

Title: Comparison of circulating tumor DNA assays for Molecular Residual Disease detection in early-stage triple negative breast cancer.

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Disclosures

N Turner reports an Investigator Initiated Research grant from Merck Sharp & Dohme during the conduct of the study. Outside the submitted work advisory board honoraria from Astra Zeneca, Bristol-Myers Squibb, Lilly, Merck Sharpe and Dohme, Novartis, Pfizer, Roche/Genentech, GlaxoSmithKline, Zentaris pharmaceuticals, Repare therapeutics, Arvinas and research funding from Astra Zeneca, BioRad, Pfizer, Roche/Genentech, Merck Sharpe and Dohme, Guardant Health, Invitae, Inivata, Personalis, Natera. C Pipinikas, P Rojas & W Emmett are employees of NeoGenomics Ltd., Cambridge, UK. I Macpherson reports consulting fees from Pfizer UK, Pierre Fabre, Eisai, Celldex, Daiichi Sankyo, Roche UK, Novartis UK, MSD, Gilead, Astra Zeneca UK, In3Bio, Stemline Therapeutics and Owkin; payment or honoraria for lectures, presentations, speakers bureaus, manuscript writing or educational events from Novartis UK, Roche UK, Genomic Health, Novartis, Eli Lilly and Astra Zeneca; support for attending meetings and/or travel from Eisai, Roche UK, Eli Lilly, Daiichi-Sankyo, Gilead and Novartis; receipt of equipment, materials, drugs, medical writing, gifts or other services from Eisai; outside the submitted work. A

Okines reports research funding grants from Roche and Pfizer; payment or honoraria from Lilly, Seagen, Esai, Pfizer, Gilead, AstraZeneca and Daiichi-Sankyo; support for attending meetings from Lilly, Roche and AstraZeneca; participation in advisory boards for Roche, Seagen, Pfizer, AstraZeneca and Daiichi-Sankyo, outside the submitted work. C Palmieri reports grant funding from Daiichi Sankyo, Pfizer and Seagen; Travel support from Gilead and Roche; Payment or honoraria for lectures, presentations, speakers bureaus, manuscript writing or educational events from AstraZeneca, Daiichi Sankyo, Eli Lilly, Exact Sciences, Gilead, Novartis, Pfizer and Roche outside the submitted work. D Wheatley reports honoraria for advisory boards from Roche, AstraZeneca, Gilead and Novartis, outside the submitted work. S Waters reports advisory consulting fees from Novartis, Sanofi-Aventis, Daiichi-Sankyo, Gilead, Eli Lilly; Meeting chair for Novartis, Gilead, outside the submitted work. MW reports Payment or honoraria for lectures, presentations from Novartis, Gilead, Roche, Eli Lilly, Pfizer, Genomic Health/Exact Sciences; outside the submitted work. J Bliss reports investigator-initiated research grants (IIR) from Merck Sharpe & Dohme and ICR/RM NIHR Biomedical Research Centre, during the conduct of the study. Outside the submitted work reports grants and non-financial support from AstraZeneca, Puma Biotechnology, Clovis Oncology, Pfizer, Janssen-Cilag, Novartis, Eli Lilly and Roche.

Abstract:

Purpose: Detection of circulating tumor DNA (ctDNA) in patients who have completed treatment for early-stage breast cancer is associated with a high risk of relapse, yet the optimal assay for ctDNA detection is unknown.

Experimental design: The cTRAK-TN clinical trial prospectively used tumor informed digital PCR (dPCR) assays for ctDNA molecular residual disease (MRD) detection in early-stage triple negative breast cancer. We compared tumor informed dPCR assays with tumor informed personalized multi-mutation sequencing assays in 141 patients from cTRAK-TN.

Results: MRD was first detected by personalized sequencing in 47.9% of patients, 0% first detected by dPCR, and 52.1% with both assays simultaneously ($p < 0.001$, Fisher's exact test). The median lead time from ctDNA detection to relapse was 6.1 months with personalized sequencing and 3.9 months with dPCR ($p = 0.004$, mixed effects Cox model). Detection of MRD at the first timepoint was associated with a shorter time to relapse compared with detection at subsequent timepoints (median lead time 4.2 vs 7.1 months, $p = 0.02$).

Conclusions: Personalized multi-mutation sequencing assays have potential clinically important improvements in clinical outcome in the early detection of MRD.

Statement of significance:

Key to the implementation of ctDNA assays for cancer MRD detection, is an understanding of which ctDNA assays characteristics are important. Through inter-assay comparison in a prospective trial, we show that increased analytical sensitivity, in this case via personalized multi-mutation sequencing, results in clinically meaningful improvements in clinical performance. These findings emphasize the importance of implementing the most sensitive ctDNA detection assays in MRD detection clinical trials.

Introduction

Substantial advances have been made in the adjuvant treatment of patients with early-stage breast cancer, with approximately 85% of patients cured by current treatment. Further improvements in outcomes for breast cancer, without substantial over-treatment of patients already cured by current therapy will require improved ways of identifying those patients with a residual risk of relapse. Detection of circulating tumor DNA (ctDNA) in plasma following the completion of treatment, often referred to as molecular residual disease (MRD) detection, is associated with a high risk of future relapse. Detection of ctDNA is most frequently done with tumor informed assays, with sequencing of the tumor tissue to identify somatic variants used to develop personalized assays that track either a few mutations with digital PCR, or multiple mutations with error corrected sequencing. Tumor agnostic assays that utilize detection of aberrant tumor specific methylation, without needing tumor tissue sequencing, are also in development. With the increasing development of multiple ctDNA methods, comparison studies are urgently required to identify the characteristics that are clinically important and identify the optimal assays to implement in clinical trials, and future clinical practice.

The c-TRAK-TN prospective clinical trial identified patients with MRD following treatment for early-stage moderate to high-risk Triple Negative Breast Cancer (TNBC) and assessed the potential activity of further adjuvant therapy with pembrolizumab after MRD detection. Other clinical trials with a similar design are ongoing, such as IMvigor011 (NCT04660344) which aims to identify and treat patients with MRD following therapy for high-risk muscle invasive bladder cancer (1). There was a higher rate of metastatic disease at the point of MRD detection with dPCR in the c-TRAK TN clinical trial than anticipated (2), emphasizing the need to assess whether ctDNA assays with better sensitivity may lengthen the lead time from MRD detection to clinical relapse, and facilitate clinical trials designed to improve patient outcomes from interventions at the point of MRD detection.

Detection of MRD with ctDNA assays is challenging, as the levels of ctDNA in these patients may be very low, requiring ultrasensitive and highly specific assays (3). A broad range of ctDNA assays are currently available, and with only a limited cross-platform comparison of these technologies (4-7), it is in general unknown whether evidence from one assay can be safely applied to other assays. An American Society of Clinical Oncology (ASCO) position paper has highlighted the need for standardization in techniques and reporting, along with more studies on cross-assay comparisons (8).

Here, we performed an initial pilot to assess the ability of the tumor informed Residual Disease and Recurrence (RaDaR[®]) personalized sequencing assays (NeoGenomics) to detect MRD in

patients with breast cancer. Next, we compared RaDaR with prospectively conducted tumor informed digital PCR (dPCR) assays in the c-TRAK TN clinical trial.

Materials and Methods.

Samples

ChemoNEAR sample collection study (REC ID: 11/EE/0063) was a biological sample collection study sponsored by the Royal Marsden Hospital and approved by a research ethics committee (11/EE/0063). All patients gave written informed consent. Patients were eligible who had early breast cancer and were scheduled to receive neo-adjuvant chemotherapy. Blood samples were collected at baseline, after the first cycle of chemotherapy, post-surgery, every 3 months for the first year, and then every 6 months up to 5 years (9, 10).

The **c-TRAK-TN clinical trial** was a phase II multi-center clinical trial sponsored by the Institute of Cancer Research (NCT03145961) and approved by a research ethics committee (17/SC/0090). All patients gave written informed consent. Patients were eligible with early-stage TNBC with either residual disease following neoadjuvant chemotherapy and surgery or with a tumor size >20mm and/or axillary lymph node involvement prior to primary surgery and adjuvant chemotherapy. Following written informed consent, a tumor tissue sample was sequenced with a targeted assay, and personalized digital PCR assays designed for 1-2 mutations per patient. Following completion of standard therapy, patients had prospective digital PCR testing every three months for 2 years. If patients had ctDNA detected during the first 12 months of testing (or extended to 15- or 18- month time points if patients missed samples due to the COVID-19 pandemic), they were randomized between observation and pembrolizumab. The trial is described in greater detail in the primary trial report (2). The co-primary endpoints of the clinical trial were i) ctDNA detection rate at 12 months and ii) sustained ctDNA clearance rate on pembrolizumab (2).

Both studies were conducted in accordance with the Declaration of Helsinki and Good Clinical Practice.

Tissue whole exome sequencing (WES).

DNA was extracted from FFPE tissue after microdissection using the QIAamp DNA Investigator Kit (Qiagen, Manchester UK, Cat. no. 56504), and from buffy coat using QIAamp DNA Blood Mini Kit (Qiagen, Manchester UK, Cat. no. 51104). Sequencing libraries were prepared using KAPA HyperPlus kit with IDT UDI 8nt Adaptors (Integrated DNA Technologies). Adapter-ligated DNA fragments were validated using Agilent TapeStation (Agilent Technologies, Palo Alto, CA, USA RRID:SCR_019547), and quantified using High Sensitivity Qubit assay on Qubit 3.0 Fluorometer

(ThermoFisher Scientific, Waltham, MA, USA). Libraries were pooled and hybridized with SureSelectXT Human All Exon V6 Kit (Agilent, Santa Clara, CA, USA) at 65°C for 24 hours. Sequencing libraries were multiplexed and sequenced on a NovaSeq6000 (Illumina, San Diego, CA, USA).

Digital Polymerase Chain Reaction (dPCR) assay

DNA was extracted utilizing the QIAamp Circulating Nucleic Acid Kit (Qiagen, Manchester UK). ctDNA testing via dPCR assay was performed as previously described (2, 10). In brief, targeted panel sequencing from 2 FFPE samples was performed using the ABC-Bio or RMH- 200 gene panels and 1-2 variants were selected for the dPCR assay (11, 12). The Thermo Scientific Custom TaqMan® SNP Genotyping Assay design tool was employed for assay design. Assay optimization was performed with a ProFlex Thermal Cycler (Applied BioSystems), Automated Droplet Generator (Bio-Rad, Pleasanton, USA) and Droplet Reader (Bio-Rad, Pleasanton, USA). Two or more FAM positive droplets were required for the sample to be called positive. If a positive result was obtained, this was independently confirmed on a second sample. Copies/mL and allele fraction were calculated as previously described (13).

RaDaR personalized sequencing analysis

As previously described (14), somatic variants from WES were prioritized using proprietary algorithms to build patient specific primer panels of up to 48 primer pairs, and combined with 21 common population single nucleotide polymorphisms (SNPs) to facilitate panel QC. Where available, an aliquot of tumor DNA from FFPE tissue or pre-capture sequencing library for progression plasma which was subjected to WES was used to validate variants.

Plasma DNA extracted from a minimum of 2mL banked plasma was sequenced with personalized RaDaR assays. Plasma DNA samples were run alongside a buffy coat DNA control sample from the patient, which was used to identify and remove germline variants, remove variants due to clonal hematopoiesis of indeterminate potential (CHIP), and as a positive amplification control. RaDaR libraries were sequenced using a Nova-Seq 6000 system (Illumina Inc., San Diego, USA) and sequencing data analyzed in a multi-step process: fastq files were demultiplexed using Illumina bcl2fastq 2.20 software. Reads were aligned using the Burrows-Wheeler Aligner (BWA) (15) and processed using proprietary software to identify primer pairs and count mutant and reference bases. Variants present in the buffy coat material or absent in the tumor tissue DNA were excluded from further analysis.

Proprietary methods were used to determine if MRD was present or absent, from the primary MRD panel variants. As previously summarized, (14) a statistical model was used to assess the statistical significance of the observed mutant counts for each variant and the information was

integrated over the entire set of personalized variants to obtain evidence of tumor presence or absence at the sample level. A sample was determined to be positive for residual disease if its cumulative statistical score was above a pre-set threshold. The tumor fraction estimated from the model was then reported (estimated variant allele frequency, eVAF) along with the mean VAF across all variants. These values were closely correlated (Supplementary Figure 1, Pearson Correlation $R=0.99$, $p<0.001$).

Statistical analysis

Survival endpoints were calculated using the Kaplan–Meier method. Time to positive ctDNA detection was calculated as a survival endpoint from start of ctDNA surveillance to the first ctDNA positive result, with patients being censored after discontinuation of ctDNA surveillance. The proportion of patients with positive ctDNA detection by 12 months from study entry was calculated with the Kaplan–Meier method including all patients with information in both assays ($n=141$). A separate sensitivity analysis was performed using calendar windows from end of treatment (date of surgery for patients with neoadjuvant chemotherapy, or date of last chemotherapy with adjuvant chemotherapy). Lead time between ctDNA detection and disease recurrence was calculated in the subset of patients with a ctDNA positive result, from the date of the first ctDNA positive result to the date of relapse. Patients were censored at date of withdrawal or at their latest follow-up date. Median lead time and recurrence rate at 12 months were reported. A mixed effect Cox regression with patient ID as a random effect, to adjust by repeated measurements, was fitted to compare the time to relapse between the two assays. As a complementary analysis, the non-parametric paired-sample Wilcoxon test was calculated only in patients with relapse to evaluate whether the differences between the time to relapse according to each assay was different from 0.

The Fisher's exact test was used to compare the proportion of patients with MRD first detected using the RaDaR or the dPCR assay. To evaluate the diagnostic performance of RaDaR to predict relapses at 24-months: the sensitivity, specificity, positive and negative predictive values were calculated. To compare the RaDaR VAF values, a linear mixed models using patients ID as random intercept was used to deal with the repeated measurement data. To compare the maximum RaDaR VAF values across sites of relapse, pairwise comparisons using the Wilcoxon test and adjusting for multiple comparisons with the Benjamini & Hochberg method were reported. No data imputation was performed. The median follow-up was calculated using the reverse Kaplan-Meier method. The threshold for statistical significance was defined as 0.05 (two-sided). Statistical analyses were performed with the R statistical software version 4.1.2 and GraphPad Prism version 6.0 (RRID:SCR_002798).

Data availability

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 to the corresponding author, or by contacting c-trak-tn-icrctsu@icr.ac.uk. Trial documentation including the protocol are available on request by contacting c-trak-tn-icrctsu@icr.ac.uk.

The ICR-CTSUs support 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-CTSUs 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-CTSUs 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.

Results

Pilot study to assess the ability of RaDaR to detect MRD in patients with breast cancer.

We initially conducted a pilot study to assess the potential of personalized sequencing with RaDaR in early-stage breast cancer, using a cohort of 170 patients recruited into a tissue collection study, to identify 17 patients who had relapsed, and five patients had no known relapse as controls. These patients included those with TNBC, hormone receptor positive and HER2 overexpressed breast cancer (Supplementary Table 1). Using a whole-exome sequencing approach, archival tissue tumor samples were sequenced to median coverage 116x (13-227x), and matched germline buffy coat sequence to median coverage of 42x (8- 100x). Personalized sequencing panels with a median of 43 (9-61) variants were designed, and a median of eight plasma timepoints (2-12) were tested per patient, with 141 plasma timepoints in total (Figure 1A).

In this pilot study, ctDNA was detected in 39.7% (56/141) of plasma timepoints and was not detected in 60.3% (85/141) of plasma timepoints. As expected, the *mean Variant Allele Frequency (mean VAF)* was significantly higher in samples where MRD was detected ($p < 0.001$), but with some overlap at lower VAF ranges, reflecting detection of MRD with an error model specific to the individual mutations tracked (Figure 1B). From patients with a known clinical relapse, RaDaR detected MRD in 100% (17/17) patients prior to clinical relapse, and MRD was not detected in 100% (5/5) of the patients with no known clinical relapse (Figure 1C). The median relapse free survival was 16.8 months (95% CI 14.7-28.9 months) for patients who had MRD detected and was not reached (NR) in patients who did not have MRD detected ($p < 0.001$) (Figure 1D). The three patients who had Central Nervous System (CNS) only relapse, had a shorter time from ctDNA detection to relapse (4.1, 5.1 and 5.7 months). We concluded that the high sensitivity and lead time of RaDaR had the characteristics to take forward to formal comparison in the cTRAK-TN trial.

Cross assay comparison in the c-TRAK TN clinical trial cohort

c-TRAK-TN enrolled 161 patients into prospective digital PCR MRD surveillance (2). For 87.5% (141/161) of patients on MRD surveillance, plasma samples were available for retrospective orthogonal testing with the RaDaR personalized sequencing ctDNA assay. The orthogonal testing set was representative of the overall study population with a median follow up of 32.5 months (95%CI 27.5 - 34.8) (Supplementary table 2, 3 and 4). A median of 2 (range 1-3) formalin-fixed paraffin-embedded (FFPE) samples per patient underwent whole exome sequencing (WES) (226 samples in total), 28.3% (64/226) diagnostic biopsies and 71.7% (162/226) surgical specimens, with a median coverage of 155x (43-361x). Matched germline buffy coat was sequenced with a median coverage of 52x (20-127x). All patients had successful assays designed, tracking a median of 47 variants (range 33-56) per patient, and a total of 899 plasma timepoints were analyzed (median of 7 timepoints per patient [range 1 - 11]) by both RaDaR and dPCR assays (Figure 2A).

In total, 839/899 of plasma timepoints tested had concordant test results, giving an overall test agreement between RaDaR and dPCR assays of 93.3% (95%CI; 91.4% – 94.8%) (Figure 2B), Similar rates of agreement were found between RaDaR and dPCR assays that tracked one or two variants (Supplementary table 5). From a per patient perspective, concordant test results occurred in 92.9% (131/141) of patients, with 58.9% (83/141) of patients not having MRD detected by either assay, and 34.0% (48/141) had MRD detected by both assays, although potentially at different timepoints (Figure 2B). Of the 48 patients who had MRD detected by both assays, MRD was detected by RaDaR at an earlier timepoint than dPCR in 47.9% (23/48) patients, MRD was first detected at the same timepoint in 52.1% (25/48) of patients, and there were no cases where MRD was first detected by dPCR (Figure 2C, $p < 0.001$ Fisher's exact test).

The rate of ctDNA detection by 12 months after the end of treatment (date of surgery for neoadjuvant chemotherapy, date of last cycle of chemotherapy for adjuvant chemotherapy) with RaDaR was 36.9% (95% CI 28.3-45.5%) and with dPCR assays was 29.8% (21.5-37.1%) (Figure 3A). By 24 months, the total duration of ctDNA testing, 39.7% (56/141) of patients had MRD detected by a RaDaR assay and 35.5% (50/141) had MRD detected by a dPCR assay. First detection of ctDNA after 12 months after the end of treatment was rare with RaDaR, and more frequent with a dPCR assay (Figure 3B). For all patients where ctDNA was detected at a plasma timepoint prior to clinical relapse, the median lead time from ctDNA detection to relapse was 6.1 (95% CI 4.2-9.0) months with RaDaR and 3.9 (95% CI 2.8-6.5) months with dPCR ($p = 0.004$, mixed effects Cox model) (Figure 3C and Supplementary Figure 2). With the RaDaR assay, detection of ctDNA at the first time point was associated with shorter time to relapse, than detection at a subsequent time point (median lead time 4.2 vs 7.1 months, $p = 0.02$) (Figure 3D).

RaDaR assay to predict relapse at 24-months

To evaluate the diagnostic performance of RaDaR personalized sequencing assays, we assess its ability to predict clinical relapses at 24 months. Patients confirmed to be free of relapse at least 24 months from the start of the study, and patients with a relapse before 24 months were included in this analysis (80.9%, 114/141). Among those 114 patients, 47 (41.2%) had a clinical relapse, and 67 (58.8%) were relapse-free at 24 months. The sensitivity of RaDaR was 95.7% (95%CI 84.3% - 99.3%), with 45 out of 47 patients with relapse having a previous ctDNA positive result. The specificity was 91.0% (95%CI 80.9% - 96.3%), 61 out of 67 relapse-free patients did not have ctDNA detection during the follow-up, although assessment of specificity is limited by follow-up. The positive and negative predictive values were 88.2% and 96.8%, respectively (Supplementary Table 6).

Analysis of discordant test results

Discordant test results between RaDaR and dPCR assays were found in 7.1% (10/141) of patients, where eight patients had MRD detected by RaDaR but not by dPCR, and two patients had MRD detected by a dPCR but not by RaDaR. Relapses were reported within the follow up period for six of the eight patients who had MRD detected by RaDaR only, and for one of the patients who had MRD detected by a dPCR assay only. The median RaDaR VAF of variants with MRD detected by both assays was higher than those samples where MRD was detected by RaDaR only (0.28% vs 0.01%, $p < 0.001$) (Figure 4A).

Sites of relapse and ctDNA detection

We investigated whether the location of relapse impacted ctDNA levels and the ability of ctDNA to detect relapse. Patients with local recurrences or lung-only relapse had lower levels of ctDNA (*mean VAF in RaDaR*) detected prior to relapse compared to patients with multiple sites of disease ($p < 0.001$, adjusted pairwise comparisons). Patients with multiple sites of relapse had the highest ctDNA levels (median value of the maximum VAF 0.0256%), followed by CNS only (Figure 4B).

In patients with relapse detected only by RaDaR prior to relapse, both local and distant recurrences were reported at relapse. In the one patient where MRD was detected before relapse by a dPCR assay and not by RaDaR, the patient had CNS disease only at relapse. One patient had a local recurrence in the absence of ctDNA detection by dPCR or RaDaR assay prior to relapse (Figure 4C).

Discussion

Multiple ctDNA assays are in clinical development for detection of MRD, and it is vital to understand whether differences between assays have clinical consequences, and whether assays give similar or different results. Here, we show that personalized multi-mutation sequencing, with the RaDaR assay, frequently detects ctDNA at an earlier timepoint than digital PCR, resulting in improved lead-time over clinical relapse, without discernable impact on specificity. The improved clinical impact of RaDaR likely reflected the ability, through tracking a larger number of variants, to confidently detect ctDNA at substantially lower ctDNA levels, below the limit of detection of digital PCR.

We demonstrate that improved sensitivity of RaDaR also reduced the required duration of ctDNA surveillance in moderate to high risk TNBC, with most relapses detected within the first 12 months of completing standard therapy, potentially negating the need for ctDNA surveillance beyond this point in this clinical setting. This has both the potential for cost savings, with fewer timepoints needing testing, and allows a patient to complete ctDNA surveillance earlier, potentially achieving an earlier time to achieving an 'all clear'.

The cTRAK-TN trial demonstrated that at the time of ctDNA detection with digital PCR, most patients had already asymptotically relapsed when imaged. In aggressive tumor types such as high-risk triple negative breast cancer, we demonstrated that RaDaR could achieve early ctDNA assay detection in patients with TNBC. In addition, in the RaDaR pilot study, that included patients with hormone receptor positive disease, the median time from ctDNA detection to relapse was long at 16.8 months. However, only a future prospective study, or studies in clinical settings where serial imaging is routinely performed, could ascertain whether imaging at the point of ctDNA detection with RaDaR will identify patients without radiological recurrence.

Emerging data suggests that sites of relapse may differ in their release of ctDNA. Patients with lung-only metastases had lower levels of ctDNA detection, than other sites of disease. However, it must be highlighted that in this study imaging was conducted on those randomized to pembrolizumab, at the point of ctDNA detection via a dPCR assay, this may reflect the increased ability to detect low levels of recurrence in the lungs, with similarly low levels of disease less easily detectable in other sites, or could reflect lower shedding into the circulation from lung metastases. Interestingly, this finding concurs with the relatively low rates of ctDNA detection in patients with stage I/II primary non-small cell lung cancer (16), suggesting that reduced shedding is a contributory factor. A relatively high number of patients in the study had apparently local-only relapse, which may reflect the ability of imaging to detect local relapse, even though conceivably these patients had imaging occult distant disease that also contributed to ctDNA detection.

We report here a longer follow-up of ctDNA detection in the cTRAK-TN study than previously reported. With this longer follow-up, we find that patients with ctDNA not detected at the first timepoint, but detected at a subsequent timepoint, had a longer lead time from ctDNA detection to relapse. Intriguingly, this group also included a set of patients with a long time to relapse, with 34% patients not having progressed at 12 months from ctDNA detection. This identifies a subgroup of triple-negative breast cancer that is more indolent, at least in the MRD setting, and a heterogeneity in clinical behavior that was not previously appreciated, although the non-uniform approach to imaging in those randomized to pembrolizumab and observation limits the study of unique characteristics of patients who did not relapse during the study. It will be interesting in future research to understand the distinct biology of this group of patients, including assessing the gene expression profiles of their primary tumors, although we speculate this may reflect immune surveillance of micro-metastases.

In summary, we have demonstrated that the increased analytical sensitivity of personalized multi-mutation sequencing assays, translated to detection of MRD with a longer median time to relapse than dPCR assays in the c-TRAK TN clinical trial. This also resulted in substantially shorter required durations of ctDNA surveillance to detect future recurrences. These findings strongly support the implementation of personalized sequencing assays in clinical trials, but also emphasize the potential benefits of further assay development to further improve analytical sensitivity and detect lower levels of ctDNA and further improve ctDNA based MRD detection.

Acknowledgements

Funding

This work was supported in part by Le Cure, the Royal Marsden Cancer Charity. It was endorsed by Cancer Research UK (CRUKE/16/024) and the ICR Clinical Trials & Statistics Unit is supported by a core programme grant (grant number C1491/A15955) from Cancer Research UK. This study represents independent research funded by the National Institute for Health Research (NIHR) Biomedical Research Centre at The Royal Marsden NHS Foundation Trust and The Institute of Cancer Research, London. The views expressed are those of the authors and not necessarily those of the NIHR or the Department of Health and Social Care. This work was supported in part by provision of drug by the Investigator Initiated Studies Program of Merck Sharp & Dohme Limited. The opinions expressed in this paper are those of the authors and do not necessarily represent those of Merck Sharp & Dohme Limited. This work uses data provided by patients and collected by the NHS as part of their care and support. We thank the patients and all investigators and research support staff at the participating centres. We also thank our patient representatives from the Independent Cancer Patients' Voice patient advocate group, members of the Trial Management Group, Trial Steering Committee and Independent Data Monitoring Committee.

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Figure 1. Assessment of personalized multi-mutation sequencing in a pilot study

1. Consort diagram of samples selected in the pilot study to assess the ability of RaDaR to detect MRD in early-stage breast cancer.
2. RaDaR ctDNA mean Variant Allele Frequency (mean VAF) in samples with MRD detected, and MRD not detected, comparison with Mann-Whitney U test, and results on the y-axis log transformed.
3. Plasma timepoints and point of clinical relapse colored by ctDNA detected, from start of ctDNA surveillance for patients shown for patients with HER2 positive (HER2+) breast cancer (n=8), Hormone Receptor positive HER2 negative (HR+HER2-) (n=9) and Triple Negative Breast cancer (TNBC) (n=5). MRD was not detected in any patient who did not have a clinical relapse reported (n=5) and was detected prior to relapse in all patients who had a clinical relapse reported (n=17).
4. Relapse free survival (RFS) in patients with and without MRD detected. The MRD detected status was treated as a time-dependent covariate to avoid the immortal time bias. The log-rank test was used to estimate survival differences.

Figure 2. Comparison of ctDNA detection between digital PCR and RaDaR in cTRAK- TN

1. Consort diagram of the samples available to perform ctDNA MRD cross assay comparison in the c-TRAK TN clinical trial.
2. Comparison of ctDNA detection in all samples analyzed with digital PCR and RaDaR. (i) Of 899 samples, 113 were concordant positive and 726 were concordant negative, leading to a test agreement of 93.3% (95% CI; 91.4-94.8%). In total 60 samples were discordant, where 58 samples had MRD detected by RaDaR, but not by dPCR, and two samples had MRD detected by dPCR and not by RaDaR. (ii) Of 114 patients, 48 were concordant positive and 83 were concordant negative, leading to a test agreement rate of 92.2% (95% CI; 87.0-96.4%), Ten patients had discordant test results, with MRD detected by RaDaR only in eight cases, and by dPCR only in two cases.
3. Plasma timepoints and point of clinical relapse colored by ctDNA detected, from start of ctDNA surveillance for patients for patients with no plasma timepoints with MRD detected (left), which includes one patient who had a clinical relapse, and for patients who had any plasma timepoint which tested positive by either assay (right).

Figure 3. Clinical validity of ctDNA detection with *digital PCR and RaDaR in cTRAK-TN*

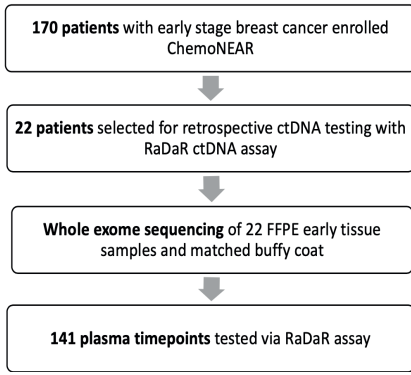
1. Time to ctDNA detection from the start of ctDNA surveillance. MRD: molecular residual disease.

2. ctDNA detection rates in calendar/time windows from the date of completion of standard treatment. The first window was for 0-1.5 months, with intervals spanning 3 months thereafter (1.5-4.5, 4.5-7.5, etc.). MRD: molecular residual disease.
3. Time from first ctDNA detection to relapse with dPCR and RaDaR assays. MRD: molecular residual disease.
4. Time from first RaDaR ctDNA detection to relapse, comparing patients with ctDNA detected at first timepoint and subsequent timepoints. MRD: molecular residual disease.

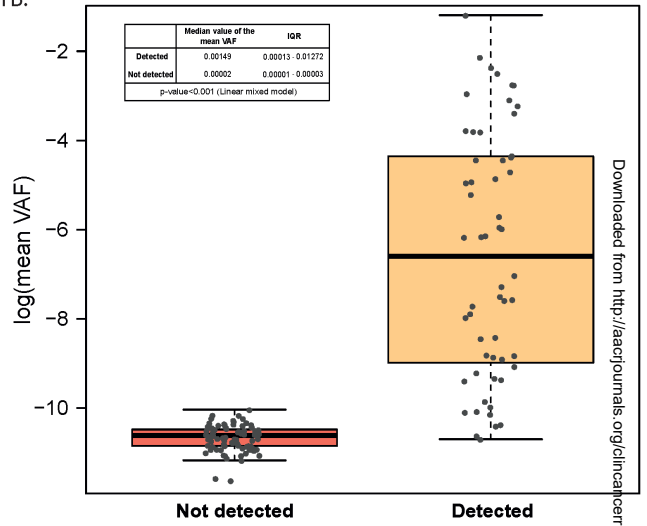
Figure 4 Analysis of factors contributing to discordance and ctDNA detection

1. RaDaR mean VAF split by timepoints, positive by dPCR only, positive by RaDaR only, or concordant results. Comparison between groups were performed using linear mixed models. Mean VAF is demonstrated on a log10 transformed axis.
2. RaDaR mean VAF by sites of relapse, in patients with site of relapse data (n=49), in patients with local-only, lung-only, CNS-only, other single sites of metastatic disease (including bone, liver and pleural), and multiple sites of disease. Pairwise comparisons using the Wilcoxon test and adjusting for multiple comparisons with the Benjamini & Hochberg were performed. For each patient the highest mean VAF across all plasma timepoints was used. Mean VAF is demonstrated on a log10 transformed axis.
3. Anatomical sites of disease at the time of relapse, for patients with MRD was not detected by either assay (n=1, local relapse), MRD detected by dPCR only (n=1, CNS relapse), and MRD detected by RaDaR only (n= 6, known sites of disease include lung, liver, and local relapses).

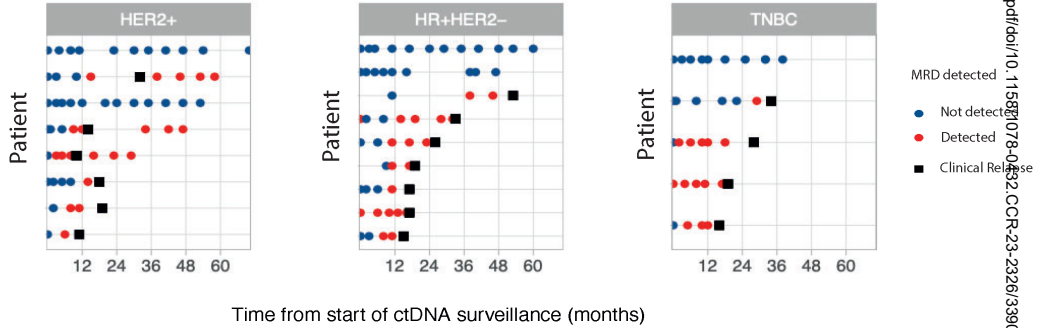
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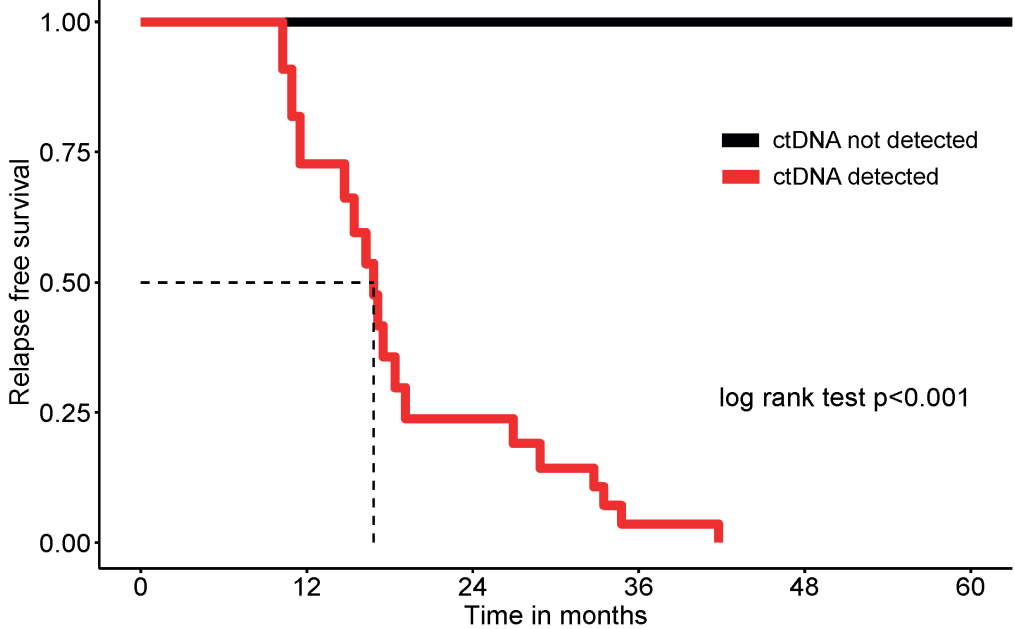
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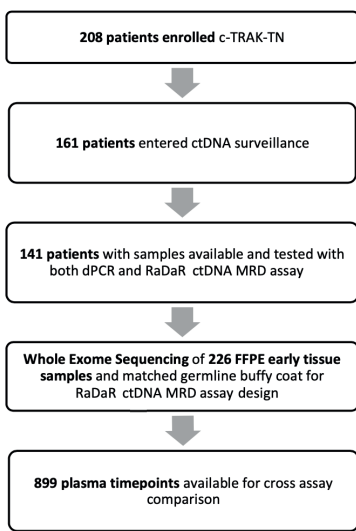
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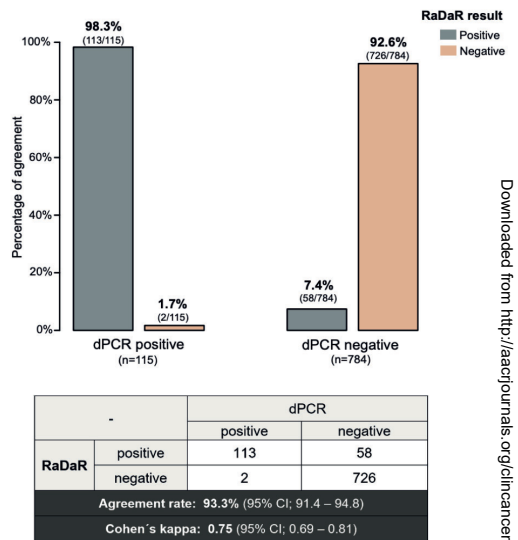
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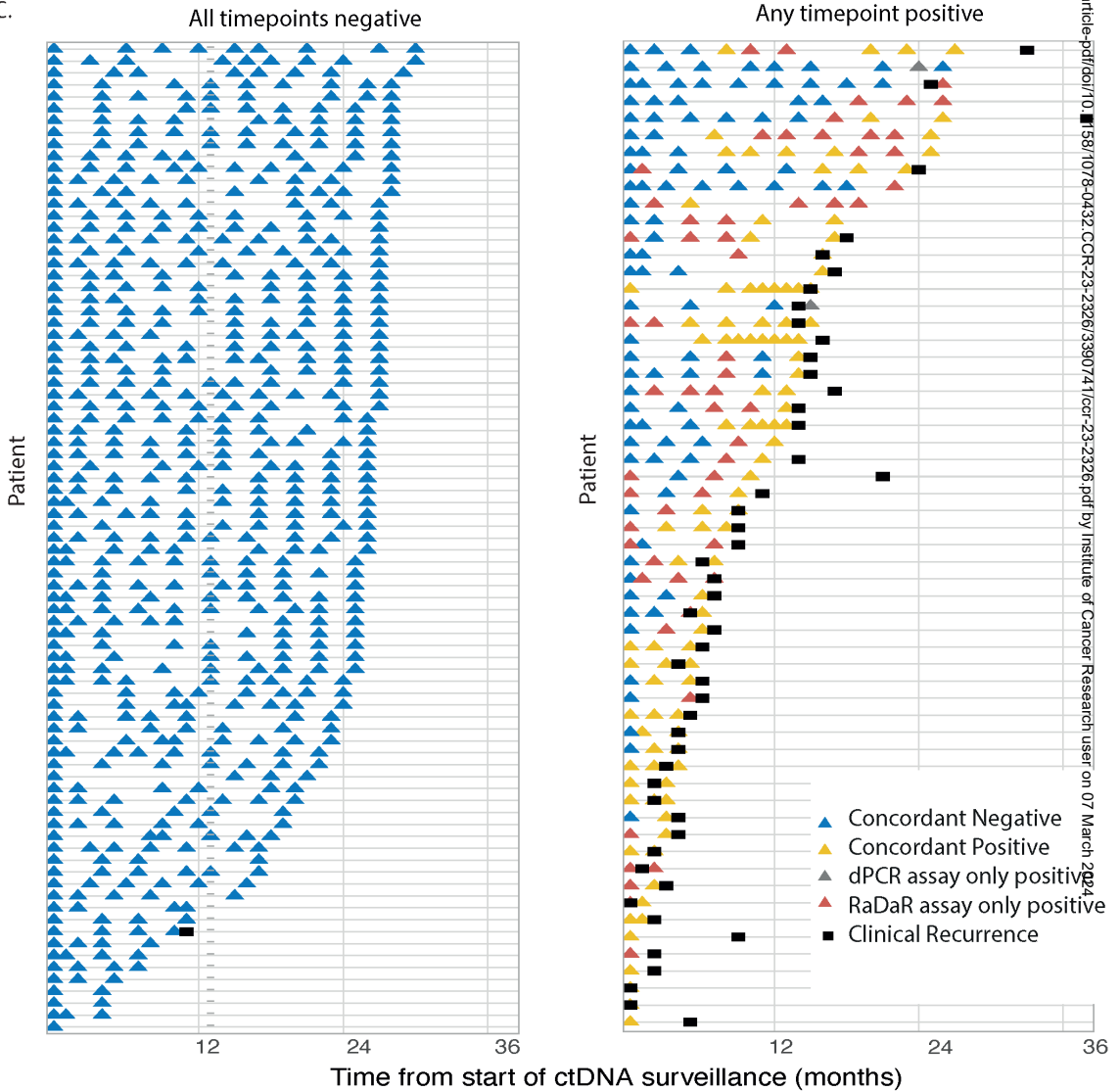
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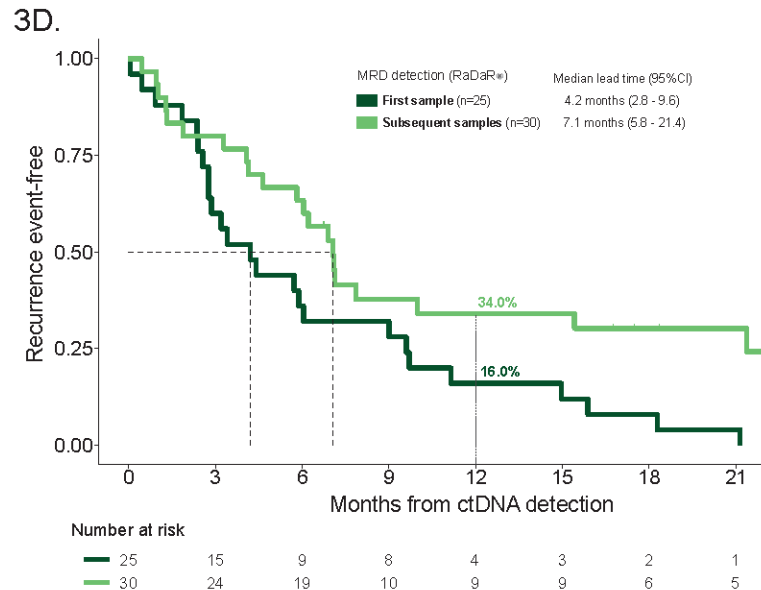
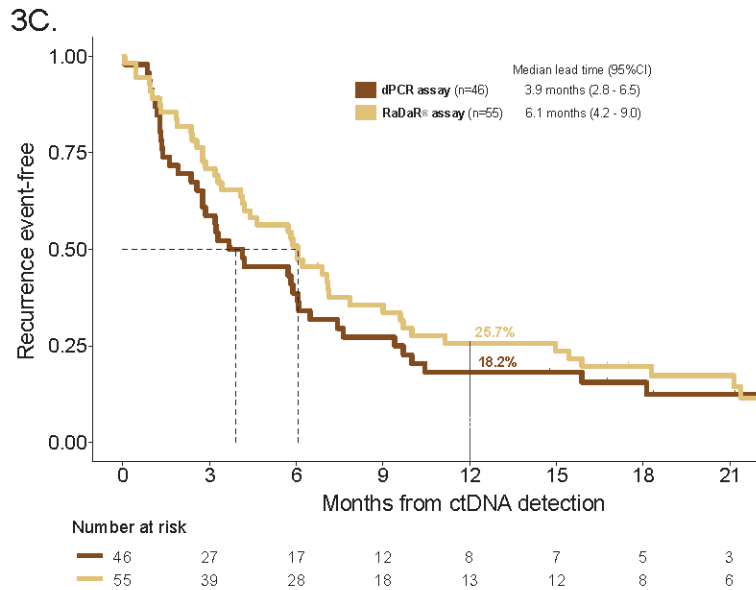
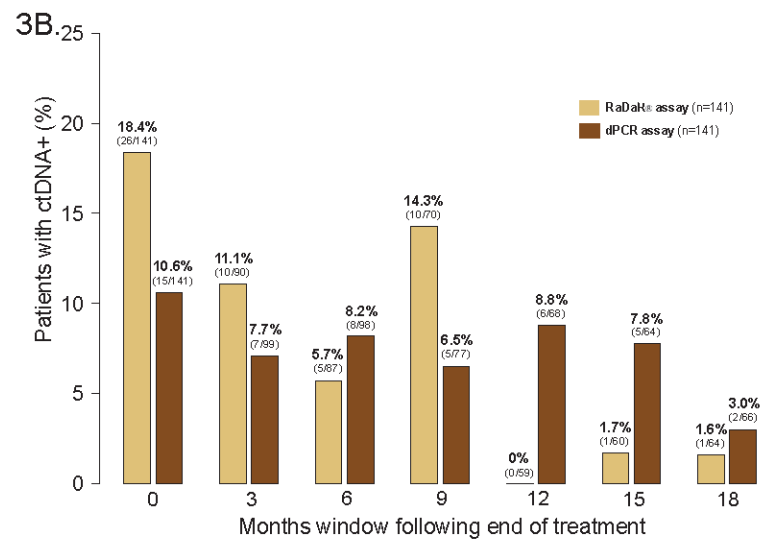
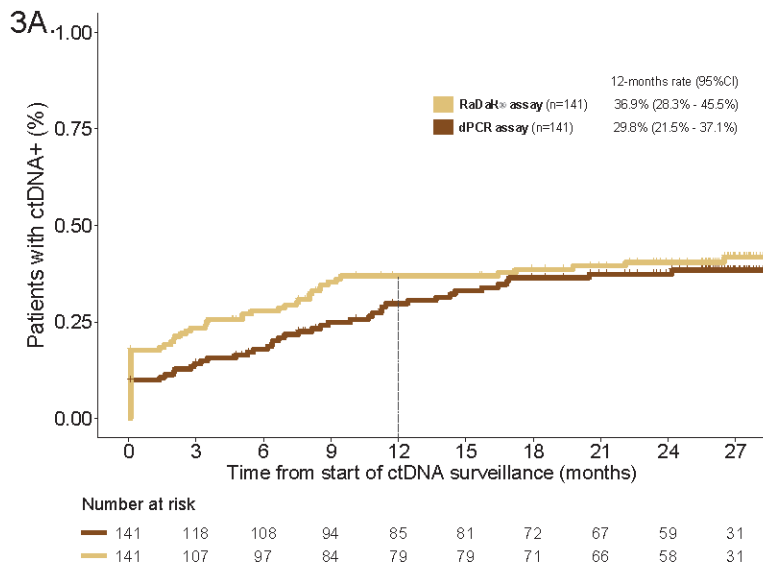


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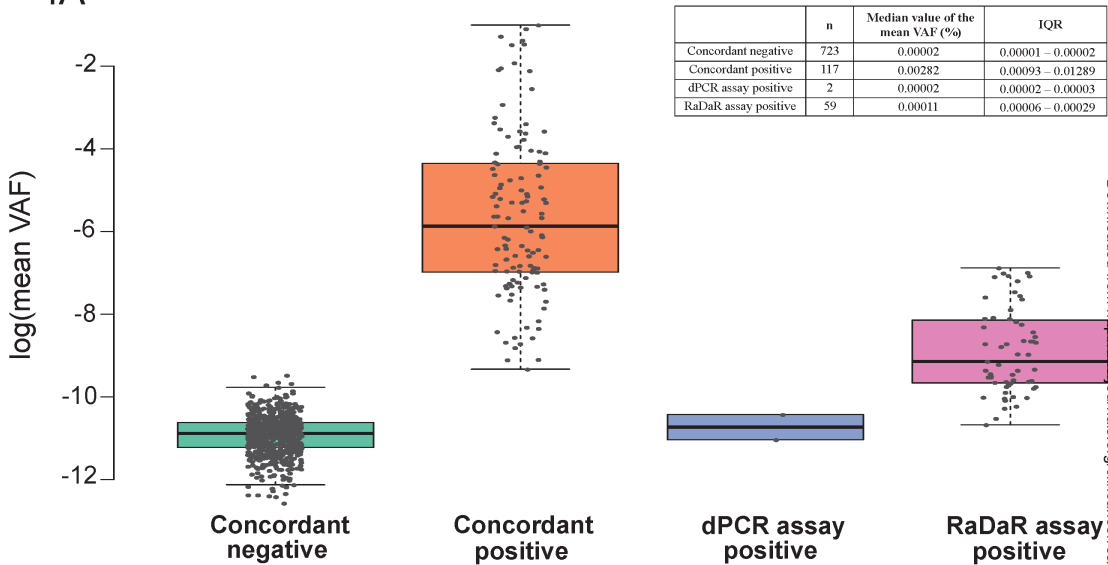


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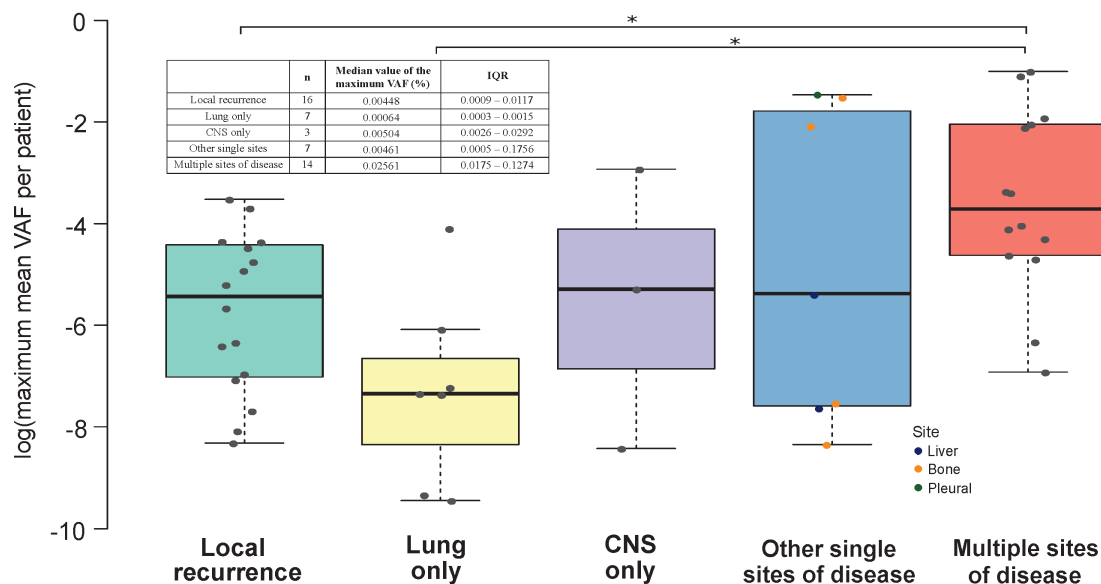




4A



4B



4C

ctDNA not detected by either assay



ctDNA detected by dPCR only



ctDNA detected by RaDaR only

