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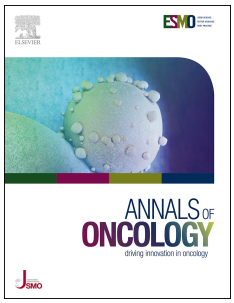

Results of the c-TRAK TN trial: a clinical trial utilising ctDNA mutation tracking to detect molecular residual disease and trigger intervention in patients with moderate and high-risk early stage triple negative breast cancer.

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Original research

Results of the c-TRAK TN trial: a clinical trial utilising ctDNA mutation tracking to detect molecular residual disease and trigger intervention in patients with moderate and high-risk early stage triple negative breast cancer.

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Clinical Trial Registries

ISRCTN:

https://www.isrctn.com/ISRCTN12569958?q=c-trak%20tn&filters=&sort=&offset=1&totalResults=1&page=1&pageSize=10

Clinicaltrials.gov:

https://clinicaltrials.gov/ct2/show/NCT03145961?term=c-trak&cond=breast+cancer&draw=2&rank=1

Plain English Summary

Cancer Research UK:

https://www.cancerresearchuk.org/about-cancer/find-a-clinical-trial/a-trial-looking-whether-ctdna-can-help-doctors-know-who-risk-breast-cancer-coming-back-pembroluzimab-reduces-ctdna-ctraktn

ABSTRACT

Background

Post-treatment detection of circulating tumour DNA (ctDNA) in early-stage triple negative breast cancer (TNBC) patients predicts high risk of relapse. c-TRAK-TN assessed the utility of prospective ctDNA surveillance in TNBC and the activity of pembrolizumab in patients with ctDNA detected (ctDNA+).

Patients and methods

c-TRAK-TN, a multi-centre phase II trial, with integrated prospective ctDNA surveillance by digital PCR, enrolled patients with early-stage TNBC and residual disease following neoadjuvant chemotherapy, or, stage II/III with adjuvant chemotherapy. ctDNA surveillance comprised three monthly blood sampling to 12 months (18 months if samples were missed due to COVID), and ctDNA+ patients were randomised 2:1; intervention:observation. ctDNA results were blinded unless patients were allocated to intervention, when staging scans were done and those free of recurrence were offered pembrolizumab. A protocol amendment (16/09/2020) closed the observation group; all subsequent ctDNA+ patients were allocated to intervention. Co-primary endpoints were i) ctDNA detection rate ii) sustained ctDNA clearance rate on pembrolizumab (NCT03145961).

Results

208 patients registered between 30/01/18 - 06/12/19, 185 had tumour sequenced, 171 (92·4%) had trackable mutations, and 161 entered ctDNA surveillance. Rate of ctDNA detection by 12 months was 27·3% (44/161,95%CI:20·6-34·9). Seven patients relapsed without prior ctDNA detection. 45 patients entered the therapeutic component (intervention n=31; observation n=14; 1 observation patient was re-allocated to intervention following protocol amendment). Of patients allocated intervention, 72% (23/32) had metastases on staging at time of ctDNA+, and 4 patients declined pembrolizumab. Of the five patients who commenced pembrolizumab, none achieved sustained ctDNA clearance.

Conclusion

c-TRAK-TN is the first prospective study to assess whether ctDNA assays have clinical utility in guiding therapy in TNBC. Patients had a high rate of metastatic disease on ctDNA detection. Findings have implications for future trial design, emphasising the importance of commencing ctDNA testing early, with more sensitive and/or frequent ctDNA testing regimes.

Key words: Breast cancer, ctDNA, pembrolizumab, molecular residual disease

Highlights

- We report the first study to prospectively assess ctDNA for molecular residual disease detection in breast cancer
- Implementation of MRD detection with personalised assays was clinically achievable
- The rapid relapsing nature of high-risk triple negative breast cancer challenged implementation of MRD detection
- Treatment with pembrolizumab did not result in ctDNA clearance, although assessment was limited by small numbers of patients
- Recommendations for implementation of MRD detection in future studies are given

INTRODUCTION

After longstanding recognition of unmet clinical need, substantial progress has been made in recent years in the management of early-stage triple negative breast cancer (TNBC). Neoadjuvant chemotherapy reduces the risk of relapse, and enables down-staging to reduce extent of surgery.[1] The addition of carboplatin to neoadjuvant chemotherapy, but not the PARP inhibitor veliparib, improves the rates of pathological complete response and disease-free survival.[2,3] The addition of pembrolizumab to a carboplatin containing regimen further improves pathological complete response rates and disease-free survival.[4,5] With improved disease-free survival rates using intensive neoadjuvant chemotherapy, the challenge now is to develop new methods to identify patients who remain at high risk of relapse after treatment completion, and who may therefore benefit from further therapy.

Retrospective studies have shown that detection of circulating tumour DNA (ctDNA) in plasma, after patients have completed therapy for early-stage breast cancer, is associated with a very high risk of relapse.[6-9] With digital PCR mutation tracking median lead-time to relapse was 10·7 (95% CI, 8·1-19·1) months overall in breast cancer, and 10·6 months in TNBC.[7] By tracking multiple mutations with sequencing, median lead-time to relapse was 8·9 months overall in breast cancer.[6] As imaging is generally not conducted in follow-up following breast cancer treatment, due to historical studies that showed no improvement in survival with imaging and tumour marker surveillance,[10, 11] these retrospective ctDNA studies have assessed the lead-time from ctDNA detection to clinical relapse. The extent to which these tests provided lead-time over radiological relapse was unknown, as is whether intervening at the point of ctDNA detection could improve outcome.

We designed the c-TRAK TN trial to establish the potential for ctDNA guided therapy, and it is to our knowledge the first prospective study to assess whether ctDNA assays have clinical utility in guiding further therapy for patients with breast cancer.

METHODS

Study design and participants

c-TRAK TN was a multi-centre phase II trial with integrated prospective ctDNA surveillance and therapeutic components. Eligible patients had early-stage TNBC and either residual disease following neoadjuvant chemotherapy and surgery, or tumour size >20mm and/or axillary lymph node involvement with primary surgery and adjuvant chemotherapy. For subsequent pre-specified analysis of ctDNA detection rates by risk of relapse, patients were divided into moderate and high risk groups. If there was residual microscopic or macroscopic invasive cancer in the axillary nodes after neoadjuvant chemotherapy, or if tumour size was >50mm with involved axillary nodes and/or ≥4 nodes were positive with primary surgery and adjuvant chemotherapy, patients were classified as being high risk. Remaining eligible patients were classified as moderate risk. TNBC was classified as oestrogen receptor (ER) negative, progesterone receptor (PgR) negative (if available) (both defined by Allred score 0/8 or 2/8 or stain in <1% of cancer cells) and HER2 negative (immunohistochemistry 0/1+ or negative by *in situ* hybridisation) as determined by the local laboratory. All patients provided two archival tumour tissue samples, where available, either from diagnostic biopsy and/or primary surgery.

Patients eligible for the study had personalised digital PCR ctDNA assays designed and were enrolled onto prospective ctDNA testing. Following assay design, patients had samples taken at baseline and three-monthly for 24 months. ctDNA surveillance commenced after patients had completed adjuvant chemotherapy and radiotherapy (if indicated), within 4 weeks of treatment end or completion of ctDNA assay design. Active ctDNA surveillance, with prospective testing, was planned for samples taken up to the 12-month timepoint. Samples from the 15-month timepoint onwards were collected for retrospective analysis, to inform on optimal duration of ctDNA surveillance in future trials. Due to the COVID-19 pandemic, ctDNA analysis was suspended from 19/03/2020 – 04/06/2020. Patients who missed active surveillance timepoints due to the suspension had active ctDNA surveillance extended up to the 18-month timepoint to replace missed timepoints, with the final timepoint of retrospective ctDNA remaining at 24 months.

The original trial design blinded ctDNA analysis. On occurrence of a ctDNA positive result, patients were allocated to the intervention or observation groups in a 2:1 ratio using minimisation, with a random element and balanced for time at which the result occurred (baseline vs. 3-12 months) and whether the patient received adjuvant capecitabine (yes vs. no). Only those allocated to intervention were notified of a positive ctDNA result, with those allocated to continuing ctDNA surveillance as per patients who remained ctDNA negative. Patients allocated to intervention were invited to complete staging investigations with the same imaging conducted at diagnosis, or by local standards for staging. Those without imaging-detected metastatic recurrence were eligible to commence pembrolizumab. Patients with imaging-detected metastatic recurrence were treated as per standard-of-care off trial. Those randomised to observation continued to provide three-monthly blood samples until 24 months with standard follow-up until relapse. As per international standard practice, regular follow-up imaging was not performed during the study. Imaging was only performed at the time of initial ctDNA detection for patients allocated to the intervention group or as clinically appropriate.

In the second part of the study, on the advice of the Independent Data Monitoring Committee (IDMC) in response to the poor outcome of patients after ctDNA detection, the observation group was closed and all subsequent ctDNA positive patients in active surveillance were allocated to intervention. Recruitment to the ctDNA surveillance component was already complete prior to the implementation of this protocol amendment (16/09/2020); at which time 40 patients had already become ctDNA positive and were randomised in the therapeutic component. Patients in the observation group who were still in the active surveillance period were re-allocated to intervention at the time of their next positive ctDNA test.

When the trial commenced, patients were able to enrol within six weeks of the end of adjuvant radiotherapy or three months of surgery following neoadjuvant chemotherapy, or three months of last cycle of adjuvant chemotherapy (capecitabine included), whichever was later. Following initial observations of high rate of metastatic disease at ctDNA detection, it was noted that these criteria resulted in patients entering later than was anticipated (Supplementary Table S1). In an

amendment implemented on 06/08/2019, when 154 patients had already been enrolled, entry criteria were updated. Subsequently patients were enrolled within six weeks of surgery following neoadjuvant chemotherapy, and before, or on the day of, the 3rd cycle of adjuvant chemotherapy. Patients were registered before or during radiotherapy, and patients who received both neoadjuvant and adjuvant chemotherapy were enrolled within six weeks of surgery and before capecitabine if given. The amendment also allowed patients to start ctDNA surveillance after completing adjuvant chemotherapy and before or during radiotherapy (if indicated).

The study was sponsored by The Institute of Cancer Research (ICR) and approved by a research ethics committee (17/SC/0090). All participants gave written informed consent before registration for ctDNA surveillance, and again before treatment. The trial was managed and analysed by the ICR Clinical Trials and Statistics Unit (ICR-CTSU) working in partnership with the clinical Chief Investigator and Trial Management Group. Emerging disease outcome, safety and activity data were reviewed regularly by an IDMC. Trial oversight was provided by an independent Trial Steering Committee.

ctDNA testing

DNA was extracted from one archival tumour sample, and subject to targeted sequencing principally with the RMH200 gene panel that sequenced 200 cancer genes,[12] or the ABC-BIO 41 gene sequencing panel.[13] One or two mutations were selected from sequencing for digital PCR design (ThermoFisher Custom Taqman Assay Design Tool), and digital PCR assays validated on the DNA from the original tumour sample, DNA extracted from the second tumour sample, and buffy coat DNA. Only assays positive on both tumour samples, to identify likely clonal mutations, and negative on buffy coat, to exclude possible clonal haematopoiesis mutations, were taken forward to ctDNA surveillance.

At each ctDNA timepoint 40mls blood was collected in Streck tubes, mailed to the central lab, and centrifuged on the day of receipt. DNA was extracted from 4mls plasma samples for each

mutation tracked using Qiagen Circulating DNA nucleic acid kit. All DNA extracted from 4mls plasma was analysed by digital PCR on a BioRad QX200 digital PCR instrument as described previously.[7] For each ctDNA timepoint analysed, DNA was additionally extracted from buffy coat, and analysed in parallel, with assays rejected if mutations were identified in buffy coat as control for clonal haematopoiesis of indeterminate potential.[14] ctDNA was detected if at least two mutant (FAM) droplets were identified, using criteria previously established.[7] For ctDNA detected samples, a separate sample from the same timepoint was analysed to confirm ctDNA detection (Supplementary Figure S1).

ctDNA surveillance initially commenced after completion of all therapy (except for adjuvant bisphosphonate if given), and within four weeks of confirmation of a trackable mutation. Following the amendment implemented on 06/08/2019, ctDNA testing commenced after completing adjuvant (non-capecitabine) chemotherapy, and could commence before or during radiotherapy, and within four weeks of confirmation of a trackable mutation. ctDNA testing commenced three months after initiation of capecitabine.

Treatment

Patients allocated to pembrolizumab treatment, without imaging-detected metastatic relapse, completed screening for adequate haematology, renal and hepatic function, and absence of previous auto-immune diseases. Patients were treated with pembrolizumab 200mg intravenously every three weeks for one year, or until disease recurrence, intolerable adverse effects, or patient request to stop treatment. Blood samples for ctDNA analysis were collected at every cycle, or three monthly if patients stopped pembrolizumab before relapse. After starting pembrolizumab, imaging was only performed if clinically indicated. Toxicity was assessed after each cycle using National Cancer Institute Common Terminology Criteria for Adverse Events version 4.

Outcomes

The study had two primary objectives, to assess whether digital PCR ctDNA assays could predict which patients were at highest risk of relapse and identify patients with molecular residual disease not visible on imaging, and to assess whether pembrolizumab resulted in a sustained clearance of ctDNA in patients who have completed treatment for moderate or high-risk early stage triple negative breast cancer, but have molecular residual disease (MRD) detected by serially assessed ctDNA analysis./span> The primary endpoints were i) rates of positive ctDNA detection by 12 and 24 months from start of ctDNA surveillance, and ii) the proportion of patients with absence of detectable ctDNA (and absence of disease recurrence) six months after commencing pembrolizumab. Additional endpoints included time to ctDNA detection, rates of detection of overt metastatic disease at the time of first ctDNA detection in patients allocated to pembrolizumab, lead-time between ctDNA detection and disease recurrence in the pembrolizumab treatment and observation group, proportion of patients with absence of detectable ctDNA (and disease recurrence) after six months in the observation group, and safety and tolerability of pembrolizumab. The study was reported once all patients had completed active ctDNA surveillance and were assessable for ctDNA clearance on pembrolizumab.

Statistical analysis

The sample size for the study was initially planned under an A'Hern single-staged design, requiring 30 patients allocated to intervention in order to achieve 90% power to detect a ctDNA clearance rate of 54% (p1) over an unacceptable clearance rate of 30% (p0), with a 10% significance level. The associated width of the 95% confidence interval detectable around p1 was ±0·17. Rejection of p0 required ≥13 patients with ctDNA clearance. In order to randomize 30 patients to intervention, 200 registrations were required for an anticipated trackable mutation rate of 75% (150 patients entering ctDNA surveillance), and a subsequent ctDNA positivity rate of 30%, in order to randomize 45 patients in a 2:1 ratio to intervention/span> and observation. Sample sizes were also calculated for a lower rate and higher rate of ctDNA positivity of 24% and 45% respectively as a contingency. The study was not powered for formal comparison between randomized treatment groups. After the protocol amendment implemented on 16/09/2020, all subsequent ctDNA positive patients (during active surveillance) were allocated to intervention.

The proportion of patients with positive ctDNA detection by 12 months, from start of ctDNA surveillance included all patients with a positive ctDNA result at any point up to the 12-month timepoint, or on extended active surveillance up to the 18 month timepoint if the 12-month sample was missed. A sensitivity analysis excluding positive cases during extended active ctDNA surveillance was conducted. This proportion was further estimated using Kaplan-Meier (KM) methods to account for censoring prior to 12 months. Patients were censored at date of recurrence, withdrawal or discontinuation of ctDNA surveillance (whichever occurred first), or at their latest follow-up date. Time to ctDNA detection was calculated from the date that the site was informed the patient could commence ctDNA surveillance. In a sensitivity analysis, interval censoring was applied in cases where ctDNA is detected immediately after missed samples. Estimates are accompanied by 95% confidence intervals (CI).

Lead-time between ctDNA detection and disease recurrence was calculated using KM methods, separately for intervention and observation groups. Lead-time in the intervention group was calculated for intention-to-treat (ITT) and per-protocol analysis populations. ITT analyses included all patients allocated to intervention regardless of whether they commenced treatment. Per-protocol analyses only included patients who commenced pembrolizumab. Patients who crossed over from observation to the intervention group were included in the observation analysis, and censored at the point the site were informed of the positive result that triggered re-allocation. Recurrences included loco-regional or distant recurrence, but excluded new primary cancers. Median lead-time is reported with 95% CI. Formal comparisons between treatment groups were not planned due to differing schedules of disease recurrence detection.

The proportion of patients with metastatic disease at the time of ctDNA detection in the intervention group were to be presented with 95% CI. Additionally, estimates of the proportion of patients who had metastatic disease at the time of starting ctDNA surveillance were calculated separately by risk group, by multiplying the rate of baseline ctDNA detection with the rate of metastatic disease detection in each group. Absence of detectable ctDNA and disease

recurrence six months after commencement of pembrolizumab was calculated using the sample collected closest to, and preceding, the six-month time point from first dose of treatment. Patients must have been negative for ctDNA at this timepoint only, and also be free of disease recurrence by this timepoint. Proportions are presented with associated exact 95% CIs.

Additional unplanned exploratory analyses were conducted to identify factors that contributed to the high rate of metastatic disease detection at ctDNA positivity. Given the unanticipated later enrolment early in the trial (Supplementary Table S1), and to take account of variability in time of study entry, ctDNA detection rates were re-analysed in calendar windows, with time from surgery in patients who received neoadjuvant chemotherapy, or from date of last cycle of chemotherapy in patients who received adjuvant chemotherapy (Figure 4). The first window was for 0 – 1·5 months to capture baseline cases, with intervals spanning three months thereafter (1·5 – 4·5, 4·5 – 7·5 etc.). To provide a more accurate analysis, the baseline samples were excluded from analysis for late-registered patients (as per the amendment 06/08/2019), as well as all samples with an immediately preceding missed sample. Furthermore, the proportion of patients with metastatic disease at time of ctDNA detection was calculated within each calendar window, with the denominator including those ctDNA positive within that calendar window and subsequently allocated to the pembrolizumab group. A separate rate of ctDNA detection was calculated for samples taken during adjuvant capecitabine treatment, with an associated 95% CI.

Safety and tolerability of pembrolizumab was assessed for the safety population; all patients who received at least one dose of pembrolizumab. Adverse events were coded by preferred term according to version 20·1 of the Medical Dictionary for Regulatory Activities Terminology (MedDRA). Adverse events were presented if >25% patients experienced any grade, or all adverse events of grade ≥2.

Results

ctDNA surveillance

A total of 208 patients were registered between 30/01/18 and 06/12/19 from 15 UK hospitals, 185/208 (88.9%) had tumour sequenced, 171/185 (92·4%) had trackable mutations, and 161/171 (94.2%) entered ctDNA surveillance (Figure 1). Of the ten who had trackable mutations but did not start ctDNA surveillance, eight relapsed before ctDNA surveillance could start, one withdrew from the study, and one was ineligible (Figure 1). Of 310 assays designed from tumour sequencing, 230 passed validation; 48 assays were positive in the germline, 24 assays failed quality control, 5 were positive in unmatched healthy plasma, and for 3 assays the mutation was not detected in the second tumour sample. Of the patients entering ctDNA surveillance, 53 had two mutations, and 108 had one mutation tracked, with the most common mutation being in *TP53*, reported in 144/161 (89.4%) patients (Supplementary Table S2). At the time of reporting, all patients had completed active ctDNA surveillance, and median follow-up in study (from the notification that patients can commence ctDNA surveillance) was 20·4 months. Clinical and pathological characteristics of the patients entering ctDNA surveillance were typical of this patient group (Table 1). The rate of ctDNA detection by 12 months (from start of surveillance) was 27·3% (44/161, 95% CI 20·6-34·9). Detection of ctDNA was highly reproducible between repeat samples (Supplementary Figure S1). ctDNA detection rates from baseline, three, six, nine, and 12 month ctDNA samples were 23/161 (14·3%), 6/115 (5·2%), 6/99 (5·1%), 7/84 (8·3%), and 2/84 (2·4%) respectively (Supplementary Table S3). An additional two patients had ctDNA detected on COVID extended active surveillance at 15 (1/51, 2%) or 18 months (1/11, 9%). These two patients were ctDNA negative at 12 months, and therefore did not contribute to the primary endpoint. Seven patients relapsed without prior ctDNA detection, although of these, two had ctDNA detected at an additional sample taken after recurrence, and the other five did not have a recurrence sample collected. Sites of recurrence in the seven patients who relapsed without prior ctDNA detection are listed in Supplementary Table S4. Kaplan-Meier estimates of ctDNA detection rates at 12 months were 26.4% (95%CI 20.1-34.1) for all patients (Figure 2A); 55·7% (95%CI 42·8-69·5) in patients with high risk disease and 11·8% (95%CI 6·9-19·8) in patients with moderate risk disease (Figure 2B). In a sensitivity analysis, accounting for patients ctDNA positive after missed samples using interval censoring, the estimate was 57·8% (95%CI 44·7-71·4) in high risk patients and was unchanged for moderate risk patients.

Forty-five ctDNA positive patients entered the therapeutic component of the trial, initially 31 to intervention and 14 to observation. One patient initially allocated to observation was re-allocated to intervention after the 09/20 amendment. Of the patients allocated to intervention, 71·9% (23/32, 95%CI: 53·3-86·3) had metastatic disease on staging at time of ctDNA detection with little difference between risk groups (69·6% and 77·8% in high risk and moderate risk patients respectively). Staging was predominantly using CT (n=16), with PET-CT (n=3), bone scan (n=2), pleural fluid cytology (n=1) and biopsy (n=1) as other methods of identification. At the time of ctDNA detection, median levels of ctDNA detected was 3·1 copies/ml (range 0·1 – 1145·6) in patients with concurrent metastatic disease on imaging, and 1·0 copies/ml (range 0·2 – 8·9) in patients without metastatic disease on imaging (Supplementary Figure S2).

Pembrolizumab treatment

Median lead-time between ctDNA detection and disease recurrence in the intervention group was 1·6 months (95%CI 1·2-4·9) (Supplementary Figure S3A-B). Of the nine patients with ctDNA detected and no metastatic disease on staging scans, four declined to start pembrolizumab. Of these four patients, two withdrew from the study and two subsequently relapsed, with ctDNA trajectories in Supplementary Figure S3C)

Five patients commenced pembrolizumab treatment, and as per the pre-specified criteria for activity evaluation none achieved ctDNA clearance at six months, and all subsequently relapsed. One patient demonstrated possible evidence of pembrolizumab activity with ctDNA falling through treatment, after an initial treatment interruption to manage an immune adverse event (Figure 3A). Pembrolizumab adverse effects were consistent with prior reports (Supplementary Table S5).

Observation group

In the patients allocated to observation, median time from ctDNA detection to recurrence was 4·1 months (95% CI: 3·2-not-defined) (Supplementary Figure S4). ctDNA clearance after six

months occurred in 21·4% (3/14, 95%CI 4·7-50·8) of the observation group. There was substantial variability in the rise of ctDNA between first detection and follow-up, ranging from 212-fold rise, to stability and fall to undetectable. In part, ctDNA clearance in the observation group likely reflected patients whose ctDNA remained approximately stable at and below the level of detection, with occasional stochastic detection and apparent clearance of ctDNA (Figure 3B). Two patients were ctDNA negative at all timepoints after initial ctDNA positive result, and at the time of data base lock had not relapsed clinically. Although unknown, these could conceivably be initial false positive results reflecting a specificity of 99·8% ((total assays run-2)/ total assays run)

Analysis of factors contributing to high rates of metastatic disease at ctDNA detection

In the exploratory calendar window analysis, of time from surgery/completing adjuvant chemotherapy to ctDNA surveillance timepoint, the highest rate of ctDNA detection occurred in the 0 – 1·5 month window with 11·3% (6/53) samples positive. Rates of metastatic disease detection were high in the early timepoints, and a general decline in metastatic disease rates was demonstrated at later timepoints, though there were small sample sizes at later timepoints (Figure 4).

We noted that high-risk patients were particularly likely to have ctDNA detected at baseline (Figure 2), with 2·8% (3/107) of moderate risk patients and 37·0% (20/54) of high risk patients ctDNA positive at baseline. Of the patients allocated to intervention on the baseline sample, 50·0% (1/2) of moderate risk patients and 76·9% (10/13) of high risk patients has metastatic disease. We therefore estimated that at least 1·4% of moderate risk patients and 28·5% of high risk patients already had metastatic disease by the time of baseline ctDNA assessment. After three months of capecitabine, approximately three months prior to scheduled end, 18·2% (4/22) patients had ctDNA detected.

DISCUSSION

The c-TRAK TN study is the first prospective study to investigate the potential of ctDNA guided therapy in the follow-up of patients with triple negative breast cancer. ctDNA was detected at the rate anticipated, with 27·3% patients having ctDNA detected during the first 12 months, but far fewer patients than anticipated started pembrolizumab treatment. This was because the rate of metastatic disease detection at the point of ctDNA detection was substantially higher than anticipated, with additional patients not wishing to start treatment in part exacerbated by the COVID-19 pandemic.

Neoadjuvant pembrolizumab is now approved for higher risk TNBC.[4] For patients with urothelial cancer, atezolizumab may improve relapse free survival in patients that are ctDNA positive after surgery.[20] Insufficient patients were treated in this study to assess potential activity of pembrolizumab guided by ctDNA, with no patients clearing ctDNA on treatment. Work published on PD(L)1 antibody therapy in treating advanced cancer, suggests that drops in ctDNA after starting therapy, not requiring full clearance, are associated with improved outcome.[21, 22] One patient in our study had a sustained fall in ctDNA on pembrolizumab, providing limited evidence of activity. Only 3 patients were *BRCA1/2* germline mutation carriers and none received adjuvant olaparib due to the timing of recruitment relative to the publication of the Olympia adjuvant olaparib phase III study results, therefore the impact of olaparib on ctDNA clearance remains unknown.

What factors contributed to the high level of metastatic disease detection?

We estimated that at least 28·5% of patients with high-risk disease who enrolled on the study already had metastatic disease at baseline ctDNA analysis. This was not the case in moderate risk patients (1·4% rate), reflecting the differing incidence of ctDNA detection at baseline. Recent analysis by Symanns and colleagues emphasises the very rapid risk of relapse of triple negative disease of high residual cancer burden after neoadjuvant chemotherapy, with approximately 50% of RCB-3 patients relapsing clinically within a year of surgery.[15] The lead-time in the observation group was shorter than in our previous study,[7] likely reflecting the recruitment of higher risk

patients into this study. Nonetheless, metastatic disease detection rates were still high during subsequent timepoints.

What are the messages for future clinical trial design in triple negative breast cancer?

Patients should be enrolled as early as possible into ctDNA testing, aiming to reduce the number of patients who have already relapsed at the time of first ctDNA sampling. For MRD monitoring, this would involve starting personalised assay design as rapidly as possible after neoadjuvant chemotherapy and surgery, and for patients who have upfront surgery during adjuvant chemotherapy so that ctDNA testing can start immediately after. Future studies could also consider imaging with high sensitivity modalities at enrolment. Alternatively, future clinical trials can investigate ctDNA monitoring during (neo)adjuvant chemotherapy, as ctDNA detection during or at the end of neoadjuvant chemotherapy, and prior to surgery, is associated with a high risk of future relapse.[18]

Assays of higher sensitivity through, for example, tracking multiple mutations per patient to allow lower level of ctDNA detection, [6, 16, 17] and with further development targeting potentially 100s of variants per panel,[17] will improve detection of very low levels of ctDNA, and will likely improve rates of detection prior to metastatic relapse. It may also be that in highly proliferative TNBC, more frequent ctDNA detection than three monthly is required in the first 0-6 months of testing. A relatively high proportion of patients tested had become ctDNA positive after three months treatment with capecitabine, indicating resistance to therapy that has developed at this timepoint, potentially demonstrating the relative inactivity of capecitabine in high-risk patients.[19] Waiting for patients to complete the full six months of treatment will lose lead-time for these patients. Finally, the rate of relapse of high-risk patients justifies testing novel therapies without the need to enrich further with ctDNA detection.

The approach we piloted in this study would benefit from further investigation, with appropriate modifications to patient population and ctDNA testing regimes, to select patients for adjuvant systemic therapy. A phase III study is assessing the potential for ctDNA to guide therapy in TNBC

and *BRCA1/2* germline mutation breast cancer with the PARP inhibitor niraparib (NCT04915755), and will provide a fuller assessment of this approach.

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References

1 Loibl S, Poortmans P, Morrow M et al. Breast cancer. Lancet 2021; 397 (10286): 1750-1769.

2 Loibl S, O'Shaughnessy J, Untch M et al. Addition of the PARP inhibitor veliparib plus carboplatin or carboplatin alone to standard neoadjuvant chemotherapy in triple-negative breast cancer (BrighTNess): a randomised, phase 3 trial. Lancet Oncol 2018; 19 (4): 497-509.

3 Geyer CE, Sikov WM, Huober J et al. Long-term efficacy and safety of addition of carboplatin with or without veliparib to standard neoadjuvant chemotherapy in triple-negative breast cancer: 4-year follow-up data from BrighTNess, a randomized phase III trial. Ann Oncol 2022.

4 Schmid P, Cortes J, Pusztai L et al. Pembrolizumab for Early Triple-Negative Breast Cancer. N Engl J Med 2020; 382 (9): 810-821.

5 Schmid P, Cortes J, Dent R et al. Event-free Survival with Pembrolizumab in Early Triple-Negative Breast Cancer. N Engl J Med 2022; 386 (6): 556-567.

6 Coombes RC, Page K, Salari R et al. Personalized Detection of Circulating Tumor DNA Antedates Breast Cancer Metastatic Recurrence. Clin Cancer Res 2019; 25 (14): 4255-4263.

7 Garcia-Murillas I, Chopra N, Comino-Mendez I et al. Assessment of Molecular Relapse Detection in Early-Stage Breast Cancer. JAMA Oncol 2019.

8 Garcia-Murillas I, Schiavon G, Weigelt B et al. Mutation tracking in circulating tumor DNA predicts relapse in early breast cancer. Sci Transl Med 2015; 7 (302): 302ra133.

9 Magbanua MJM, Swigart LB, Wu HT et al. Circulating tumor DNA in neoadjuvant-treated breast cancer reflects response and survival. Ann Oncol 2021; 32 (2): 229-239.

10 Impact of follow-up testing on survival and health-related quality of life in breast cancer patients. A multicenter randomized controlled trial. The GIVIO Investigators. JAMA 1994; 271 (20): 1587-1592.

11 Rosselli Del Turco M, Palli D, Cariddi A et al. Intensive diagnostic follow-up after treatment of primary breast cancer. A randomized trial. National Research Council Project on Breast Cancer follow-up. JAMA 1994; 271 (20): 1593-1597.

12 Pascual J, Lim JSJ, Macpherson IRJ et al. Triplet therapy with palbociclib, taselisib and fulvestrant in PIK3CA mutant breast cancer and doublet palbociclib and taselisib in pathway mutant solid cancers. Cancer Discov 2020.

13 Pearson A, Proszek PZ, Pascual J et al. Inactivating NF1 mutations are enriched in advanced breast cancer and contribute to endocrine therapy resistance. Clin Cancer Res 2019.

14 Razavi P, Li BT, Brown DN et al. High-intensity sequencing reveals the sources of plasma circulating cell-free DNA variants. Nat Med 2019; 25 (12): 1928-1937.

15 Symmans WF, Yau C, Chen YY et al. Assessment of Residual Cancer Burden and Event-Free Survival in Neoadjuvant Treatment for High-risk Breast Cancer: An Analysis of Data From the I-SPY2 Randomized Clinical Trial. JAMA Oncol 2021.

16 Cutts Rea. Molecular residual disease detection in early stage breast cancer with a personalized sequencing approach. Cancer Research 2021; Proceedings: AACR Annual Meeting 2021: Abstract 536.
17 Parsons HA, Rhoades J, Reed SC et al. Sensitive Detection of Minimal Residual Disease in Patients Treated for Early-Stage Breast Cancer. Clin Cancer Res 2020; 26 (11): 2556-2564.
18 Magbanua MJM, Li W, Wolf DM et al. Circulating tumor DNA and magnetic resonance imaging to predict neoadjuvant chemotherapy response and recurrence risk. NPJ Breast Cancer 2021; 7 (1): 32.
19 Mayer IA, Zhao F, Arteaga CL et al. Randomized Phase III Postoperative Trial of Platinum-Based Chemotherapy Versus Capecitabine in Patients With Residual Triple-Negative Breast Cancer Following Neoadjuvant Chemotherapy: ECOG-ACRIN EA1131. J Clin Oncol 2021; 39 (23): 2539-2551.
20 Powles T, Assaf ZJ, Davarpanah N et al. ctDNA guiding adjuvant immunotherapy in urothelial carcinoma. Nature 2021; 595 (7867): 432-437.
21 Zhang Q, Luo J, Wu S et al. Prognostic and Predictive Impact of Circulating Tumor DNA in Patients with Advanced Cancers Treated with Immune Checkpoint Blockade. Cancer Discov 2020; 10 (12): 1842-1853.
22 Cabel L, Riva F, Servois V et al. Circulating tumor DNA changes for early monitoring of anti-PD1 immunotherapy: a proof-of-concept study. Ann Oncol 2017; 28 (8): 1996-2001.

Table 1. Baseline clinical and pathological characteristic of patients enrolled into ctDNA surveillance

Figure 1. Consort Diagram of c-TRAK TN study

Figure 2. ctDNA detection over time

A. Rates of ctDNA detection in the whole study population, months from start of ctDNA surveillance

B. Rates of ctDNA detection in moderate and high risk patients, months from start of ctDNA surveillance

Figure 3. ctDNA trajectories in patients on pembrolizumab treatment and on observation.

A. ctDNA trajectories in patients on pembrolizmab treatment. One patient (pink), had had an interruption of pembrolizumab to manage adverse events, with a subsequent fall in ctDNA. No clearance of ctDNA was observed

B. ctDNA trajectories in patients on observation.

Figure 4. Time interval analysis of ctDNA detection rates

ctDNA detection rate (blue) and overt metastatic disease detection of imaging (red), with samples re-analysed from time from surgery, for patients who had neoadjuvant chemotherapy, or time from last cycle of adjuvant chemotherapy, for patients who had primary surgery. Calendar windows defined as ±1·5 months from each time point

Appendix A:

Study collaborators: Professor S. Chan (Nottingham University Hospital), Dr A. Armstrong (The Christie), Dr F. Raja (University College London Hospital), Professor P. Schmid (St Bartholomew's), Professor A. Tutt (Guy's and St Thomas', Dr R. Roux (Churchill Hospital, Oxford), Dr S. Cleator (Imperial College Healthcare NHS Trust).

Appendix B:

Data sharing

De-identified individual participant data, together with a data dictionary defining each field in the set, will be made available to other researchers on request. Trial documentation including the protocol are available on request by contacting c-trak-tn-icrctsu@icr.ac.uk.

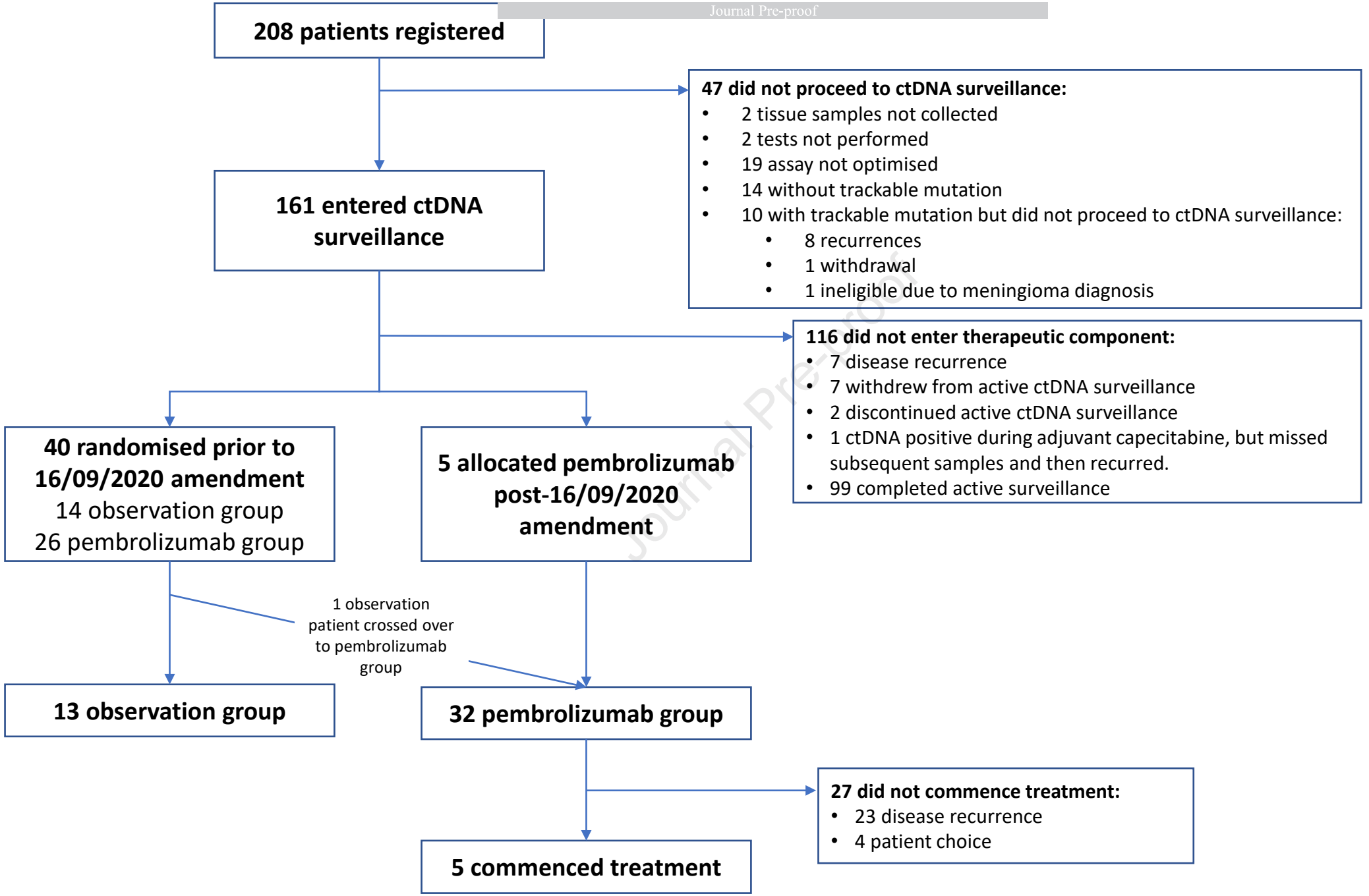
The ICR-CTSU supports the wider dissemination of information from the research it conducts, and increased cooperation between investigators. Trial data is collected, managed, stored, shared and archived according to ICR-CTSU Standard Operating Procedures to ensure the enduring quality, integrity and utility of the data. Formal requests for data sharing are considered in line with ICR-CTSU procedures, with due regard given to funder and sponsor guidelines. Requests are via a standard proforma describing the nature of the proposed research and extent of data requirements.

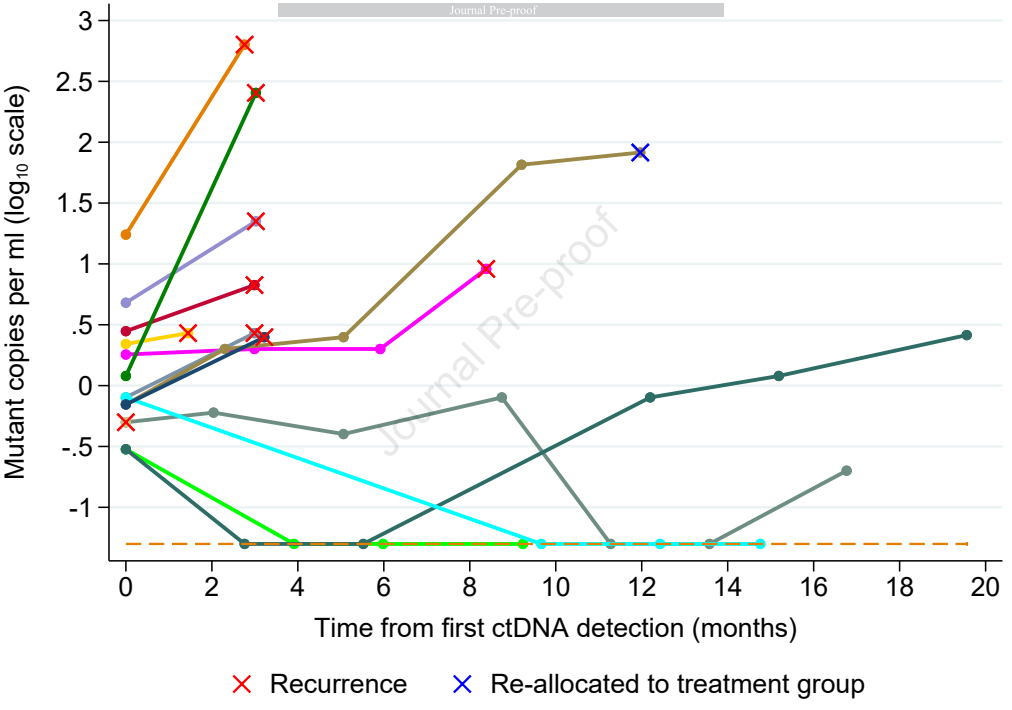
Data recipients are required to enter a formal data sharing agreement, which describes the conditions for release and requirements for data transfer, storage, archiving, publication and Intellectual Property. Requests are reviewed by the Trial Management Group (TMG) in terms of scientific merit and ethical considerations including patient consent. Data sharing is undertaken if proposed projects have a sound scientific or patient benefit rationale, as agreed by the TMG and approved by the Independent Data Monitoring and Steering Committee, as required. Restrictions relating to patient confidentiality and consent will be limited by aggregating and

anonymising identifiable patient data. Additionally, all indirect identifiers that may lead to deductive disclosures will be removed in line with Cancer Research UK Data Sharing Guidelines.

Table 1. Baseline clinical and pathological characteristic of patients enrolled into ctDNA surveillance

		High risk (N=54)		Moderate risk (N=107)		Total (N=161)	
		n	%	n	%	n	%
Type of definitive surgery	Mastectomy	30	55·6	25	23·4	55	34·2
	Breast conserving surgery (BCS)	24	44·4	82	76·6	106	65·8
Tumour grade at surgery	1 - Low	0	0·0	1	0·9	1	0·6
	2 – Intermediate	11	20·4	18	16·8	29	18·0
	3 – High	42	77·8	87	81·3	129	80·1
	Unobtainable	1	1·9	1	0·9	2	1·2
Tumour size at surgery (mm)	Median (IQR)	30 (17 – 52)		22 (14 – 30)		24 (15 – 38)	
Nodal status at surgery	Negative	0	0·0	82	76·6	82	50·9
	Microscopic disease	16	29·6	3	2·8	19	11·8
	Macroscopic disease	38	70·4	22	20·6	60	37·3
Chemotherapy received	Neoadj only	28	51·9	36	33·6	64	39·8
	Adjuvant only	8	14·8	38	35·5	46	28·6
	Neoadj & adjuvant capecitabine	12	22·2	29	27·1	41	25·5
	Neoadj and other adjuvant chemo	6	11·1	4	3·7	10	6·2
BRCA germline mutation result	No known mutation	28	96·6	67	93·1	95	94·1
	BRCA1 mutation	1	3·4	2	2·8	3	3·0
	BRCA2 mutation	0	0·0	3	4·2	3	3·0




Journal Pre-proof
Mutant copies per ml (log10 scale)
3
2.5
2
1.5
1
.5
0
-.5
-1
0
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4
6
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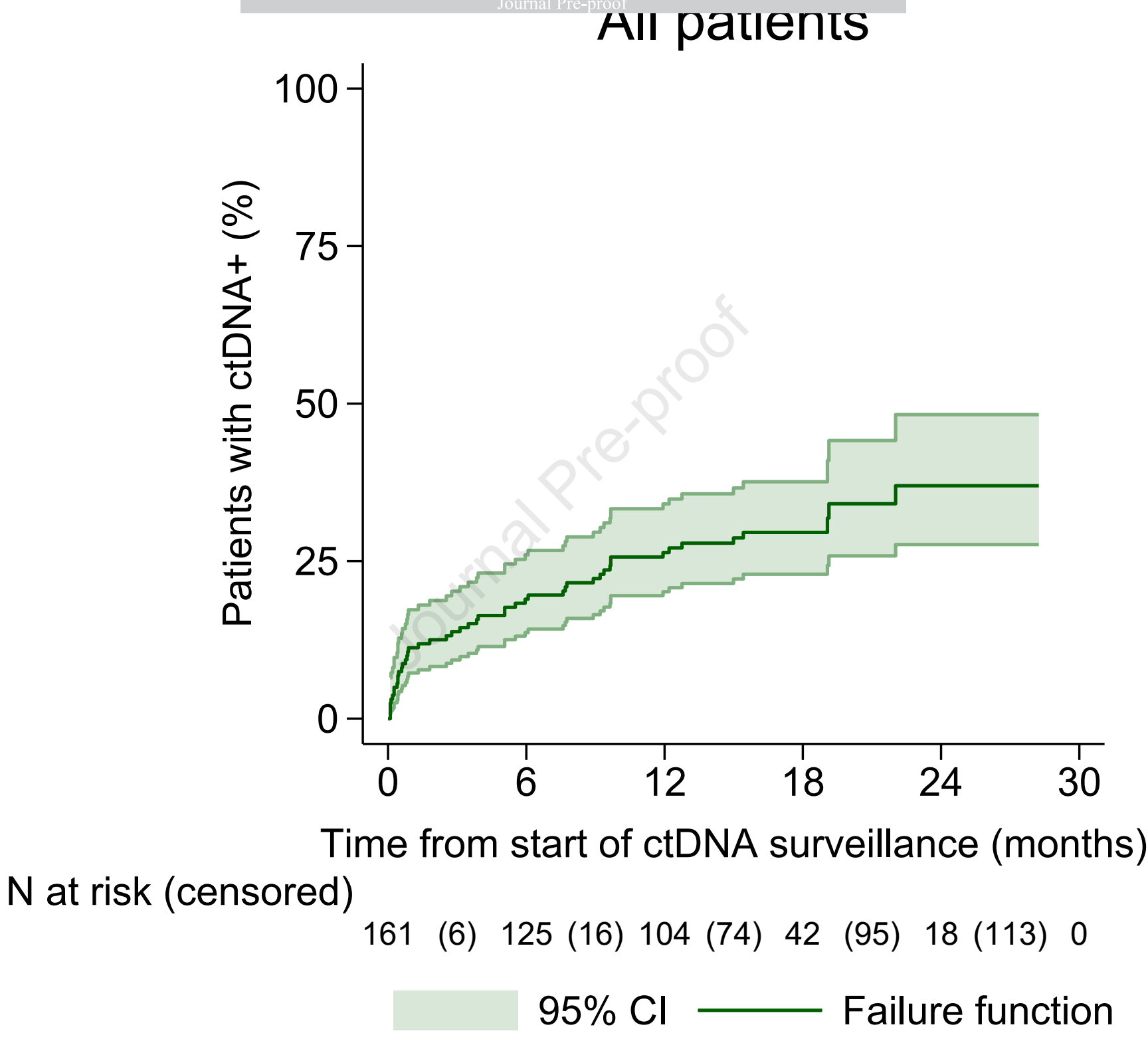
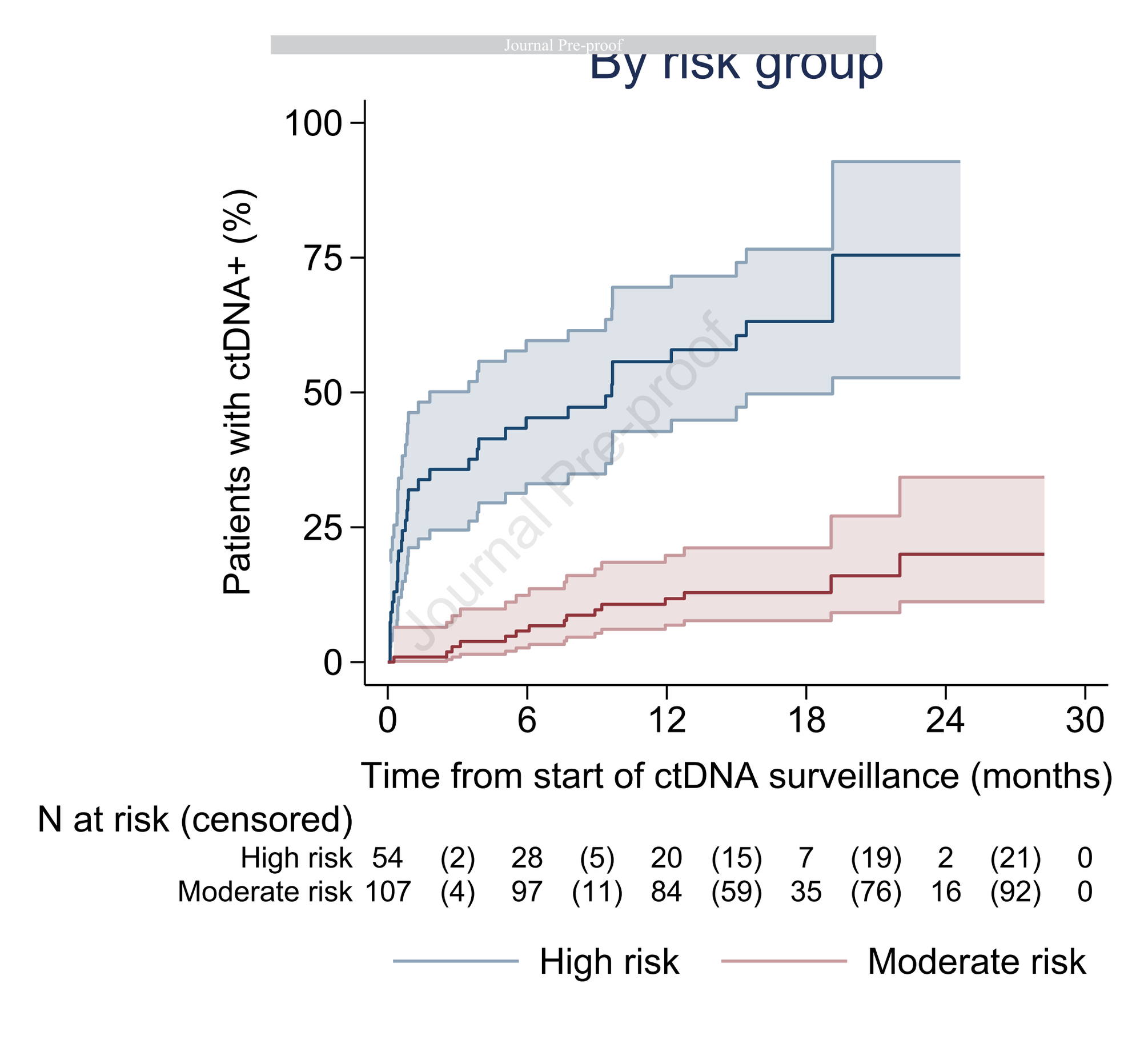
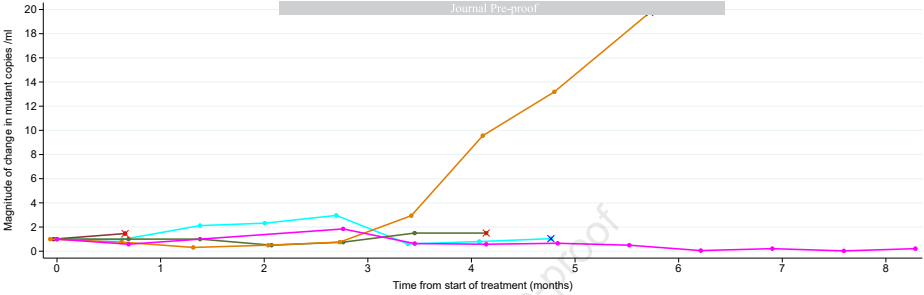
10
12
14
16
18
20
Time from first ctDNA detection (months)
Recurrence
Re-allocated to treatment group







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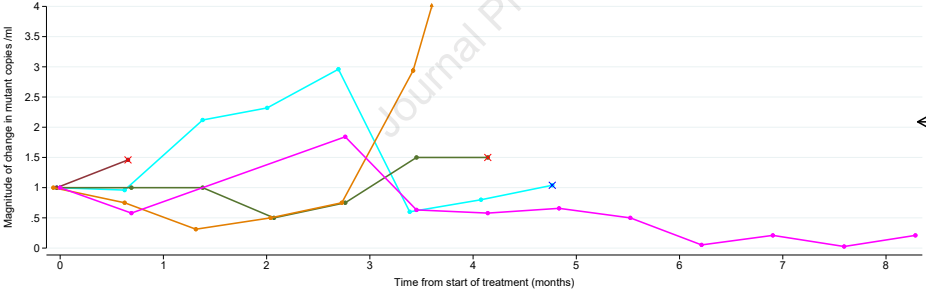

Discontinued treatment due to:

× Disease progression × Adverse event