# Serial Postoperative Circulating Tumor DNA Assessment Has Strong Prognostic Value During Long-Term Follow-Up in Patients With Breast Cancer

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## ABSTRACT

**PURPOSE** Here, we report the sensitivity of a personalized, tumor-informed circulating tumor DNA (ctDNA) assay (Signatera) for detection of molecular relapse during long-term follow-up of patients with breast cancer.

**METHODS** A total of 156 patients with primary breast cancer were monitored clinically for up to 12 years after surgery and adjuvant chemotherapy. Semiannual blood samples were prospectively collected, and analyzed retrospectively to detect residual disease by ultradeep sequencing using ctDNA assays, developed from primary tumor whole-exome sequencing data.

**RESULTS** Personalized Signatera assays detected ctDNA ahead of clinical or radiologic relapse in 30 of the 34 patients who relapsed (patient-level sensitivity of 88.2%). Relapse was predicted with a lead interval of up to 38 months (median, 10.5 months; range, 0-38 months), and ctDNA positivity was associated with shorter relapse-free survival ($P < .0001$) and overall survival ($P < .0001$). All relapsing triple-negative patients (n = 7/23) had a ctDNA-positive test within a median of 8 months (range, 0-19 months), while the 16 nonrelapsed patients with triple-negative breast cancer remained ctDNA-negative during a median follow-up of 58 months (range, 8-99 months). The four patients who had negative tests before relapse all had hormone receptor–positive (HR+) disease and conversely, five of the 122 nonrelapsed patients (all HR+) had an occasional positive test.

**CONCLUSION** Serial postoperative ctDNA assessment has strong prognostic value, provides a potential window for earlier therapeutic intervention, and may enable more effective monitoring than current clinical tests such as cancer antigen 15-3. Our study provides evidence that those with serially negative ctDNA tests have superior clinical outcomes, providing reassurance to patients with breast cancer. For select cases with HR+ disease, decisions about treatment management might require serial monitoring despite the ctDNA-positive result.

ACCOMPANYING CONTENT

Data Sharing Statement

Data Supplement

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## INTRODUCTION

Breast cancer is the most commonly diagnosed cancer in men and women combined and is the leading cause of cancer-related deaths in women.[1] The current standard of care for women with early-stage breast cancer for most patients consists of surgery and (neo)adjuvant chemotherapy and/or endocrine therapy with the aim of eliminating microscopic minimal residual disease (MRD).[2,3] However, up to 30% of patients with breast cancer ultimately relapse with life-threatening metastases after their primary treatment.[4] Hence, there is an urgent need to develop more sensitive technologies capable of detecting MRD and following patients with breast cancer after primary treatment with the aim of identifying whether interventions in patients with MRD might be helpful in improving outcomes. Personalized circulating tumor DNA (ctDNA) measurements have been shown to predict relapse in lung and

## CONTEXT

**Key Objective**
To determine the value of a personalized, tumor-informed circulating tumor DNA assay (Signatera) for early detection of relapse in patients with breast cancer.

**Knowledge Generated**
The assay predicted relapse in 30 of 34 patients with variable lead intervals. In patients with triple-negative breast cancer, the test was consistently predictive, but a minority of patients with hormone receptor–expressing breast cancer were not detected and others in this subtype had a positive test with no evidence of relapse as yet.

**Relevance**
The test is highly predictive of recurrence in patients with breast cancer, especially the triple-negative subtype. For patients with hormone receptor–positive breast cancer, the test needs to be used with care since a small proportion of patients relapse with a negative test and others whose test is positive have not yet relapsed.



colon cancers,[5,6] and there is some evidence that treatment given to patients showing a positive test in the absence of overt recurrence might be of benefit.[7]

For patients with breast cancer, several groups—including our own—have shown that ctDNA detection can antedate metastatic recurrence.[8-10] We presented the preliminary results of the Exploratory Breast Lead Interval Study (EBLIS) in 2019.[11] After 2 years of follow-up, approximately 50% of the predicted events (18 relapses) had occurred. After an interim analysis, the trial management group recommended laboratory assessment of serial plasma in the first 50 patients. Of these, 49 patients had successful tumor whole-exome sequencing (WES) enabling ctDNA (Signatera bespoke, multiplex PCR next generation sequencing) assay design, wherein we reported a lead interval of up to 2 years (median of 8.9 months; range, 0.5-24 months) between detection of ctDNA and clinical detection of overt metastatic disease. The results indicated that the tumor-informed, ctDNA assay was prognostic of recurrence in the majority of patients (16/18).[11]

Concerning the late adjuvant setting, in a recent report, 83 patients with hormone receptor–positive (HR+) breast cancer were followed up for a median of 10.4 years from diagnosis. Eight patients had a positive ctDNA test; six were MRD-positive before overt clinical recurrence, with a median lead interval of 12.4 months, while two ctDNA-positive patients had not relapsed at the time of last follow-up.[12] However, this study only had a median of two samples per patient, and as patients with HR+ breast cancer remain at risk for many years, more information is needed on ctDNA dynamics in HR+ breast cancer.

Here, we report results for the entire EBLIS cohort, to our knowledge, the largest breast cancer cohort with the longest ctDNA based follow-up to date, where a total of 156 patients with primary breast cancer were followed for up to 12 years with semiannual blood sampling. A total of 1,136 plasma samples from the 156 patients were profiled for ctDNA detection with personalized Signatera assays following our previously validated approach.[11]

## METHODS

### Patients and Samples

EBLIS is a multicenter, prospective cohort study, funded by Cancer Research UK and the National Institute for Health Research that opened to recruitment in 2012. Patients must meet all the inclusion criteria to be considered eligible for this study. All patients provided written informed consent before entry into the study. None of the assay results were shared with either clinicians or patients. Patients were age 18 years or older, have had histologically confirmed breast cancer, and must have completed all surgery and chemotherapy within 3 years of entry into the study. They had to have an Adjuvant! Online risk of relapse at >65% relapse or mortality of >50% at 10 years. The trial protocol was approved by the Riverside Research Ethics Committee (REC:13/LO/115; IRAS:126462). The primary objective was to determine the lead interval between detection of ctDNA in plasma and clinical detection of overt metastatic disease. A cohort of 188 patients were monitored with semiannual blood sampling for ctDNA analysis, along with concomitant clinical examination as described previously (Fig 1A; Table 1; Data Supplement, Tables S1-S2d).[11] The study census date (last date of follow-up) was December 31, 2021. All patients had provided consent for the publication of the study.

### Signatera Assay Design and Analysis

Personalized, tumor-informed Signatera ctDNA assays were developed, from primary tumor WES data, targeting 16 high-ranked, clonal, somatic single-nucleotide variants (SNVs) that were used to detect ctDNA in plasma. Details of the methodology and workflow have been reported

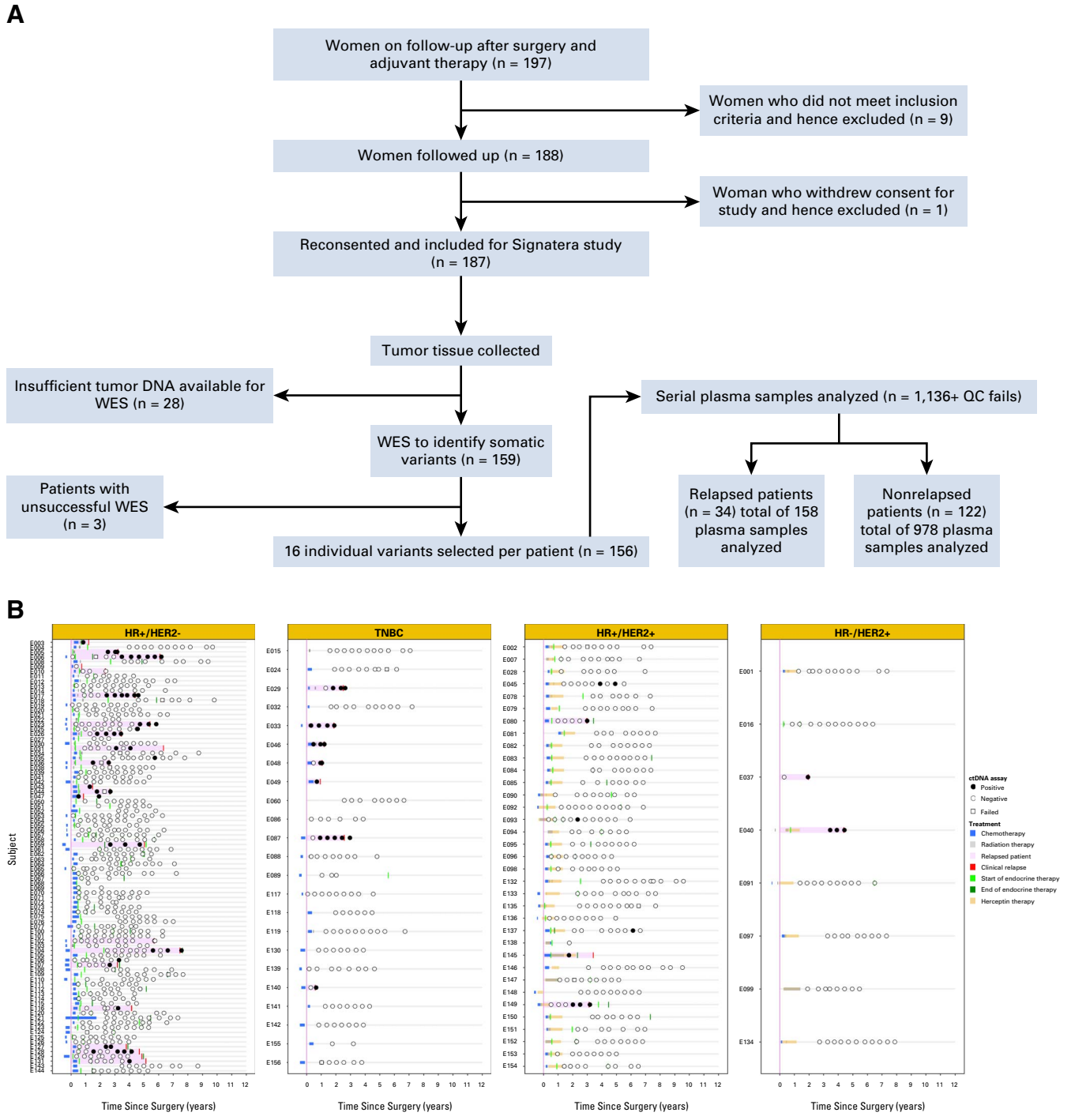


**FIG 1.** EBLIS study flow diagram and patient timeline summaries showing detection of ctDNA ahead of clinical relapse. (A) Patient recruitment and collection of clinical samples. For the 156 women with breast cancer monitored in this study, exonic alterations were determined through paired-end sequencing of FFPE tumor-tissue specimens and matched normal DNA. Patient-specific Signatera assays were designed to include 16 somatic mutations identified from whole-exome sequencing data. Serial plasma samples were analyzed with the corresponding custom assay panels using the Signatera workflow in a blinded manner in a CLIA-certified laboratory. A total of 1,141 plasma samples were analyzed for ctDNA detection. (B) Each patient's time since surgery showing longitudinal ctDNA assay samples, treatment and relapse status and results summary of each patient's (n = 156) treatment regimen by subgroup along with results of serial plasma samples (n = 1,136) analyzed. CLIA, Clinical Laboratory Improvement Amendments; ctDNA, circulating tumor DNA; EBLIS, Exploratory Breast Lead Interval Study; FFPE, formalin-fixed paraffin-embedded; QC, quality control; TNBC, triple-negative breast cancer; WES, whole-exome sequencing.

**TABLE 1.** Patient and Tumor Baseline Characteristics

| | Molecular Subtype (No.) | | | | |
|---|---|---|---|---|---|
| Variable | HR+/HER2− (90) | HR+/HER2+ (35) | TNBC (23) | HER2+ (8) | All Subjects (156) |
| Age, years | | | | | |
| ≤40 | 8 | 8 | 2 | 0 | 18 |
| 41-60 | 57 | 18 | 14 | 5 | 94 |
| 61-80 | 25 | 9 | 5 | 3 | 42 |
| >80 | 0 | 0 | 2 | 0 | 2 |
| Age at diagnosis, years | | | | | |
| Mean (SD) | 53.9 | 51.8 | 57.1 | 61.1 | 54.3 |
| Median | 54 | 50 | 57 | 57 | 54 |
| Min-max | 26-80 | 29-79 | 34-87 | 48-80 | 26-87 |
| Size of tumor, mm | | | | | |
| Mean (SD) | 41.1 | 35.9 | 24.8 | 34.5 | 37.1 |
| Median | 32 | 30 | 25 | 31 | 30 |
| Min-max | 5-150 | 1-100 | 5-50 | 27-60 | 1-150 |
| Tumor type | | | | | |
| IDC | 73 | 34 | 22 | 6 | 135 |
| ILC | 16 | 1 | 0 | 1 | 18 |
| Other | 1 | 0 | 1 | 1 | 3 |
| Type of surgery | | | | | |
| Mastectomy | 56 | 21 | 10 | 5 | 92 |
| Breast conservation | 30 | 14 | 13 | 2 | 59 |
| Bilateral mastectomy | 2 | 0 | 0 | 0 | 2 |
| Other | 2 | 0 | 0 | 1 | 3 |
| Tumor grade | | | | | |
| 1 | 1 | 0 | 0 | 0 | 1 |
| 2 | 57 | 3 | 2 | 0 | 62 |
| 3 | 32 | 32 | 21 | 8 | 93 |
| Histology | | | | | |
| Left | 46 | 22 | 13 | 5 | 86 |
| Right | 44 | 13 | 10 | 3 | 70 |
| HER2 status | | | | | |
| Positive | 0 | 35 | 0 | 8 | 43 |
| Negative | 90 | 0 | 23 | 0 | 113 |
| ER status | | | | | |
| Positive | 89 | 33 | 0 | 0 | 122 |
| Negative | 1 | 2 | 23 | 8 | 34 |
| PgR status | | | | | |
| Positive | 64 | 12 | 0 | 0 | 76 |
| Negative | 24 | 20 | 23 | 8 | 75 |
| Not documented | 2 | 3 | 0 | 0 | 5 |
| Staging | | | | | |
| IA | 1 | 0 | 6 | 0 | 7 |
| IIA | 2 | 3 | 2 | 0 | 7 |
| IIB | 26 | 12 | 9 | 5 | 52 |
| IIIA | 36 | 16 | 4 | 2 | 58 |
| IIIB | 0 | 0 | 0 | 0 | 0 |
| IIIC | 24 | 4 | 2 | 1 | 31 |
| Unknown | 1 | 0 | 0 | 0 | 1 |

NOTE. After screening and recruitment, patients were followed up with six monthly blood samples for up to 10 years. HER2 status was determined by using IHC and FISH. A patient was considered to have HER2-positive cancer if IHC had a HER2 3+ score and/or a positive FISH test.
Abbreviations: ER, estrogen receptor; FISH, fluorescence in situ hybridization; HER2, human epidermal growth factor receptor 2; HR+, hormone receptor–positive; IDC, infiltrating ductal carcinoma; IHC, immunohistochemical; ILC, infiltrating lobular carcinoma; PgR, progesterone receptor; SD, standard deviation; TNBC, triple-negative breast cancer.

previously.[5,6,11] All tests were carried out in a Clinical Laboratory Improvement Amendments–certified laboratory.

### Statistical Analyses

The study sample size was described previously.[11] Clinical characteristics of patients were summarized using descriptive statistics, including means, medians, or range for continuous variables, and frequency and percentage for categorical variables. The Wilcoxon matched-pairs signed rank test was used to compare mean variant allele frequency (VAF) at the first ctDNA-positive time point and the last time point before relapse. Sensitivity was defined as the number of patients with preclinical metastasis detected by ctDNA over the total number of patients with clinical relapse. Specificity was defined as the number of patients who were ctDNA-negative during the clinical follow-up period over the total number of patients who have not relapsed. The primary and secondary outcome measures were relapse-free survival (RFS) and overall survival (OS), respectively. RFS was assessed by standard radiologic criteria measured from date of surgery to verified first radiologic recurrence (local or distant). OS was defined as the time from date of surgery to the date of death or last follow-up date (December 31, 2021, or death). Primary associative analysis used a univariable approach with categorical ctDNA status (negative and positive). RFS and OS were compared between patients with positive and negative ctDNA status at the first blood sample time point (baseline) and any time point by using Kaplan-Meier and log-rank tests. Hazard ratios (HRs) for RFS and OS were estimated using a univariable Cox proportional hazard model. Multivariable Cox regression models were used to determine the impact of ctDNA on RFS and OS while controlling for clinicopathologic factors. An exploratory analysis was performed in a subgroup of the cohort with complete data on cancer antigen (CA)15-3 and ctDNA. Fisher's exact test was used to evaluate the correlation between the measurements of ctDNA and CA15-3. All statistical tests were two-sided. A $P$ value of <.05 was regarded as statistically significant for results. All statistical analyses were performed using R (R Foundation for Statistical Foundation, R version 4.0.1, *survival* [version 3.2-7] and *survminer* [version 0.4.8], R Core Team, Vienna, Austria).

## RESULTS

Here, we report full results from EBLIS, to our knowledge, the largest breast cancer cohort with the longest ctDNA follow-up to date. A total of 188 patients with primary breast cancer, recruited after surgery and adjuvant chemotherapy, were followed up with semiannual blood sampling for ctDNA analysis. After review of all available formalin-fixed paraffin-embedded surgical tissue blocks, 29 patients did not have sufficient residual tumor for WES. In the remaining 159 patients, paired tumor and genomic DNA samples were subjected to WES; samples from two patients failed WES quality control requirements, and tumor WES for a third patient identified too few somatic variants, leaving 156 patients for ctDNA testing (see the flow diagram: Fig 1A). The landscape of somatic mutations detected in the 156 primary tumor DNA samples was similar to other breast cancer series, with *TP53* and *PIK3CA* being the most commonly mutated genes (Data Supplement, Fig S1).

The clinicopathologic characteristics of the patient cohort are presented in Table 1. The median follow-up was 77 months (range, 8-140 months; Table 2). Patient clinical characteristics, blood sample time points, CA15-3 levels, tests to confirm metastasis, and treatment schedules are provided in the Data Supplement (Tables S1-S2d).

### Long-Term Postoperative Follow-Up of Patients With Breast Cancer With the Signatera Residual Disease Test

Multiple plasma samples (n = 1,136) for ctDNA evaluation were available from all 156 patients, with a median of 8

**TABLE 2.** Median Follow-Up and Lead Interval by Molecular Subtype

| | Molecular Subtype (No.) | | | | |
|---|---|---|---|---|---|
| Variable | HR+/HER2− (90) | HR+/HER2+ (35) | TNBC (23) | HER2+ (8) | All Subjects (156) |
| Follow-up months, median (range) | 94 (27-131) | 73 (8-140) | 58 (8-99) | 86 (25-113) | 77 (8-140) |
| Treatment, No. (%) | | | | | |
| NACT | 29 (32) | 8 (23) | 10 (43.5) | 1 (12.5) | 48 |
| ACT | 57 (63) | 27 (77) | 10 (43.5) | 5 (62.5) | 99 |
| None | 4 (6) | 0 (0) | 3 (13) | 2 (25) | 9 |
| Patients who relapsed, No. (%) | 22 (24.4) | 3 (8.6) | 7 (30.4) | 2 (25) | 34 (21.8) |
| Relapses detected, No. (%) | 18 (81.8) | 3 (100) | 7 (100) | 2 (100) | 30 (88.2) |
| Lead interval months, median (range) | 13 (2-38) | 6 (4-13) | 8 (0-19) | 15.7 (11.6-19.8) | 10.5 (0-38) |
| No. of blood samples per patient, median (range) | 8 (1-11) | 8 (1-11) | 6 (1-11) | 9.5 (3-11) | 8 (1-11) |

NOTE. Median follow-up was from the date of surgery (months). Lead interval was from detection of ctDNA in plasma to clinical detection of overt metastatic disease.

Abbreviations: ACT, adjuvant chemotherapy; ctDNA, circulating tumor DNA; HER2+, human epidermal growth factor receptor 2–positive; HR+, hormone receptor–positive; NACT, neoadjuvant chemotherapy; TNBC, triple-negative breast cancer.

(range, 1-11) samples per patient. These included 121 plasma samples from continued follow-up of 31 patients who had not relapsed at the first reporting census date (interim analysis, June 30, 2018).[9] In the full cohort, time from surgery to first blood sample ranged from 3 to 57 months (median, 16 months; Data Supplement, Table S2a). Personalized Signatera assays detected ctDNA in a total of 46 of the 1,136 plasma samples (Fig 1B; Data Supplement, Tables S3a and S3b).

Thirty-four patients (21.7%) had been diagnosed with clinical recurrence at the last date of follow-up (Table 3). Plasma ctDNA was detected ahead of clinical or radiologic relapse in 30 of the 34 relapsed patients, with a patient-level sensitivity of 88.2% (Table 2; Fig 1B). Considering molecular subtypes, the patient-level sensitivity was 81.8% for the HR+/human epidermal growth factor receptor 2 (HER2)− group; however, 100% of relapses were detected through ctDNA in the HR+/HER2+, triple-negative breast cancer (TNBC), and HER+ groups (Table 2). Metastatic relapse was predicted with a lead interval between ctDNA detection and relapse of up to 38 months (median, 10.5 months; range, 0-38 months), updating the lead interval of 2 years (median, 8.9 months; range, 0.5-24 months) reported in the first 49 patients.[9] The longest lead time to molecular relapse was observed in HR+/HER2− patients (median, 13 months; range, 2-38 months) and HR−/HER2+ patients (median, 15.7 months; range, 11.6-19.8 months). Patients with HR+/HER2+ (median, 6 months; range, 4-13 months) and TNBC (median, 8 months; range, 0-19 months) had the shortest time to molecular relapse (Fig 1B; Table 2). Of note, there were no new positive ctDNA results in the TNBC cohort after 19 months and during a median follow-up of 58 months (range, 8-99 months).

The four relapsed patients not detected in the study (E09, E010, E0102, and E0129) were all HR+/HER-; two had bone recurrence (one with axillary lymph node involvement), one had a malignant pleural effusion and no other sites of metastasis, and one had an isolated local recurrence to bone (Fig 1B; Table 3; Data Supplement, Table S2c). Of the remaining 122 nonrelapsed patients, 116 patients were consistently ctDNA-negative across 941 plasma tests over up to 12 years after their primary surgery. Four patients (E035, E093, E106, and E137) had a single ctDNA-positive sample detected with low VAF, followed by a negative test, three with two variants and one with five variants detected (Fig 1B; Data Supplement, Tables S3a-S3c). Another patient (E045) had two of nine plasma samples that were termed ctDNA-positive, each of which was followed by a negative test. All five of these patients had HR+ breast cancer, and none had relapsed by the study census date (December 31, 2021). The disease status for these five patients was subsequently reviewed; at April 30, 2023, none had yet relapsed.

One other patient with a ctDNA-positive result (E025) was diagnosed with primary lung cancer. Her last blood sample on study had two variants detected, raising the possibility of recurrent breast cancer as opposed to primary lung cancer but the patient withdrew participation on the study, precluding access to the lung cancer tissue for molecular comparison.

### Rising ctDNA VAF and Mean Tumor Molecules/mL Antedates Relapse

The mean tumor molecules per mL (MTM/mL) was calculated on the basis of the mean of ctDNA molecules detected per mL of the patient's plasma. There was a positive correlation (rho, 0.75; $P < .001$) between the MTM/mL and VAF (Data Supplement, Table S3c). The number of variants, mean VAF, and MTM/mL varied between patients, with significantly higher VAF ($P = .0028$) and MTM/mL values ($P < .001$) observed at the time closest to relapse compared with the first ctDNA-positive sample. Moreover, patients who relapsed showed significantly higher median MTM/mL values compared with the five patients who did not relapse (0.60 [0.15-5.70] *v* 0.12 [0.06-128.2], $P = .011$). Although statistically significant, this trend is based on a small number of patients who did not relapse.

### Association Between Circulating Tumor DNA and Clinical Outcomes

The impact of ctDNA status on clinical outcomes was assessed. Patients with a positive ctDNA test had poorer RFS (HR, 52.98 [95% CI, 18.32 to 153.20]; $P < .0001$) and a significantly reduced OS (HR, 53.69 [95% CI, 7.01 to 411.49]; $P < .0001$; Figs 2A and 2B). This includes those patients with ctDNA detected in the first postsurgical plasma sample (HR, 30.15 [95% CI, 13.76 to 66.05]; $P < .0001$ for RFS) and (HR, 19.32 [95% CI, 6.66 to 56.01]; $P < .0001$ for OS; Figs 2C and 2D). Moreover, in multivariable models incorporating clinicopathologic variables, ctDNA status remained the most significant factor associated with RFS and OS ($P < .0001$; Data Supplement, Table S4).

### Circulating Tumor DNA and Other Monitoring Tests

Concurrent ctDNA analyses and CA15-3 measurements were available for 100 patients. CA15-3 status was defined as positive and negative at a cutoff value of 30 U/mL. The Fisher's exact test showed a borderline significant correlation between ctDNA status and CA15-3 status ($P = .053$; Data Supplement, Table S5a). Multivariate analysis indicated that ctDNA was independent of CA15-3 in predicting RFS and OS. Here, positive ctDNA status was significantly associated with shorter RFS [HR, 30.89 [94% CI, 10.05 to 94.99]; $P < .001$) and OS (HR, 35.52 [95% CI, 4.41 to 285.96]; $P < .001$), whereas CA15-3 was not (Data Supplement, Table S5b).

## DISCUSSION

The Signatera assay detected ctDNA up to 3 years before overt breast cancer relapse in the EBLIS patient population. The prognostic association is particularly striking for

**TABLE 3.** Clinical and ctDNA Characteristics in Patients With Clinical Relapse

| Publication ID | Type of Recurrence | Site of Metastasis | Time From Surgery to Relapse, Days | Lead Time, Days | ctDNA-Positive at First Plasma Time Point | ctDNA-Positive at Any Plasma Time Point |
|---|---|---|---|---|---|---|
| E003 | Metastatic | Pleura, lymph nodes, liver, and bone[a] | 435 | 133 | Yes | Yes |
| E005 | Metastatic | Nodal disease right hilum and mediastinum[a] | 1,183 | 263 | Yes | Yes |
| E006 | Metastatic | Right mediastinum and bilateral cervical nodes | 2,242 | 973 | No | Yes |
| E009 | Metastatic | Sternum, pelvis, and vertebrae[a] | 256 | Not available | No | No |
| E010 | Local | Sternum | 857 | Not available | No | No |
| E017 | Metastatic | Sternoclavicular joint, skin, and lung | 1,611 | 721 | No | Yes |
| E023 | Metastatic | Liver | [b] | Not available | No | Yes |
| E026 | Metastatic | Spine | 1,263 | 611 | No | Yes |
| E029 | Metastatic | Lung[a] | 918 | 258 | No | Yes |
| E031 | Metastatic | Skin on right lower back[a] | 1,428 | 301 | No | Yes |
| E033 | Metastatic | Intraclavicular fossa and sentinel lymph nodes | 680 | 570 | Yes | Yes |
| E036 | Metastatic | Bone and bladder[a] | 951 | 405 | Yes | Yes |
| E037 | Metastatic | Lung[a] | 717 | 610 | Yes | Yes |
| E040 | Metastatic | Bone[a] | 1,617 | 259 | Yes | Yes |
| E043 | Metastatic | Liver, lung, bone, and bile duct[a] | 535 | 68 | Yes | Yes |
| E044 | Local | Local nodes and bone mets[a] | 968 | 323 | Yes | Yes |
| E046 | Metastatic | Bone, liver, and pleura | 439 | 263 | Yes | Yes |
| E047 | Metastatic | Local nodes and intrapulmonal nodes | 302 | 114 | Yes | Yes |
| E048 | Metastatic | Bone and pleura | 372 | 199 | Yes | Yes |
| E049 | Metastatic | Not known[a] | 336 | 79 | Yes | Yes |
| E059 | Metastatic | Bone | 1,849 | 856 | Yes | Yes |
| E080 | Metastatic | Skin and bone mets | 1,232 | 137 | No | Yes |
| E087 | Local | Unresectable nodal recurrence[a] | 940 | 596 | No | Yes |
| E102 | Metastatic | Axillary LNs and bone mets | 2,092 | Not available | No | No |
| E104 | Metastatic | LNs, liver, and spinal mets | 2,753 | 695 | No | Yes |
| E107 | Metastatic | Bone mets at L2 and L5 of spine | 1,164 | 194 | No | Yes |
| E116 | Metastatic | Bone mets | 1,524 | 116 | No | Yes |
| E127 | Metastatic | Bone, pleural, and nodes outside axilla[a] | 1,389 | 512 | No | Yes |
| E128 | Metastatic | CNS, bone, and liver mets | 1,722 | 1,147 | No | Yes |
| E129 | Metastatic | Pleural | 1,786 | Not available | No | No |
| E131 | Metastatic | Bone and liver mets | 1,887 | 421 | No | Yes |
| E140 | Metastatic | CNS | 206 | 0 | No | Yes |
| E145 | Metastatic | Skin, bone, pleural, and liver[a] | 827 | 188 | Yes | Yes |
| E149 | Metastatic | Skin and bone | 1,148 | 408 | No | Yes |

NOTE. Lead time refers to the time (in days) from the first positive plasma sample to clinical occurrence.
Abbreviations: ctDNA, circulating tumor DNA; LN, lymph node.
[a]Patients are deceased.
[b]Excluded as dates affected by COVID-19.



patients with TNBC, as all seven patients with TNBC who relapsed had a positive ctDNA result before overt relapse. Additionally, none of the other patients with TNBC became ctDNA-positive after 19 months of monitoring, and during a median follow-up of 58 months (range, 8-99 months), which corresponds to the expected time frame of breast cancer recurrences in this subtype.[13]

The correlation between ctDNA detection and recurrence from HR+ breast cancer is also strong, but discordances were observed that may be attributable to underlying tumor biology—of note, four HR+ patients developed recurrent disease despite persistent ctDNA negativity. Additionally, five HR+ patients had one or two positive ctDNA samples with no diagnosis of recurrence despite prolonged follow-up, as shown using a similar personalized ctDNA technology in a smaller series for two HR+ patients.[10] Although these cases are technically considered false-positive results, one could postulate that these are situations in which indolent micrometastatic disease is present and transiently sheds

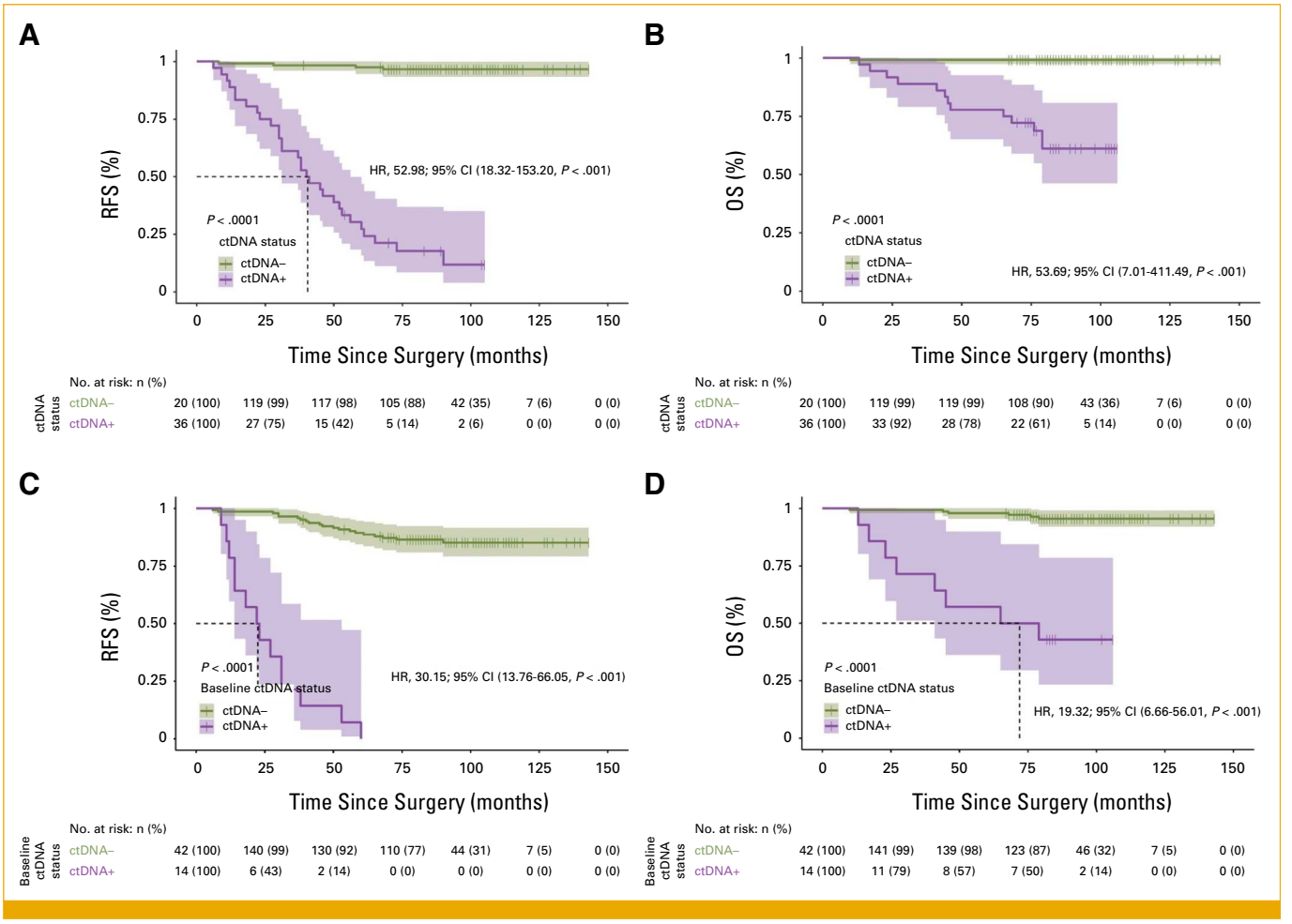


**FIG 2.** Personalized ctDNA detection in serial plasma samples predicts relapse-free survival and overall survival. (A) Relapse-free survival according to the detection of ctDNA in any follow-up plasma sample after surgery (HR, 52.98 [95% CI, 18.32 to 153.20]; $P < .0001$). (B) Overall survival according to the detection of ctDNA in any follow-up plasma sample after surgery (HR, 53.69 [95% CI, 7.01 to 411.49]; $P < .0001$). (C) Relapse-free survival according to the detection of ctDNA in the first postsurgical plasma sample (HR, 30.15 [95% CI, 13.76 to 66.05]; $P < .0001$). (D) Overall survival according to the detection of ctDNA in the first postsurgical plasma sample (HR, 19.32 [95% CI, 6.66 to 56.01]; $P < .0001$). n = 156 patients. ctDNA, circulating tumor DNA; HR, hazard ratio; OS, overall survival; RFS, relapse-free survival.



ctDNA as a result of biologic changes (eg, holding endocrine therapy). The presence or absence of ctDNA detection should be interpreted differently in the context of HR+/HER2− disease and might require serial monitoring; ctDNA positivity can serve as a measure of tumor activity, which among other things can be affected by ongoing treatment (eg, endocrine therapy). With milder phenotype, prolonged treatment duration, underestimated adherence to therapy, relapse destinations such as bone brain, or local progression (associated with lower ctDNA availability rate), HR+/HER2− disease is more challenging than other breast cancer subtypes for ctDNA detection. Although more evidence is needed to better understand the significance of occasional ctDNA positivity followed by serially negative results during the course of treatment, it is possible that more information on therapy adherence could help resolve this question. The results presented here support the use of ctDNA in clinical trials to determine if this technology can improve outcomes. Similar to colorectal cancer, the velocity of ctDNA concentration increase in subsequent tests,[14] known to be associated with time to clinical progression, might provide useful insights into the biology of HR+ breast cancer subtype.

Several studies have been published that studied ctDNA in the setting of breast cancer surveillance monitoring,[15–17] but these focus on patients with TNBC, which is known to be associated with higher levels of ctDNA.[9] These and other studies have evaluated smaller numbers of patients, and/or used other assays, so, it is difficult to compare our results with other groups. In general, however, the findings reported herein support the use of ctDNA defined by clonal somatic SNVs (Signatera), hotspot mutations,[9,10] breakpoint junctions,[10] or amplifications[18] to detect MRD and predict relapse. Importantly, serial longitudinal assessments are helpful to confirm the trajectory of ctDNA changes—particularly in patients with indolent HR+ breast cancer. Persistently negative ctDNA results strongly correlated with

the lack of disease recurrence and may therefore provide reassurance to patients.

In conclusion, the EBLIS study demonstrates that serial postoperative ctDNA analysis has strong prognostic value and allows for earlier detection of recurrence than by scans in many patients, while repeated negative tests can provide reassurance to patients. This provides a potential window that could enable the design of trials to assess the impact of earlier therapeutic interventions, which may lead to improved clinical outcomes, particularly in the setting of more aggressive subtypes (ie, TNBC). For patients with HR+ breast cancer, who remain at risk of relapse for many years, a negative test does not rule out the possibility of relapse, and for those where ctDNA is detected, a repeated Signatera test may be needed to confirm a positive test. In particular, confirming ctDNA concentration increase in subsequent tests might be more informative. Earlier intervention opportunities may allow better and more timely treatment with switch of endocrine therapy, but properly controlled randomized studies will be needed to determine if this is the case. Our study has some limitations: all blood tests were assayed retrospectively. Thus, it is not possible to state conclusively that patients did not have evidence of metastatic disease on conventional scanning as was shown in a recent study.[16]

All told, however, our results suggest that ctDNA testing may add to existing recommendations for symptom assessments, physical examination, and routine breast imaging as a means of monitoring patients with breast cancer after completion of definitive local therapy with or without adjuvant chemotherapy.



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E.K., H.S., D.R., and B.Z. are employees of Natera, Inc, and own stock, or options to stock, in the company. E.C.d.B., R.M., and D.S. are employees of AstraZeneca and hold AstraZeneca shares. B.A. is now an employee of Inivata Ltd. R.H. is now an employee of Nonacus Ltd. D.F.-G. is now an employee of GEICAM.

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I = Immediate Family Member, Inst = My Institution. Relationships may not relate to the subject matter of this manuscript. For more information

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