Residual ctDNA after treatment predicts early relapse in patients with early-stage non-small cell lung cancer

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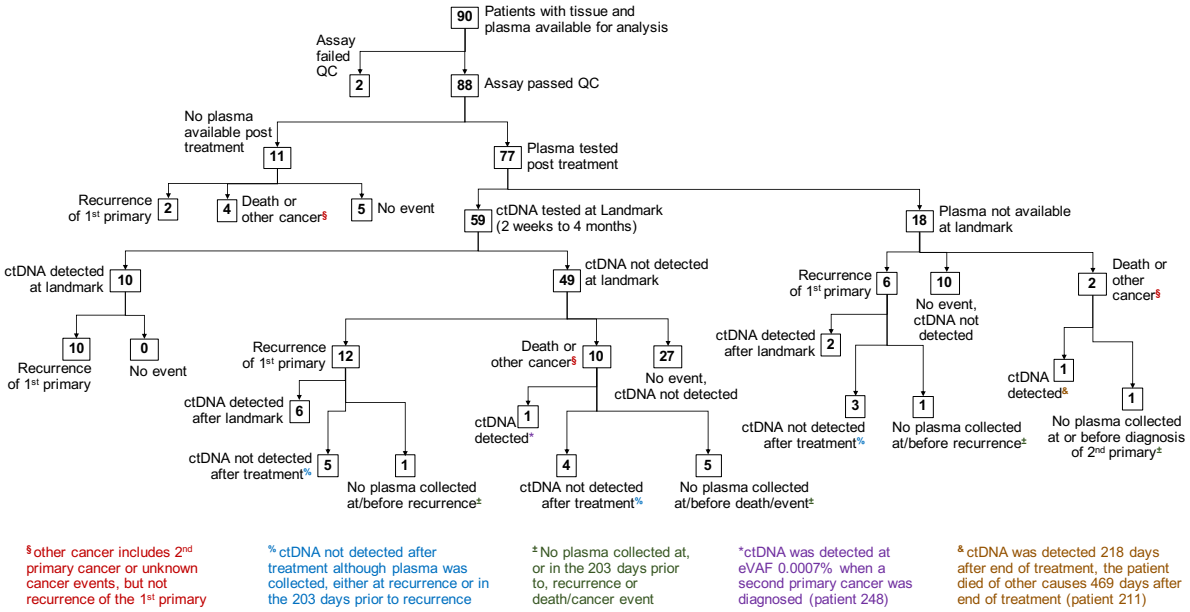
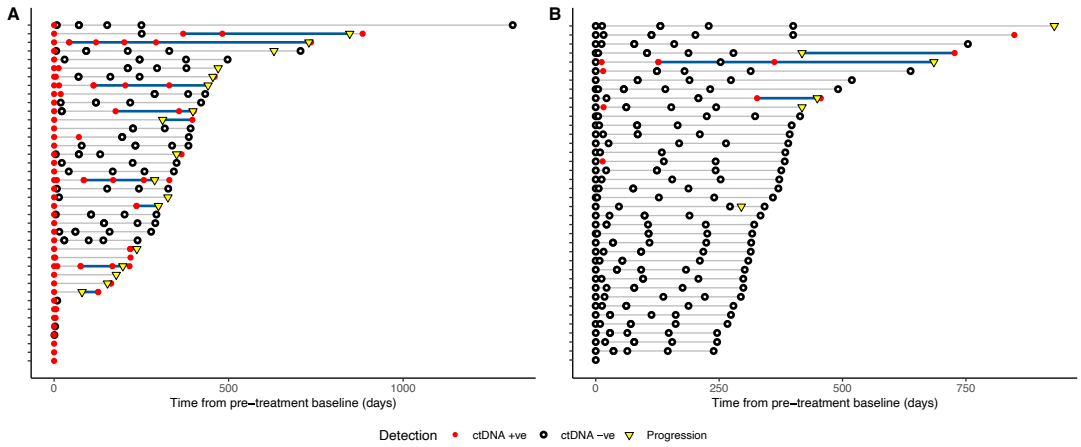
Table S3: ctDNA RaDaR™ data for all 363 timepoints for all 88 patients.

Supplementary Figures



Supplementary Figure S1: CONSORT flow diagram of patient disposition, sample availability and results of ctDNA analysis. Showing sample availability and ctDNA detection using the RaDaR™ assay for 90 patients for whom tumor samples were available for sequencing (out of 100 eligible patients in LUCID (30)).

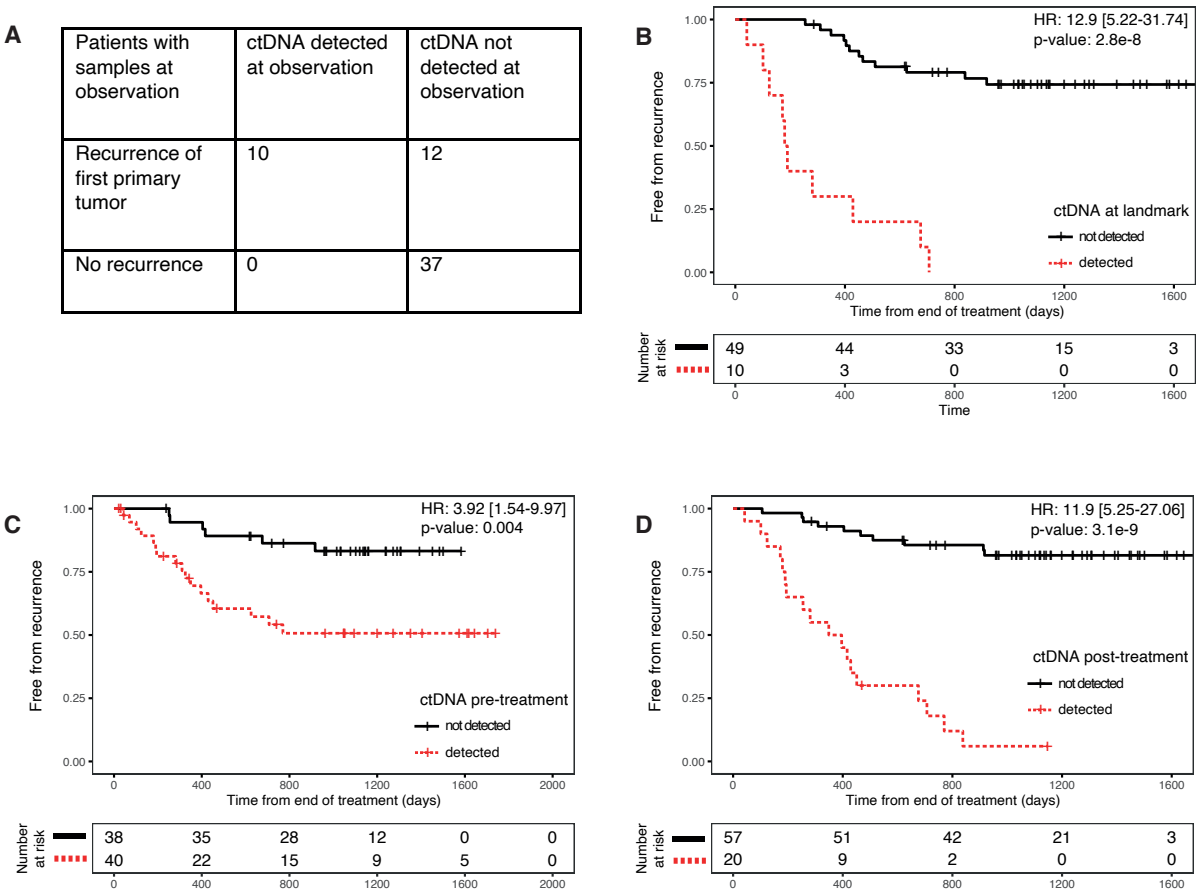


Supplementary Figure S2: Time-strand plots split by ctDNA detection prior to treatment. Red dots indicate samples with ctDNA detected (ctDNA positive), and black circles indicate samples in which ctDNA was not detected (ctDNA negative). Clinical recurrence is indicated with an inverted yellow triangle. The lead time between ctDNA detection ≥2 weeks from end of treatment and clinical recurrence is indicated with a solid blue line. Time is indicated (in this figure only) from the time of collection of the baseline (pre-treatment) sample. A) 40 patients with ctDNA detected at baseline, B) 38 patients with ctDNA not detected at baseline.

Supplementary Figure S3 [provided as a separate .pdf file]: Longitudinal monitoring of ctDNA levels in plasma for all 88 patients. Red dots indicate samples with ctDNA detected, and black circles indicate samples in which ctDNA was not detected, when analyzed using patient-specific RaDaR™ assays. Time is measured (horizontal axis) from the end of treatment. Black arrows above the plot indicate the time of surgery, and clinical recurrence of the first primary tumor if this occurred during the study is indicated by an inverted yellow triangle. “C”, “D” or “E” above the axes indicate other Cancer events, Death, or End of follow-up. Time of these events is indicated on the horizontal axis. Bold vertical lines are a guide to the eye and highlight the lead time (indicated in text above the axes) between the earliest detection of ctDNA, ≥2 weeks after the end of treatment, and when clinical recurrence of the first primary was first recorded for each patient.

A

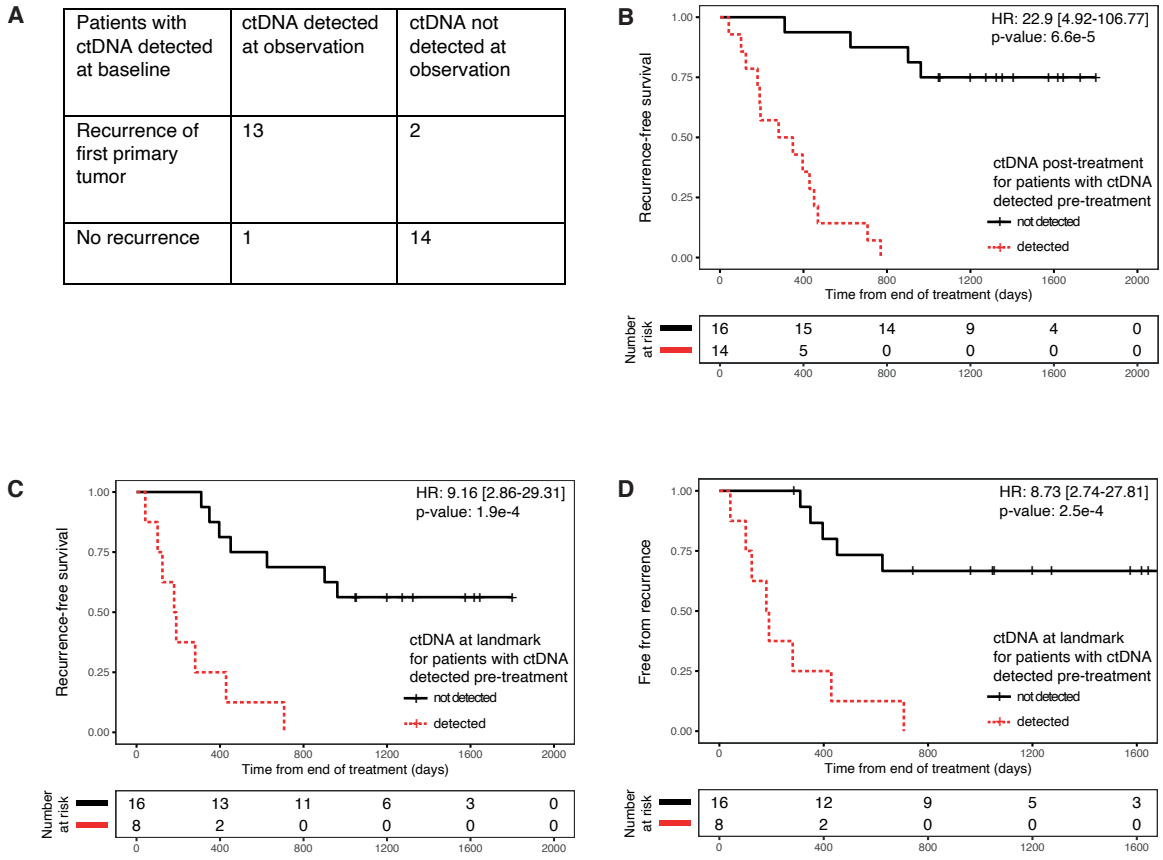
Patients with samples at observation	ctDNA detected at observation	ctDNA not detected at observation
Recurrence of first primary tumor	10	12
No recurrence	0	37



Supplementary Figure S4: Fraction of patients who remain free from recurrence (FFR). A) Concordance between ctDNA detection at any timepoint during observation ≥2 weeks after the end of treatment, and recurrence of the patients' first primary tumor. See data for individual patients in **Supplementary Table S1**. B-D) Kaplan-Meier analysis showing the fraction of patients without recurrence as a function of time. Patients right-censored due to loss of information are shown as vertical tick-marks. Patient subgroups are defined based on ctDNA detection using the RaDaR™ assay at different timepoints (see individual panels). Patients with ctDNA detected are shown in red dotted lines, and those with ctDNA not detected at the respective timepoints are shown in black solid lines. The numbers of patients remaining at risk are shown below each graph. B) Patients are split by ctDNA detection at Landmark (samples available for 59 patients). Hazard Ratio (HR): 12.9 (95% CI: 5.22-31.74, p-value: 2.8e-08). C) Patients are split by ctDNA detection at baseline (samples available for 78 patients). HR: 3.92 (95% CI: 1.54-9.97, p-value: 4.1e-03). D) Patients are split by ctDNA detection at any timepoint during observation ≥2 weeks after end of treatment (samples available for 77 patients). HR: 11.9 (95% CI: 5.25-27.06, p-value: 3.1e-09).

A

Patients with ctDNA detected at baseline	ctDNA detected at observation	ctDNA not detected at observation
Recurrence of first primary tumor	13	2
No recurrence	1	14



Supplementary Figure S5: Concordance with recurrence and survival analysis for patients with ctDNA detected prior to treatment. A) Concordance between ctDNA detection at any timepoint during observation ≥2 weeks after the end of treatment, and recurrence of the patients' first primary tumor, for patients with ctDNA detected prior to treatment. See data for individual patients in **Supplementary Table S1**. B-D) Kaplan-Meier analysis showing the fraction of patients without events as a function of time. Patients right-censored due to loss of information are shown as vertical tick-marks. Patient subgroups are defined based on ctDNA detection using the RaDaR™ assay at different timepoints (see individual panels), within the group of 40 patients who had ctDNA detected prior to start of treatment (at baseline). Patients with ctDNA detected are shown in red dotted lines, and those with ctDNA not detected at the respective timepoints are shown in black solid lines. The numbers of patients remaining at risk are shown below each graph. B) Recurrence-free survival (RFS, counting as events either recurrence of the first primary tumor or death if not preceded by a second primary tumor) of patients for whom ctDNA was detected prior to treatment, split by ctDNA detection at any timepoint during observation ≥2 weeks after the end of treatment (samples available for 30 patients). HR: 22.9 (95% CI: 4.92-106.77, p-value: 5.5e-06). C) RFS of patients split by ctDNA detection at the Landmark timepoint, which is the first plasma sample available in the window ≥2 weeks and ≤4 months after the end of treatment, for patients for whom ctDNA was detected prior to treatment (samples available for 24 patients). HR: 9.16 (95% CI: 2.86-29.31, p-value: 1.9e-04). D) Fraction of patients who remain free from recurrence (FFR) split by ctDNA detection at the Landmark timepoint, which is the first plasma sample available in the window ≥2 weeks and ≤4 months after the end of treatment, for patients for whom ctDNA was detected prior to treatment (samples available for 24 patients). HR: 8.73 (95% CI: 2.74-27.81, p-value: p-value: 2.5e-04).

Supplementary Methods

Patient cohort

Demographics for the full cohort of 100 patients recruited to LUCID, based on clinical data at the time of diagnosis, were recently reported by Heider et al. (30). For patients who underwent surgery, cancer stages and histological subtypes were updated after surgery (**Fig.1B**, **Supplementary Table S1**). 8 patients received further treatment after surgery (**Supplementary Table S1**), including radiotherapy and/or chemotherapy (Cisplatin and Vinorelbine, or Carboplatin and Vinorelbine). Of the 88 patients for whom patient-specific assays were successfully designed and validated (see below), 69/88 (78.4%) underwent surgery, 43/88 patients (48.9%) had stage I disease, 25/88 (28.4%) stage II, and 20/88 (22.7%) stage III. The majority of the patients had adenocarcinoma (55/88, 62.5%), and most were ex-smokers (63/88, 71.6%). Nine percent had never smoked.

Sample collection and processing

FFPE tissue blocks were cut as 8-µm sections and tumor-enriched regions recovered by macrodissection based on regions marked on an adjacent haematoxylin-and-eosin-stained section by the study pathologist. DNA was extracted using the QIAamp® DNA FFPE Tissue Kit (Qiagen, Germany) according to manufacturer's instructions, except that DNA was incubated at 56°C and 500 rpm overnight and samples were eluted twice through the columns using 20 µL ATE buffer. Samples were quantified using the Qubit® dsDNA BR Assay Kit (ThermoFisher Scientific, US), and for samples with more than 800 ng DNA, DNA repair was carried out using the NEBNext® FFPE DNA Repair Mix (New England Biolabs, UK) according to the manufacturer's instructions.

DNA library preparation from tumor and buffy coat samples

FFPE tumor DNA and buffy coat DNA samples were sheared to ~200 bp in length using the Covaris LE220 (Covaris, US), and fragmentation sizes were confirmed on a subset of samples by capillary electrophoresis using Bioanalyser (Agilent, US), as previously described (30). Tumor and buffy coat next generation sequencing libraries were prepared using the ThruPLEX® DNA-seq kit (Takara Bio, Japan) using 100 ng sheared FFPE tumor DNA and 50 ng of sheared buffy coat DNA, with the number of library amplification cycles adjusted based on manufacturer's recommendations. Libraries were quantified by qPCR using NEBNext® Library Quant Kit for Illumina® using the ROX low setting (New England Biolabs, UK), and library size and quantification was also assessed using the TapeStation® (Agilent, US).

Exome capture

Following library preparation of tumor and buffy coat libraries, exome capture was performed using a TruSeq® Exome Kit including a 45 Mbp bait set (Illumina, US) (30). 250 ng of each DNA library were combined in two-plex to four-plex reactions, with tumor and buffy coat DNA kept in separate reactions. 1 µL of i5 and i7 TruSeq HT xGen universal blocking oligonucleotides (IDT, US) were added at the hybridization step to ensure compatibility with the ThruPLEX libraries, and the volume of CT3 buffer adjusted to 51 µL. Two rounds of hybridization, each for 24 hours, were performed. After exome capture, samples were quantified and library size checked as above, and sequencing performed on a HiSeq4000 (Illumina, US) using PE150 sequencing and tumor and buffy coat libraries sequenced on separate lanes. Libraries prepared from tumor samples collected at the time of progression were sequenced on a NovaSeq 6000.

Patient-specific assay development

RaDaR™ is a personalized ctDNA assay built on the InVision® platform, which utilizes multiplex PCR and targeted next generation sequencing (NGS) (35). Patient-specific primer panels were designed targeting 48 amplicons per patient covering tumor-specific somatic variants selected from the somatic variants identified in the exome sequencing (47 variants for Patient 283; 26 of the amplicons covered paired variants; see **Supplementary Table 2**). The patient-specific primer-panels were manufactured (IDT) and combined with a fixed primer panel of common population-specific single nucleotide polymorphisms (SNPs), which functioned as an internal sample QC during the NGS testing. An aliquot of tumor DNA from FFPE tissue was used for primer panel qualification to confirm the accuracy and performance of the RaDaR™ panel design. Bespoke panels were considered successful if at least 32 of the patient-specific primer pairs passed a minimal coverage threshold, set to 40,000 expected reads. Buffy coat DNA control samples from the same patient were used to confirm the absence of germline variants, for removal of variants for example due to CHIP, and further amplification control. For Patient 221 and Patient 236, buffy coat control data was not available for most variants.

Sequencing analysis of patient-specific multiplex PCR libraries

Following primer panel qualification, RaDaR™ assays were used to analyze plasma cfDNA. Libraries were sequenced using the Nova-Seq 6000 system and sequencing data analyzed in a multi-step process: fastq files were demultiplexed using *bcl2fastq*, reads were then aligned to the GRCh38/hg38 assembly of the human genome using the *bwa-mem* alignment software (38) and processed to identify primer pairs and count mutant and reference bases. Variants that were present in buffy coat sequencing data or absent in tumor DNA sequencing data were excluded from analysis. A statistical model was used to assess the 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 ctDNA presence or absence at the sample level. The variant allele fraction was estimated from this model.

ctDNA detection at recurrence and lead time analysis

The lead time was determined to be the time difference (in days) between the first date of ctDNA detection in a sample taken ≥2 weeks after the end of treatment and the date of clinical progression. When available, samples taken at recurrence were used in the analysis. Due to occasional irregular scheduling in patient clinic visits and sample collection, some recurrence samples were collected shortly after clinical recurrence was confirmed.

Individual patient timecourses with longitudinal detection of ctDNA over time

Individual patient timecourses showing the dynamics of ctDNA over time were plotted from the time from treatment end. ctDNA levels were estimated as part of the calling procedure, using 5 ppm as the minimum level. ctDNA negative samples were classed as ND (not detected) and plotted at the bottom of the plot, corresponding to a level of 1 ppm on the y-axis (considering that the minimum detected ctDNA possible would be at 5 ppm, this point would always be below variant allele fraction values for detected ctDNA). The dotted lines between samples, and the annotated ctDNA level at progression, are purely visual indicators of temporal trends. Black vertical bars indicate the beginning and end of treatment, if available.

Data availability

Primary data including patient demographics, mutation lists and assay designs, sample collections times and ctDNA detection is available in **Supplementary Tables**.

Tumor and buffy coat sequencing data are available through the EGA (EGAD00001008502).

Supplementary Information

Longitudinal monitoring of ctDNA to detect residual disease and recurrence

Personalized RaDaR™ assays were used to analyze serial samples from each patient (**Fig.3A**), analyzing a total of 363 plasma samples (**Fig.3B**, **Supplementary Figure S2**). Overall, ctDNA was detected by these assays in 56% of patients (49/88), and in 26% of all samples (94 of 363), at a median eVAF of 0.042% for positive samples. Excluding baseline samples, ctDNA was detected in 19% samples (54 of 285) with median eVAF 0.026%, 63% of samples (34 of 54) had eVAF<0.1% and 41% (22 of 54) had eVAF<0.01%.

We evaluated time courses of ctDNA detection (**Supplementary Figure S3**) and highlight several illustrative examples. In Patient 218, who had stage IA adenocarcinoma, ctDNA was detected prior to surgery, not detected 20 days after surgery, but detected again approximately 6 months after surgery, preceding the clinical identification of recurrence by 222 days (**Fig.3C**). In Patient 267, who had stage IIB adenocarcinoma, ctDNA was detected before and after surgery, but following adjuvant chemotherapy and radiotherapy given for post operative stage and positive margins, respectively, ctDNA was not detected. The patient had no clinical or radiological progression in a follow up period spanning 1047 days after the end of treatment (**Fig.3D**). For Patient 297 with stage IIA adenocarcinoma and Patient 276 with stage IIA squamous cell carcinoma, ctDNA was detected at all timepoints analyzed. ctDNA was detected immediately post-surgery (1 day and 2 days respectively) at eVAF of 10.6 ppm (equal to 0.00106%) and 8.9 ppm (0.00089%). In samples collected ≥2 weeks after the end of treatment for those patients, there was a lead time of 203 and 328 days respectively for ctDNA detection ahead of clinical identification of recurrence (**Fig.3E**, **Fig.3F**).

In 48 samples collected 1-3 days after surgery, ctDNA was detected in 12 patients (25%), with a median eVAF of 26 ppm. Half of those 12 patients had later recurrence of their primary tumor with median time to recurrence 416.5 days after surgery. In 6 of the 12 patients ctDNA was detected after surgery at extremely low concentrations with eVAF<15 ppm and 4 of those patients had later recurrence of their primary tumor (e.g. **Fig.3E**, **Fig.3F**). However, 6 of the 12 patients with ctDNA detected in the first 3 days after surgery had no recurrence of the primary tumor during a median follow-up time of 543.5 days and up to 1047 days (**Fig.3D**). These observations support an interpretation that ctDNA may be transiently present in the blood at very low concentrations also in cases where the disease may have been removed.

We focused analysis on 230 plasma samples collected from 77 patients during observation, defined here as all timepoints ≥2 weeks after the end of treatment. ctDNA was detected during observation in 40 samples from 20 patients (26% of the 77 patients). Of these, 38 samples were from 18 patients who had recurrence of their first primary tumor, and 2 were from the set of 152 samples collected during observation from 49 patients who did not have recurrence of their first primary tumor (**Supplementary Figure S1**, **Supplementary Table S1**). Of these two samples, one sample from Patient 248 had ctDNA detected at an eVAF of 0.0004% (4 ppm). This sample was collected at the time of clinical progression, clinically indicated as a second primary cancer, and supported by data from WES analysis of the new cancer which had no overlapping mutations with the first primary cancer. The patient was subsequently lost to follow-up 313 days after this sample was collected. The other sample with ctDNA detected was from Patient 211, for whom ctDNA was detected at an eVAF of 0.0666% (666 ppm) 251 days before the patient died of other causes. ctDNA was not detected in

150 other samples from 47 patients who did not have recurrence of their first primary tumor, including 8 patients who had a clinical diagnosis of a second primary cancer. Considering these two samples described above, where there was no confirmed recurrence of the primary tumor, as possible false positives, the PPV of ctDNA detection during observation for recurrence of the first primary tumor was 95% (38/40 positive samples), and the specificity was 98.7% (150/152 samples).

Across the cohort, 28 patients who had samples collected during observation experienced clinical recurrence of their first primary tumor. ctDNA was detected during observation in 18 of these 28 patients (64.3%) (**Supplementary Figure S1**), at eVAFs ranging from 0.0011% (11 ppm) to >10% (**Supplementary Figure S3**), with median time to detection of 117.5 days from the end of treatment.

According to the study protocol, samples were serially collected only in the first few routine clinical visits up to approximately one year after the end of treatment (range 211-412 days), and for a subset of patients also at/after disease progression (**Supplementary Table S3**). Of 19 patients for whom samples were collected at/after recurrence of their first primary tumor, ctDNA was detected at/after recurrence in 14 patients (73.7%). For the other 5 patients, 18 samples were collected in total across all timepoints and ctDNA was detected only in one sample (collected pre-treatment from Patient 233, more than 700 days prior to recurrence). Only one of the 5 patients (Patient 233 with squamous cell carcinoma) had a biopsy collected at the time of progression to confirm recurrence, and no biopsy was available for the other 4 patients (all of whom had adenocarcinoma).

For 12 patients, ctDNA was detected in samples collected during observation prior to recurrence, in the first ~9 months after end of treatment. In those cases the median lead time between ctDNA detection and clinical recurrence was 212.5 days (**Supplementary Table S1**). In 6 patients, ctDNA was detected during observation only in the plasma samples collected after clinical recurrence. Of these patients, 5 had no samples available during observation in the period of 210 days before clinical recurrence, including 3 patients who had recurrence less than 200 days after end of treatment and for whom samples were available only after recurrence. For one patient, ctDNA was detected after recurrence but was not in a sample collected 139 days prior to recurrence. This patient (Patient 277) was initially diagnosed with a second primary cancer, but analysis of the recurrent cancer by WES indicated it was a recurrence of the first primary tumor. Overall, the data is consistent with a lead time of approximately 200 days from ctDNA detection to recurrence when ctDNA is detected.

In 10 of the 28 patients who developed clinical recurrence of their first primary tumor, ctDNA was not detected in samples collected ≥2 weeks after the end of treatment. For 5 of the 10, no samples were collected in the 200 days prior to clinical recurrence, while for the remaining 5 the median time from the last sample collected to clinical recurrence was 91 days. Of the 28 patients who had clinical recurrence of their first primary, 8 had recurrence <200 days after the end of treatment, and in 7 of those 8 cases ctDNA was detected either before or after recurrence. In the 20 patients who had clinical recurrence more than 200 days after end of treatment, ctDNA was detected prior to recurrence in 8 cases (40%), and in those cases the median lead time for ctDNA detection was 402.5 days. In the remaining 12 cases the median time from the last sample to recurrence was 192 days.

Of the 28 patients, 21 had samples available prior to treatment. Of those 21, 15 patients had ctDNA detected prior to treatment ("baseline-positive"), and in 6 patients ctDNA was not detected. In the baseline-positive patients, ctDNA was detected during observation in 13 of the 15 patients who experienced clinical recurrence of their first primary tumor (86.7%).

ctDNA detection ≥2 weeks post-treatment is predictive of clinical disease relapse

We compared the detection of ctDNA at baseline, at a Landmark timepoint defined as the first sample collected in a time-frame between 2 weeks and 4 months from the end of treatment, or during observation defined as any time ≥2 weeks after the end of treatment, to clinical outcomes including overall survival (OS, counting as events death from any cause), recurrence-free survival (RFS, counting as events either recurrence of the first primary tumor, or death if not preceded by a second primary tumor), and freedom from recurrence (FFR, counting as events only recurrence of the first primary tumor) (**Fig.4**). We used Kaplan-Meier and log rank tests to evaluate the predictive value of ctDNA detection.

Of 59 patients that had a plasma sample collected at a Landmark timepoint, 10 (17%) had ctDNA detected, and all 10 had recurrence of their first primary tumor (**Supplementary Figure S1**). These patients had a shorter OS and RFS compared to patients for whom ctDNA was not detected at Landmark, with a Hazard Ratio (HR) of 5.48 and 14.8 respectively (p-value 0.00029 and 1.4e-08; **Fig.4AB**). Of the 49 patients with ctDNA not detected at Landmark, 17 patients had RFS events (recurrence or death, **Fig.4B**) of whom 12 patients had recurrence of their first primary tumor (**Supplementary Figure S4AB**, **Supplementary Table S1**). Analysis at Landmark therefore had 100% specificity and PPV but missed 12/22 patients with clinical recurrence (54.5%) and 17/27 patients (63.0%) with recurrence or death, resulting in a NPV of 75.5% (37 of 49) for recurrence and 65.3% (32 of 49) for recurrence or death.

Analysis of ctDNA pre-treatment, in 78 patients where samples were available, was also prognostic. Of 23 patients with recurrence of their primary tumor and samples at baseline ctDNA was detected in 17 (73.9%) and was missed in only 6 patients (26.1%), however specificity was lower and only 17/40 (42.5%) of patients with ctDNA detected at baseline had recurrence of their original primary tumor (**Supplementary Figure S4C**, **Supplementary Figure S2**, **Supplementary Table S1**). Patients for whom ctDNA was detected pre-treatment also had shorter OS and RFS compared to patients for whom ctDNA was not detected at baseline; however, the hazard ratios and p-values were much lower (HR=2.97 and 3.14, p-values 0.01 and 0.003 respectively; **Fig.4CD**). A stronger difference in RFS was observed when comparing patients in which ctDNA was detected vs. not detected at any timepoint during observation after treatment (HR=9.81, p-value 7e-10; **Fig.4E**). Analysis at any timepoint during observation identified 18 of 28 events of clinical recurrence (64.3%) with high specificity (18/20, 90%; **Supplementary Figure S4D**, **Supplementary Table S1**).

In the subset of patients with ctDNA detected pre-treatment, both sensitivity and specificity were high for detection of ctDNA after treatment (**Fig.4F**, **Supplementary Figure S5**). Only two of 16 patients for whom ctDNA was detected pre-treatment but not detected during observation had a clinical recurrence of their first primary tumor (**Fig.4F**), corresponding to a NPV of 87.5% (14/16). Of the 15 patients who had a clinical recurrence of their first primary tumor ctDNA was detected during observation in 13 (sensitivity of 86.7%) and only one of 14 patients who had ctDNA detected during observation did not experience clinical recurrence (PPV of 92.9%), resulting in a significant hazard ratio for recurrence and RFS for ctDNA detection at observation (HR=18.2, p-value 0.00022; **Fig.4F**) and at Landmark (**Supplementary Figure S5**). Of patients with ctDNA not detected at baseline, on the other hand, ctDNA was detected at Landmark in only 1 of 29 patients with a Landmark sample available (Patient 261 who had clinical recurrence of the primary tumor 558 days after the positive Landmark sample), and in only 4 of 37 patients in samples collected during observation (**Supplementary Table S1**).

Summary of results of previous studies

In a study by Abbosh et al. published in 2017, the TRACERx team used personalized sequencing assays targeting 16-25 variants per patient and found that ctDNA detection could predict relapse in post-operative lung cancer patients an average of 70 days sooner than imaging (39). In an update presented in 2020, the same group used assays that targeted up to 200 variants per patient (40). They reported a specificity of 99.3%, and found that ctDNA was detected at or before relapse in 37/45 patients who experienced relapse of their primary tumor. The median lead time from ctDNA detection to clinical relapse for these 37 patients was 151 days. Similar to our observations, ctDNA was not detected in patients who were diagnosed with second primary cancers during follow-up using personalized assays designed to target mutations identified in the first primary tumor.

Chaudhuri et al. used personalized hybrid-capture panels to detect ctDNA after treatment in a population of patients with predominantly stage II-III disease treated by radical radiotherapy. They reported ctDNA detection in the first post-treatment blood sample to be highly prognostic, with detection in 94% of evaluable patients experiencing recurrence. ctDNA detection post-treatment preceded radiographic progression in 72% of patients by a median of 5.2 months (21).