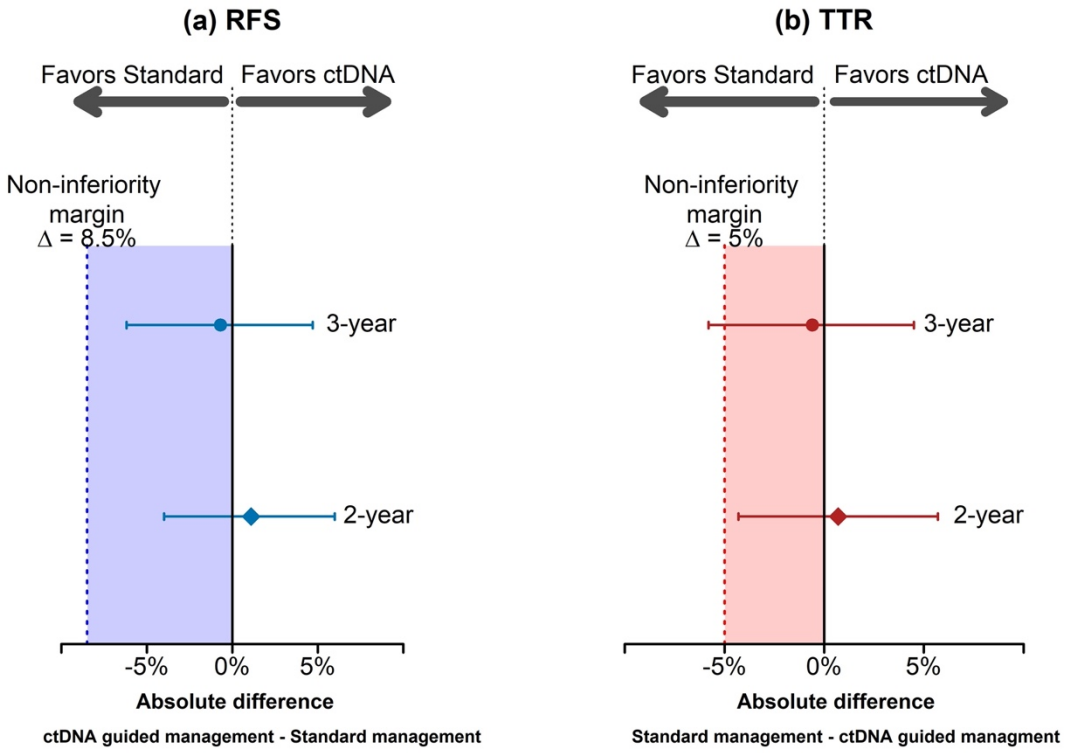

Figure S3. Time-To-Recurrence Difference During Follow-Up and Non-Inferiority Margin for the Intention-to-Treat Population

The point-estimate difference in recurrence rate over time between ctDNA-guided and standard management is indicated by the blue line. The associated 95% confidence interval (CI) is indicated by the grey shading. The non-inferiority margin of -5% is indicated by the pink shading. The upper bound of the 95% confidence interval at 2-year lies above the -5% margin, confirming non-inferiority of the ctDNA-guided management to standard management.

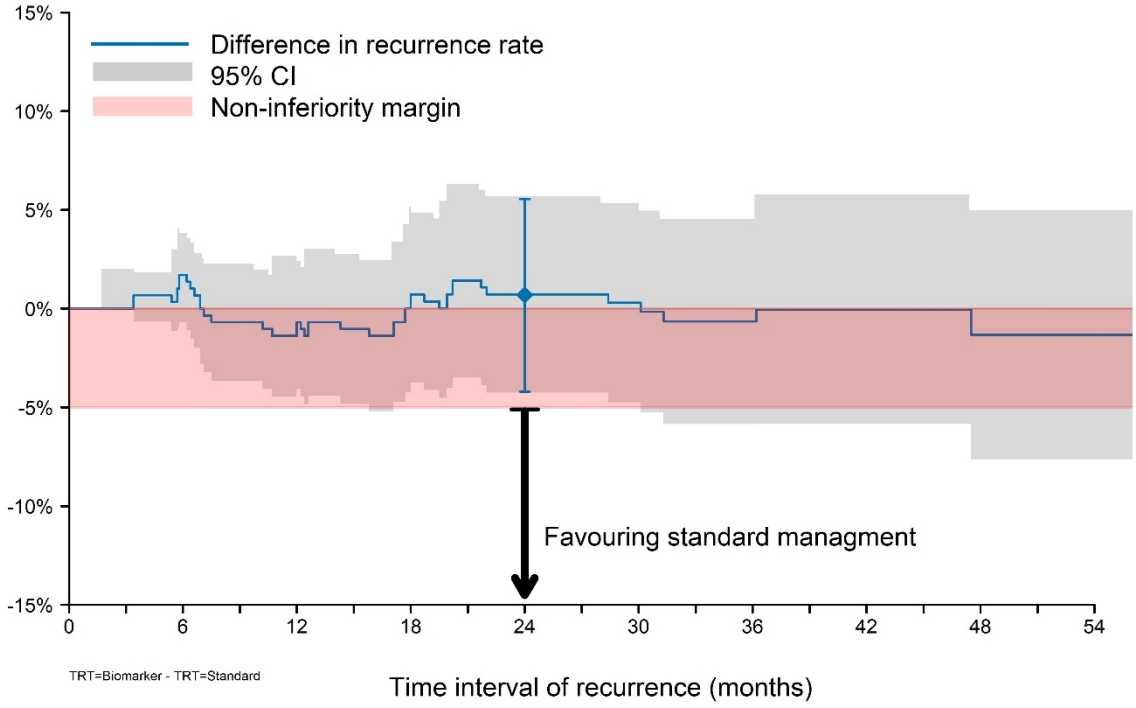

Figure S4. Cumulative Incidence of Recurrence by Management Group for the Intention-to-Treat Population

Cumulative incidence rates of recurrence at 1-year, 2-year and 3-year landmarks are shown.

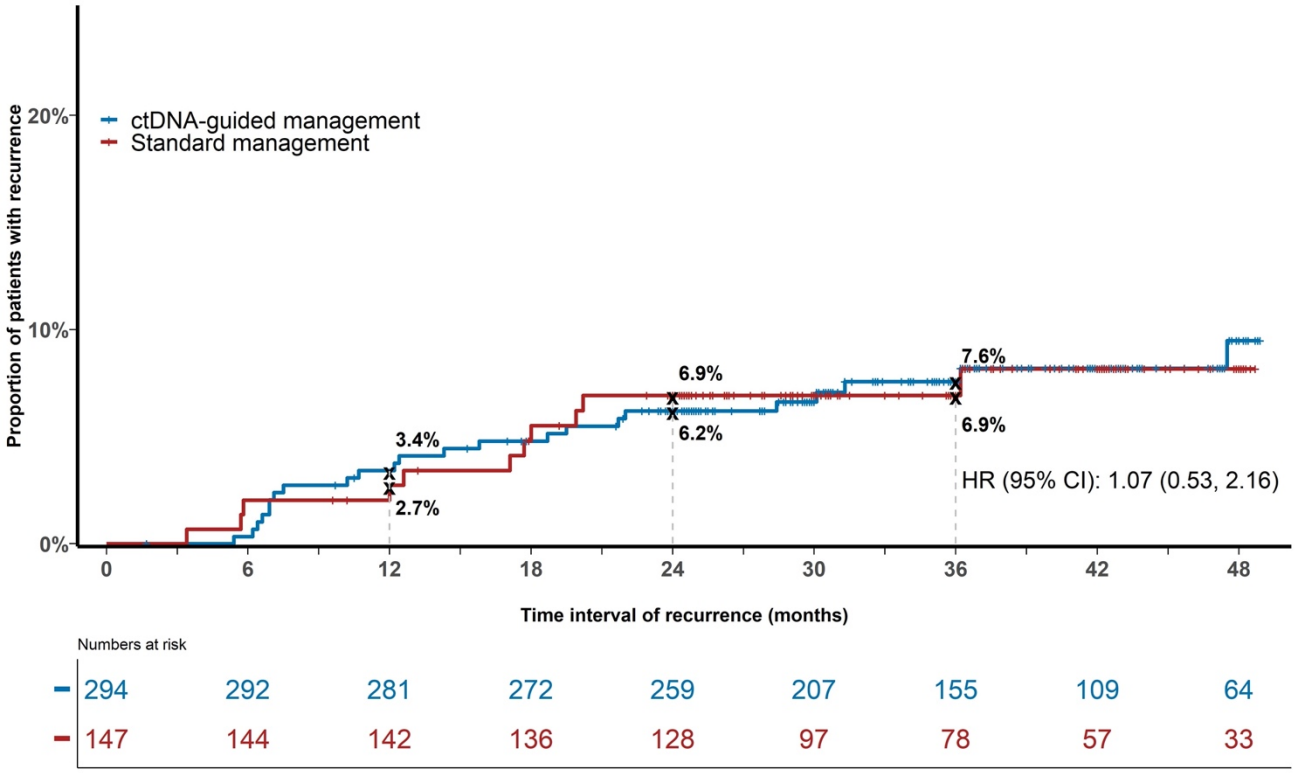

Figure S5. Recurrence-Free Survival for the Intention-to-Treat Population According to Subgroup

Hazard ratios and 95% confidence intervals (CIs) according to subgroups are shown.

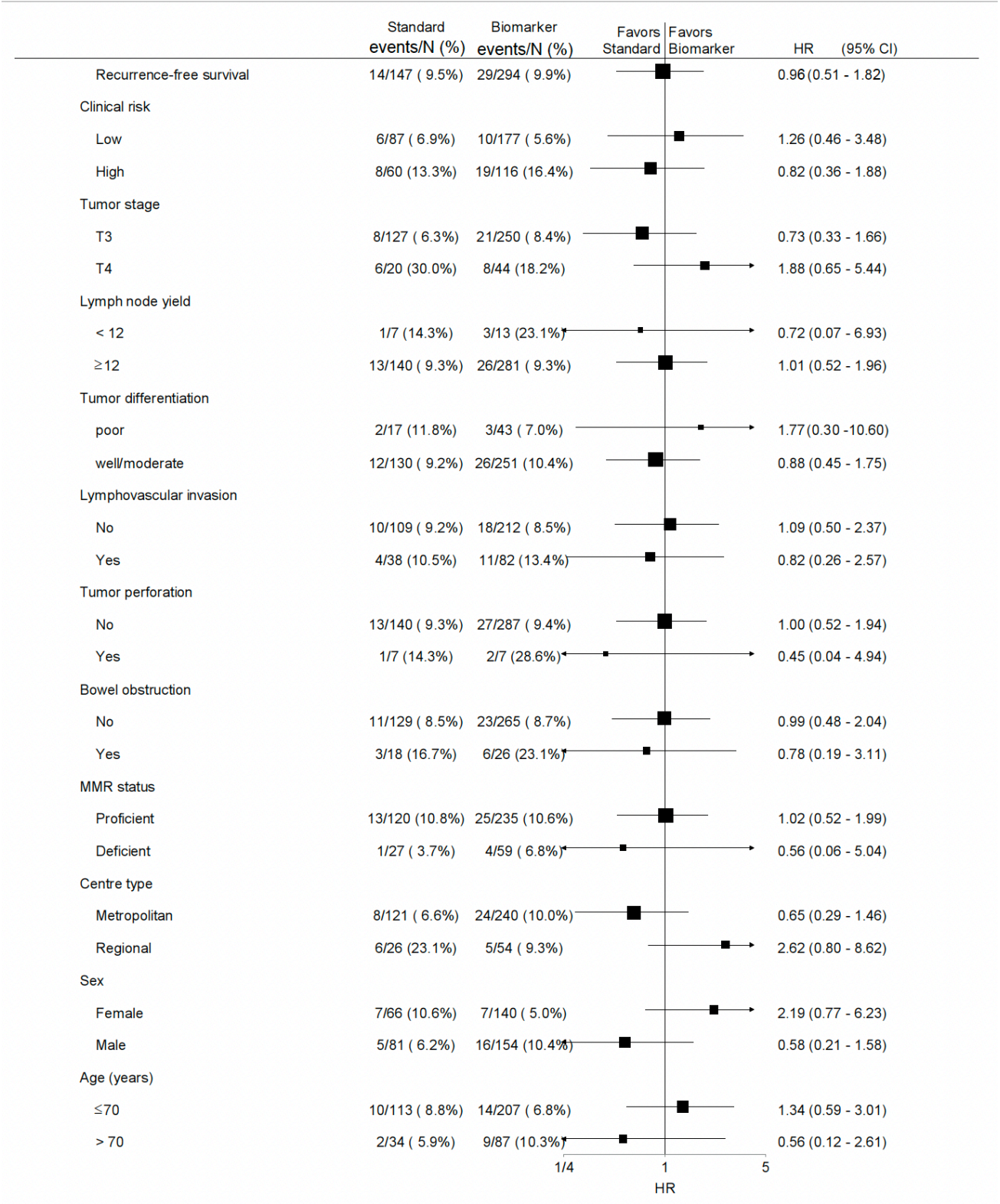

Figure S6. Recurrence-Free Survival Difference During Follow-Up and Non-Inferiority Margin for the Per-Protocol Population

The point estimate difference in recurrence-free survival rate over time between ctDNA-guided and standard management is indicated by the blue line. The associated 95% confidence interval (CI) is indicated by the grey shading. The non-inferiority margin of -8.5% is indicated by the pink shading. The upper bound of the 95% confidence interval at 2-year lies above the -8.5% margin, confirming non-inferiority of the ctDNA-guided management to standard management.

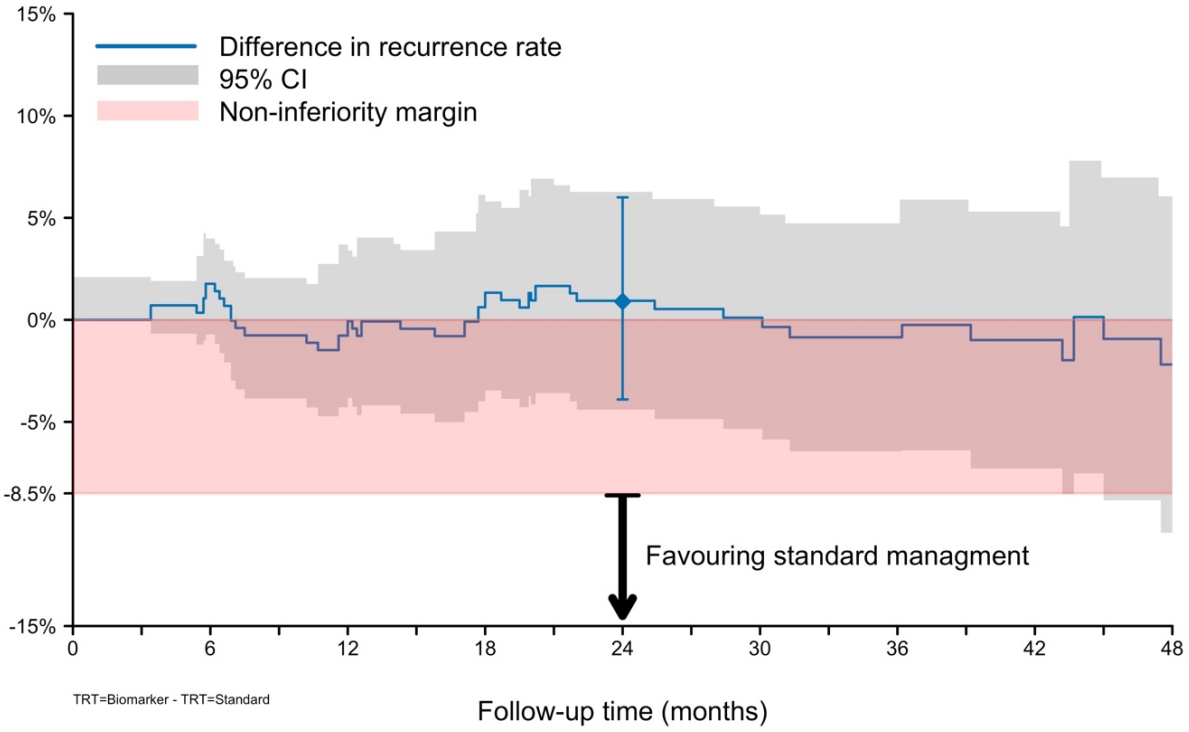

Figure S7. Time-to-Recurrence Difference During Follow-Up and Non-Inferiority Margin for the Per-Protocol Population

The point estimate difference in recurrence rate over time between ctDNA-guided and standard management is indicated by the blue line. The associated 95% confidence interval (CI) is indicated by the grey shading. The non-inferiority margin of -5% is indicated by the pink shading. The upper bound of the 95% confidence interval at 2-year lies above the -5% margin, confirming non-inferiority of the ctDNA-guided management to standard management.

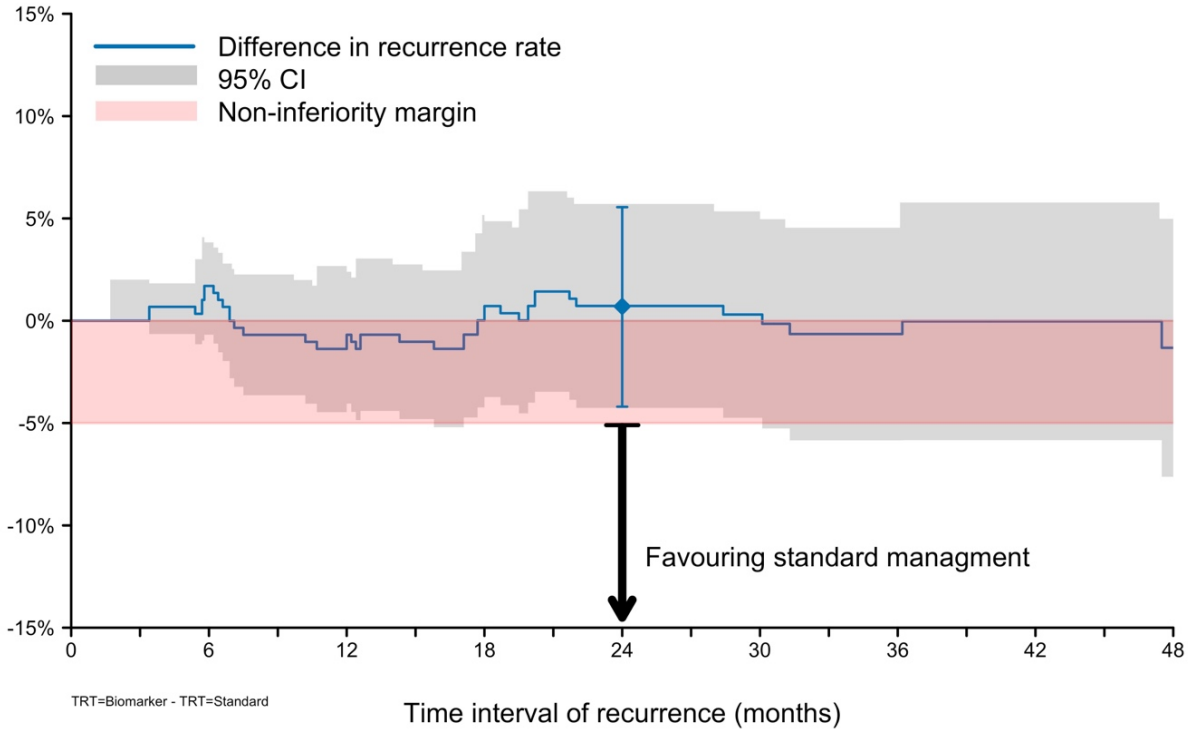

Figure S8. Cumulative Incidence of Recurrence According to ctDNA Status in ctDNA-Guided Group

Kaplan-Meier estimates of recurrence rates at 1-year, 2-year and 3-year landmarks for ctDNA-positive and ctDNA-negative patients are shown. The 3-year rate of recurrence was 13.6% in treated ctDNA-positive patients and 6.6% in the untreated ctDNA-negative patients, indicating worse prognosis in ctDNA-positive patients despite adjuvant treatment.

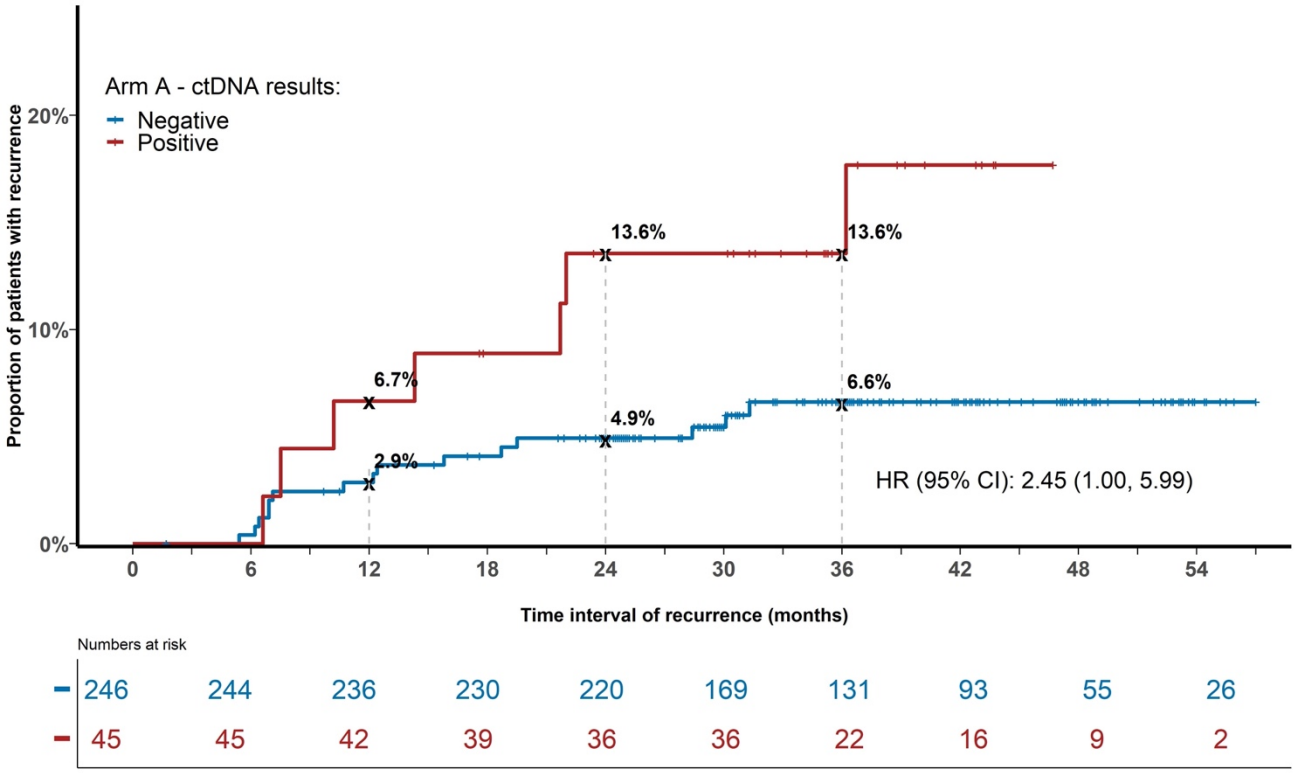

Figure S9. Recurrence-Free Survival for ctDNA-Guided Patients, according to ctDNA Status and Clinical Risk

Kaplan–Meier estimates of recurrence-free survival according to ctDNA results (positive or negative) and clinical risk group for ctDNA-negative patients. The effect of ctDNA-positive status by clinical risk was not examined due to the small total number of patients with a ctDNA-positive result. By design, ctDNA-positive patients received adjuvant chemotherapy while ctDNA-negative patients (regardless of clinical risk) did not. 96.7% of patients with low-risk clinic-pathologic features and a ctDNA-negative result were alive and disease-free at 3 years.

ctDNA-Guided Patients

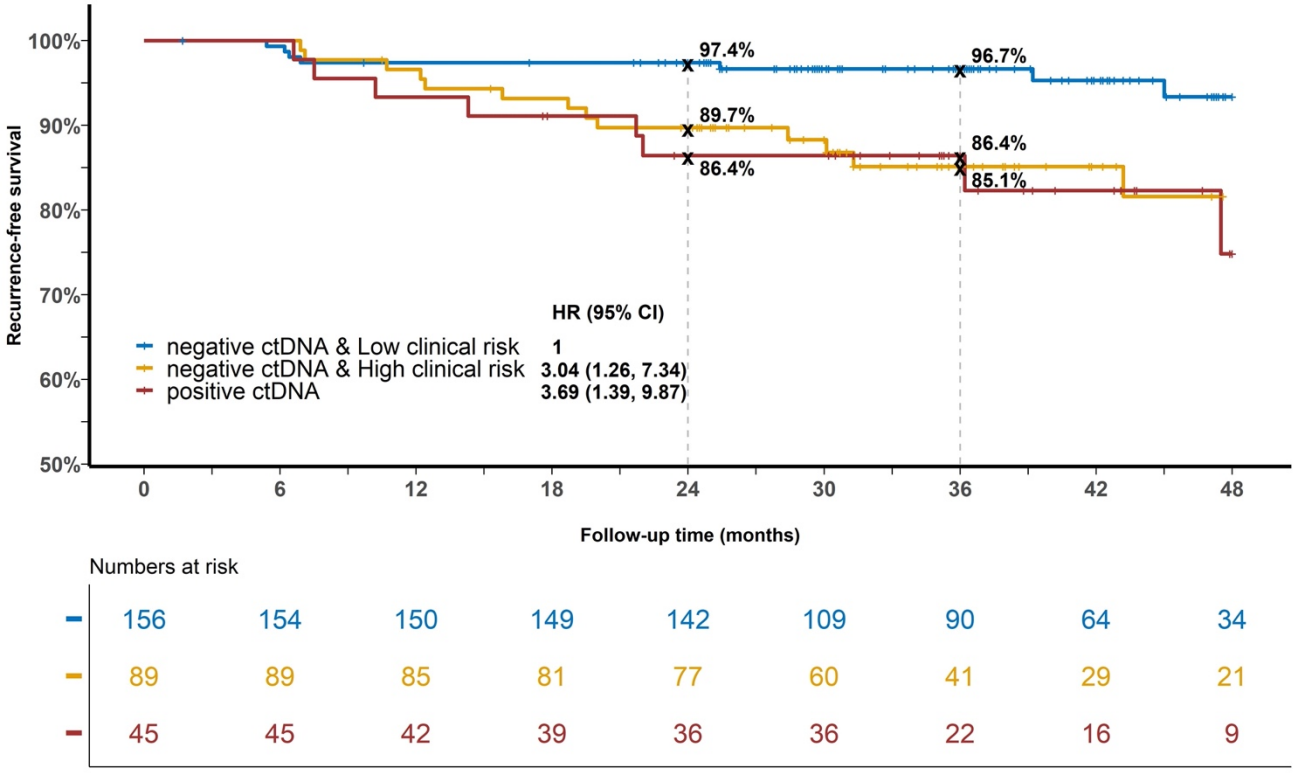

Figure S10. Recurrence-Free Survival for ctDNA-Guided Patients, according to ctDNA Status and T-stage

Kaplan–Meier estimates of recurrence-free survival according to ctDNA results (positive or negative) and T stage for ctDNA-negative patients. The effect of ctDNA-positive status by T stage was not examined due to the small total number of patients with a ctDNA-positive result. By design, ctDNA-positive patients received adjuvant chemotherapy while ctDNA-negative patients did not. 94.2% of patients with T3 tumor and a ctDNA-negative result were alive and disease-free at 3 years.

ctDNA-Guided Patients

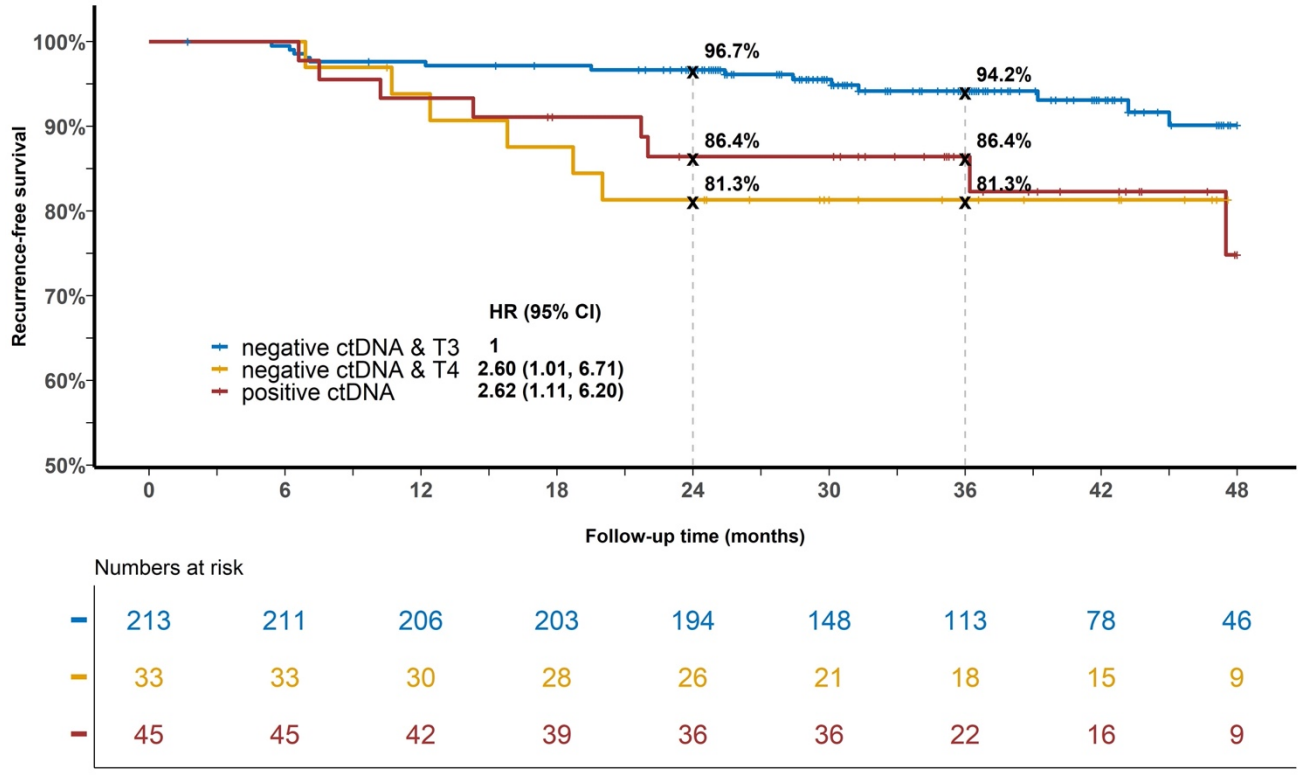

Table S1. Chemotherapy Regimens

Regimen Name	Regimen Dose	Schedule
De Gramont (modified)	Leucovorin 50 mg IV Fluorouracil 400 mg/m^2 IV Fluorouracil 2400 mg/m^2 CIV pump over 46 hours	Every 2 weeks
QUASAR (modified)	Leucovorin 50 mg IV Fluorouracil 375-450 mg/m^2 IV (dose per institutional standard)	Every week
Roswell Park (modified)	Leucovorin 50 mg IV Fluorouracil 500 mg/m^2 IV	Every week for 6 weeks followed by 2-week break
Capecitabine	Capecitabine twice daily orally days 1 to 14 (dose as per institutional standard of care)	Every 3 weeks
Modified FOLFOX6	Oxaliplatin 85 mg/ m^2 IV Leucovorin 50 mg IV Fluorouracil 400 mg/m^2 IV Fluorouracil 2400 mg/m^2 CIV pump over 46 hours	Every 2 weeks
CAPOX/XELOX	Oxaliplatin 130 mg/ m^2 IV Capecitabine 1000mg/m^2 twice daily orally days 1 to 14	Every 3 weeks

Table S2. Patient Characteristics at Baseline for the Per-Protocol Population

Characteristics	Standard management (N = 141)	ctDNA-guided management (N = 277)	Overall (N = 418)
Gender			
Female	44.7% (63/141)	48.0% (133/277)	46.9% (196/418)
Male	55.3% (78/141)	52.0% (144/277)	53.1% (222/418)
Age at randomisation (years)			
n, mean (sd)	141, 62 (11)	277, 63 (12)	418, 63 (11)
Median (range)	62 (28, 84)	65 (30, 87)	64 (28, 87)
ECOG status			
0	83.7% (118/141)	78.3% (216/276)	80.1% (334/417)
1	14.2% (20/141)	21.0% (58/276)	18.7% (78/417)
2	2.1% (3/141)	0.7% (2/276)	1.2% (5/417)
Centre type			
Metropolitan	82.3% (116/141)	82.7% (229/277)	82.5% (345/418)
Regional	17.7% (25/141)	17.3% (48/277)	17.5% (73/418)
Primary tumor site			
Left-sided colon/rectum	53.9% (76/141)	41.2% (114/277)	45.5% (190/418)
Right-sided colon	46.1% (65/141)	58.8% (163/277)	54.5% (228/418)
Tumor stage			
T3	85.8% (121/141)	85.2% (236/277)	85.4% (357/418)
T4b	14.1% (20/141)	14.8% (41/277)	14.6% (61/418)
Tumor differentiation			
Poorly	9.9% (14/141)	14.8% (41/277)	13.2% (55/418)
Well	90.1% (127/141)	85.2% (236/277)	86.8% (363/418)
LN yield			
< 12	5.0% (7/141)	4.3% (12/277)	4.5% (19/418)
≥ 12	95.0% (134/141)	95.7% (265/277)	95.5% (399/418)
Tumor perforation			
No	95.0% (134/141)	97.8% (271/277)	96.9% (405/418)
Yes	5.0% (7/141)	2.2% (6/277)	3.1% (13/418)
Pre-operative bowel obstruction			
No	87.2% (123/141)	90.9% (249/274)	89.6% (372/415)
Yes	12.8% (18/141)	9.1% (25/274)	10.4% (43/415)
Lymphovascular invasion			
No	73.8% (104/141)	72.6% (201/277)	73.0% (305/418)
Yes	26.2% (37/141)	27.4% (76/277)	27.0% (113/418)
Perineural invasion			
No	95.0% (134/141)	94.6% (262/277)	94.7% (396/418)
Yes	5.0% (7/141)	5.4% (15/277)	5.3% (22/418)
MMR status			
Abnormal/Deficient	17.7% (25/141)	20.6% (57/277)	19.6% (82/418)
Normal/Proficient	82.3% (116/141)	79.4% (220/277)	80.4% (336/418)
Clinical risk*			
High	41.8% (59/141)	39.5% (109/276)	40.3% (168/417)
Low	58.2% (82/141)	60.5% (167/276)	59.7% (249/417)
Time from surgery to randomization (days)			
Median (IQR)	33 (28, 41)	32 (28, 39)	32 (28, 39)

Table S3. Representativeness of the DYNAMIC Study Participants

Category	
Disease under investigation	Colorectal cancer
Special considerations related to	
Sex and gender	Colorectal cancer affects men more than women (ratio of 5:4)
Age	Prevalence increases steeply with age, although the incidence in younger individuals age under 50 is increasing in several countries including Australia and the United States
Race or ethnic group	Colorectal cancer incidence is higher in African Americans than other race/ethnic groups; colorectal cancer incidence in Asians and Hispanics is lower than non-Hispanic whites
Overall representativeness of this trial	The demographics of the participants in the DYNAMIC study were consistent with those expected of this stage II colon cancer patient population treated in clinical trials. The median age and ratio of men to women was consistent with other clinical trials in this setting. Biologic sex was reported by the participants; options were female and male. Consistent with other adjuvant colorectal cancer trials involving chemotherapy, the proportion of older individuals (age > 70) is under-represented in this trial compared to the broader population. Information on race or ethnic group was not specifically collected on this study but given the Australian population is predominantly non-Hispanic whites, we would anticipate an under-representation of Black, Asians and Hispanics in this study.

References

1. Tie J, Wang Y, Tomasetti C, et al. Circulating tumor DNA analysis detects minimal residual disease and predicts recurrence in patients with stage II colon cancer. Sci Transl Med 2016;8:346ra92.
2. Kinde I, Wu J, Papadopoulos N, Kinzler KW, Vogelstein B. Detection and quantification of rare mutations with massively parallel sequencing. Proc Natl Acad Sci U S A 2011;108:9530-5.
3. Tie J, Cohen JD, Wang Y, et al. Serial circulating tumour DNA analysis during multimodality treatment of locally advanced rectal cancer: a prospective biomarker study. Gut 2018.